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Bachelor Thesis

Interactions of *Trypanosoma brucei* F_OF_1 ATP Synthase Subunits - An Application of Yeast Two Hybrid

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Annotation

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Anotace

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Abstract

The F_OF_1-ATP synthase has a unique composition and function in *Trypanosoma brucei*, a flagellated parasite endemic to developing regions of the world where it causes Human African Trypanosomiasis (HAT) and Nangana in cattle. Currently there are no good treatments to cure the disease as most of the medicine is antiquated, difficult to administer and highly toxic. Because of the unique properties of the *T. brucei* F_OF_1-ATP synthase, this enzyme is considered a possible drug target. This final component of the oxidative phosphorylation pathway actually works in reverse during the infectious stage of this parasite as it needs to hydrolyze ATP to maintain the essential mitochondrial membrane potential in the absence of a cytochrome mediated respiratory chain. In addition to the well conserved eukaryotic subunits, this large complex is also comprised of several subunits that have no known homology outside of kinetoplastids, an ancient group of protists with a unique mitochondrial DNA structure. To further explore the composition and organization of this potential drug target, we employed the technique of yeast two-hybrid to map the protein-protein interactions of the individual subunits. Our preliminary results suggest that important information about the organization of the *T. brucei* F_OF_1-ATP synthase can be gleaned from this experimental approach.

Abstrakt

Trypanosoma brucei je jednobuněčný parazit způsobující závažnou spavou nemoc u lidí a nemoc zvanou nagana u zvířat. Tato onemocnění jsou endemická v rozvojových zemí subsaharské Afriky. I když v poslední době klesá počet nakažených lidí, spavá nemoc je letální pokud je neléčena. Bohužel i dostupná léčba je silně nevyhovující z důvodů své toxicity, efektivity vůči rezistentním kmenům a náročnosti na aplikaci. Proteinový komplex F_oF_1 ATPsyntáza je životně důležitý pro buňku parazita a jeho složení a funkce je rozdílná od komplexu buňky lidské. Kromě velmi dobře konzervativních podjednotek F_oF_1 ATP syntázy tento komplex také obsahuje nové podjednotky, které nemají homology mimo skupinu trypanozóm. I funkce tohoto komplexu je jedinečná, jelikož tento komplex nevytváří ATP, jak jeho jméno napovídá, ale tuto molekulu hydrolyzuje za účelem vytvoření membránového potenciálu a tudíž funguje jako F_oF_1 ATPáza. Díky těmto vlastnostem je tento proteinový komplex považován za možný cíl pro vývoj nových chemoterapeutik. Detailní zkoumání tohoto komplexu může osvětlit funkci nových podjednotek a také jejich pozici v rámci komplexu. Z tohoto důvodu jsme se rozhodli studovat proteinové interakce známých i nových podjednotek in vivo metodou „yeast-two-hybrid“. Z dostupných výsledků je patrné, že tento přístup je možný a přínosný a pomůže vytvořit mapu proteinových interakcích podjednotek F_oF_1 ATPázového komplexu u *T. brucei*.

Abbreviations

3AT *(3-amino triazole)*; AA *(Amino Acid)*; AD *(Activation Domain)*; ATP *(Adenosine triphosphate)*; BD *(Binding Domain)*; cV *(F_OF_1-ATP Synthase)*; DNA *(Desoxy ribonucleic acid)*; dNTP *(Desoxy ribonucleotides)*; fwd. *(Forward sequence strand)*; HAT *(Human African Trypanosomiasis)*; kbp *(kilo base pair)*; Mbp *(mega base pairs)*; nt *(nucleotide)*; Nt# *(position of the nucleotide)*; PCR *(Polymerase chain reaction)*; rev. *(Reverse sequence strand)*; RNA*(ribonucleic acid)*; SD-Leu-Trp-His *(Syntetically defined media lacking Leucine and/or Tryptophan and/or Histidine)*; SDS-PAGE *(Sodium dodecyl sulfate - polyacrylamide gel electrophoredsis)*; SRA *(Serum resistance associated)*; *T. b. b.* or *r.* or *g. (Trypanosoma brucei brucei or rhodesiense or gambiense);* VSG *(Variant surface Glycoprotein)*; YPD *(Yeast peptone dextrose)*; YPDA *(Yeast peptone dextrose with adenine hemisulfate)*; Y2H *(Yeast two Hybrid)*;

Additionally the common single letter nucleotide abbreviations for the nucleotides and the three letter amino acid abbreviations for the amino acids were used throughout.

Keywords

Trypanosoma brucei; F_OF_1-ATP Synthase; yeast two hybrid; protein interactions; Human African Trypanosomiasis; drug target

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1 Introduction

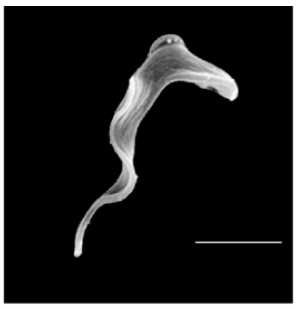

Figure 1.1: Electron micrograph of T. brucei (Hammarton et al. 2007)

Trypanosoma brucei, a unicellular eukaryote parasitizing animals as well as humans, causes african human trypanosomiasis (HAT) (Stuart et al. 2008) and nagana in animals (Biryomumaisho et al. 2013). These diseases occur mostly in sub saharan africa and are considered as big of a threat to the local economy as well as to people's lives. Infection of mammalian hosts occurs via the Tsetse fly (*Glossina spp*). (Etet et Mahomoodally, 2011) Although the numbers of infections have declined continuously in recent years, medication is still inconvenient, being complex in application and posing the risk of grave side effects (WHO, 2012).

In the past years, research has gained a lot of knowledge about *T. brucei*. One of these revelations was the special function of the F_OF_1-ATP synthase (cV) in *T. brucei'*s bloodstream stage (BS). (Schnaufer et al. 2005). Further, it was found that this complex has several additional subunits, that don't possess any homlogues in species not closely related to *T. brucei* (Ziková et al. 2009). The uniqueness of these characteristics makes cV a potential target for drug development. By employing the yeast two-hybrid method to identify interactions between T. brucei cV subunits, additional knowledge about this crucial protein complex can be deepened.

1.1 The organism Trypanosoma brucei

1.1.1 Taxonomy

From the taxonomic point of view, *T. brucei* belongs to the eukaryotic "superclade" of Excavata, which splits into several subgroups. The Euglenozoan group is considered to be the home of Trypanosomes. Generally, Euglenozoa are characterized by one or two flagella which originate in a flagellar pocket. The clade of Euglenozoa is then further split into three subclades, the Euglenida including hereotrophic as well as phototrophic taxa, the Diplonemea being strictly heterotrophic and possessing "pseudovanes" for feeding and the Kinetoplastea. The commmon trait of Kinetoplastida (Stuart et al. 2008) is an aggregation of DNA in the single mitochondrion called kDNA. The kDNA is in many cases situated in proximity to the origin of the flagella. The genus of trypanosoma includes other pathogens besides *T. brucei*, such as *T. cruzi,* which causes chagas disease (Stuart et al. 2008). This genus is situated in the kinetoplastean taxum of Trypanosomatida and this clade holds solely parasitic species. Apart from Trypanosoma, the genus Leishmania is also a well known human pathogen, being responsible for the various forms of Leishmaniasis (Stuart et al. 2008) (Adl

et al. 2005).

The species *Trypanosoma brucei* is itself divided into three subspecies namely *Trypanosoma brucei brucei, T. b. gambiense* and *T. b. rhodesiense,* whereby the latter two are infectious for humans. *T. b. brucei.* does not infect humans because the parasite cells are lysed by special human serum proteins. The two other subspecies express the so called SRA (serum resistance associated) gene in order to prevent this lysis (Xong, Vanhamme et al. 1998). Despite this, it is discussed that *T. b. brucei* and *T. b. rhodesiense* are just "phenotypic variants" (Balmer et al. 2011).

1.1.2 Morphology and cell characteristics

From the morphologic perspective, *T. brucei* has longish cells that are kept in shape by a cytoskeleton made from microtubules. This cytoskeleton also determines the exact position of most organelles. Many of those organelles are present as just a single copy. Some of these are the mitochondrion with the kinetoplast, the flagellum and the golgi apparatus.(Matthews, 2005) The mitochondrion is very large and runs through the whole cell. During the mammalian stages it doesn't contain an active mitochondrial cytochrome dependent respiration chain. Hence, it can't perform oxidative phosphorylation and therefore no mitochondrial cristae are observed.

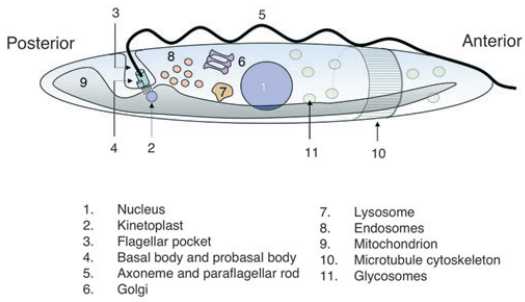

Figure 1.2: Basic body plan of T. brucei, (Mattews,2005)

Furthermore, most of the Krebs-cycle enzymes are not expressed(Hannaert et al. 2003). For energy production in this stage, *T brucei* relies on the abundant glucose from the hosts' blood (Matthews, 2005), which is processed by so called glycosomes. These are modified peroxisomes capable of performing glycolysis (Stuart et al. 2008). Another important feature of *T. brucei* is the flagellar pocket. Obviously, it is the origin of the single flagellum that is connected to the outside of the cell body and enables movement. Additionally, it is the place where endo- and exocytosis occur. Close to this is where the kinetoplast is situated. It consists of two kinds of DNA, namely maxicircles, which encode mitochondrial proteins and minicircles encoding guide RNAs that are templates in RNA editing. This is a process where RNAs transcribed from maxicircle DNA are modified by the addition or deletion of uridines via a special protein complex called the editosome. This type of RNA editing is found exclusively in kinetoplastids.

The nucleus, which is found in the middle of the cell, consists of 11 big chromosomes containing DNA in the mega-basepair range and more than 100 small chromosomes of about 50 kbp (Matthews, 2005).

One additional prominent property of *T. brucei* is its ability of genetic recombination, although it is not essential and the normal way of reproduction is cell division. Sexual recombination happens in the salivary glands of the Tsetse fly, by a still not entirely understood mechanism (Gibson et al. 2008).

1.1.3 Its Life cycle

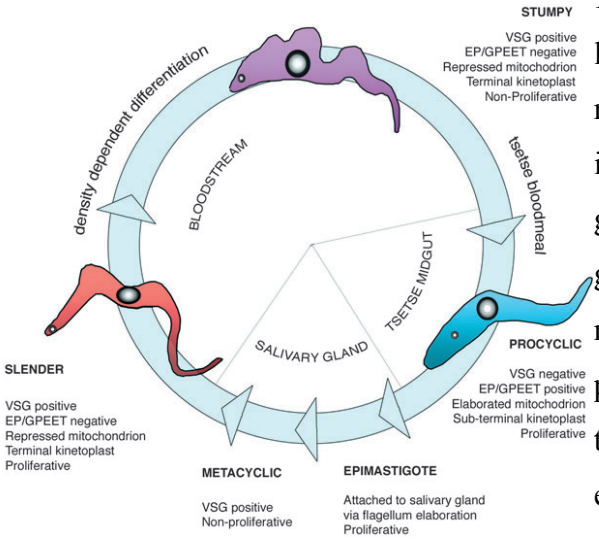

Figure 1.3: Illustration of the life cycle of T. brucei, (Mattews,2005)

T. brucei undergoes a variety of changes during its life-cycle due to the transition from insect to mammalian host. In the Tsetse fly, *T. brucei* is initially in its procyclic form reproducing in the mid-gut of the insect. In this stage a coat of surface glycoproteins termed procyclins is present on the cell membrane (Acosta-Serrano et al. 1999), probably as protective shield against damaging influences from the host such as proteases (Pays et Nolan, 1998). The energy supply in this form is maintained by mitochondrial respiration.

In the next stage, the trypanosomes invade the salivary glands of the Tsetse fly and differentiate into epimastigotes. There they cling to the salivary glands with their flagellar membranes and multiple by sexual reproduction (Gibson et al. 2008). Next, the epimastigotes transform into metacyclics, which build up a dense coat of variant surface glycoproteins (VSG). These are antigens to which the mammalian host's immune system responds. By rapidly varying the surface coat (it can be completely replaced in just 12 minutes), the host's immune response is dodged. For this strategy, the genome of *T. brucei* bears about 1000 different genes that encode VSGs. Metacyclics don't proliferate anymore and wander into the lumen of the salivary gland where they are ready to be transmitted into the mammalian host. It takes the parasites four weeks to reach this stage in the insect. Hence, just one in 1000 Tsetse flies has these metacyclics in its salivary gland and is therefore infective.

Once in the mammalian bloodstream, the cells differentiate into the slender bloodstream form. This form is characterized by a switch in energy metabolism. The oxidative phosphorylation pathway at the inner mitochondrial membrane is turned down and ATP is synthesized as already mentioned via glycolysis in the glycosomes. During this stage, the parasite multiplies in the host's bloodstream and lymph system. With increasing amount of cells, differentiation into another form

starts, termed the stumpy bloodstream form. In this stage, *T. brucei* do not reproduce anymore because unlimited growth in the host would cause the parasites to die earlier due to a lack of nutrients and the probability for the infection of a new Tsetse fly would therefore be decreased. Furthermore, when growth is halted, it allows for the coordinated cellular processes necessary for infection of the insect vector to begin. That is where the cycle starts over again: *T. brucei* is taken up by a Tsetse fly, exchanges its VSG coat for a procyclin one and starts to settle as procyclics in the insect gut.

During the whole life-cycle of *T. brucei,* the kinetoplast along with the flagellar pocket changes its position in the cell. Although it can only be speculated why this happens, it is a good characteristic to distinguish between the different stages of the life cycle along with the general morphological traits (Matthews, 2005) (Stuart et al. 2008).

1.2 The Diseases: Nagana and Sleeping Sickness

The diseases caused by *T. brucei* occur in 36 african countries located between the Sahara and the Kalahari desert. This region is referred to as *"Tsetse fly belt"*. In this zone the more than 20 subspecies of *Glossina* (Stuart et al. 2008) transmit trypanosomiasis to humans and animals. (Etet et Mahomoodally, 2011) Additionally, trypanosomiasis can be also transmitted from the mother to the unborn and by other blood nourishing insects. However, these modes of infection have unknown impact (WHO, 2012). In the following sections, there is an overview of the animal and human form of trypanosomiasis, as well as the available preventive measures and medicinal treatments.

1.2.1 Human African Trypanosomiasis (HAT) alias Sleeping Sickness

When *T. brucei* was discovered to be the pathogen of sleeping sickness and nagana around the beginning of the 20th century, the diseases were strongly present in many African countries with several hundreds of thousands of people dying (Kennedy, 2012). In the following decades, huge efforts were undertaken to gain control over HAT. These resulted in an extreme reduction of cases at the beginning of the 1960s. However, due to a loss of interest in a disease that seemed to be eradicated (Simarro et al. 2011) and the social and political unrest in the region, HAT reappeared and peaked with an estimated three hundred thousand infections in 1998. In recent years this trend was again reversed resulting in 7139 reported cases in 2010, which corresponds roughly to thirty thousand cases in total. (WHO, 2012)

As already mentioned, HAT is caused by *T. b. rhodesiense* and *T. b. gambiense,* whereby the latter is responsible for 97% of all cases. (Kennedy, 2012) Infections with *T. b. gambiense* are

considered to be more chronic, whereas those with *T. b. rhodesiense* are reported to be acute. In either case, an infection ends fatally if not treated (Stuart et al. 2008), although there are rare reports of self-healing in *T. b. gambiense* HAT patients (Kennedy, 2012).

By and large, an infection with *T. brucei* can be divided into two stages. The early stage (the hemolymphatic stage) first appears one to three weeks after the initial infection and is accompanied by rather unspecific symptoms such as fever, headache, fatigue and weight loss. The parasite in this stage rapidly reproduces in the bloodstream and the lymph system of the host. At a certain point the parasite crosses the blood brain barrier and invades the central nervous system. This marks the second late stage (CNS stage), which is accompanied by severe symptoms such as the name giving sleeping disorders in 74% of the patients, mental disorders including confusion and behavioural changes, as well as motoric deficiencies. Finally, the second stage ends with the death of the patient if they are not under medical treatment (WHO, 2012) (Kennedy, 2012).

1.2.2 Animal Trypanosomiasis alias Nagana

Trypanosomiasis in wild, as well as in domestic animals, is caused not only by the *T. brucei* subspecies but also by other *Trypanosoma* species named *T. vivax, T. congolense, T. simiae* and *T. suis* (Biryomumaisho et al. 2011). For the human pathogenic Trypanosomes, animals represent a reservoir that is most important for *T. b. rhodesiense* (WHO, 2012) Along with different wild animals, important livestock such as cattle, sheep, goats and pigs are threatened by nagana. A diverse set of symptoms can occur upon infection and therefore complicates diagnosis. The only reliable clinical sign for animal trypanosomiasis is considered to be anaemia, which only becomes apparent once the disease becomes chronic. Additionally, anaemia is found frequently in uninfected animals, often due to poor general living conditions. Therefore, serological tests and PCR based methods are used widely to detect nagana (Biryomumaisho et al. 2011).

The impact of this animal disease on the local human population, which mostly lives in rural areas that depend on agriculture, is quite grave. Infected domestic animals produce less milk and lose weight to the point that they are not capable of reproducing or performing demanding physical work. That decisively deteriorates the economical perspectives of an endemic region and hampers agricultural prosperity in large areas (Biryomumaisho et al. 2011).

1.2.3 Prevention and Cure

One of the main measures to prevent new infections with *T. brucei* is vector control. This means that by decimating the populations of Tsetse flies, it is possible to reduce *T. brucei* infections.

This is done using insecticides, special traps for the flies or by infertile insects. Another measure incorporates extensive surveillance programs that detect infections and treats the patient (Stuart et al. 2008) (Simarro et al. 2011).

Diagnosis of sleeping sickness is nevertheless a rather complex issue, because the clinical signs for HAT are often mistaken for malaria (Etet et Mahomoodally, 2011) and there is a lack of infrastructural facilities in many of the affected African areas. Moreover, the diagnostic methods available are often difficult to apply in the field or suffer from insensibility (Kennedy, 2012). Despite all of this, the reduction in cases of sleeping sickness was quite successful in the recent years and this has resulted in a historic minimum in 2010.

Nevertheless, many people still get infected and for those patients, medical treatment is rather complicated and may be ineffective or even dangerous. Currently, therapy is based on five compounds including one auxiliary substance. None of the substances can be administered orally (Kennedy, 2012) and all of them give rise to quite pronounced side effects (Stuart et al. 2008). Furthermore, three of the four actual preparations were introduced at least a century ago (Etet et Mahomoodally, 2011) and therefore increasing resistance against certain compounds are being reported (Baker et al. 2013).

For treatment of the first stage of HAT, two compounds are available, called suramin and pentamidine. Suramin is the oldest of the drug currently used as it was developed in the 1920s. It is a polysulfonated naphthalene derived compound. How suramin acts on *T. brucei* is still unknown, although there are several hypotheses. There were reports of resistance in *T. b. gambiense* infections in the 50s of the last century. Since then it has been used mainly for treating *T. b. rhodesiense* HAT. Treating a patient with suramin generally involves 5 intravenous injections every 3 to 5 days for four weeks.(Etet et Mahomoodally, 2011) The possible side effects include renal failure, skin lesions or anaphylactic shock (Kennedy, 2012).

Pentamidine, an aromatic diamidine, was first used in the 1930s. It's mode of action is also still under discussion. However, it is known that pentamidine damages the kinetoplast, but this is not believed to be its only mode of action. Curing with pentamidine is usually performed by a seven day period of daily intramuscular injections. Pentamidine shows a cross resistance with the second stage treatment of melarsoprol, which is probably due to reduced uptake of the drugs by changes in membrane channels (Baker et al. 2013). Adverse effects of pentamidine feature gastrointestinal disorders, hypertension and hypo- as well as hyperglycaemia (Kennedy, 2012).

In the CNS stage, the substances melarsoprol and eflornithine are used independently for

treatment, whereas nifurtimox, initially a preparation against Chagas disease, is employed together with eflornithine for a combination therapy. Generally, it can be said that treatment of this stage is more problematic due to the high toxicity of these compounds.

Melarsoprol is the last of the class of arsenic containing organic compounds that is still employed to fight second stage HAT. It was first used in 1949 (Kennedy, 2012) in order to replace more toxic arsenic derived medicine (Baker et al. 2013). It is the only effective drug against second stage *T. b. rhodesiense* HAT and can also be used against *T. b. gambiense*. Melarsoprol aims at a specific trypanosoma molecule, trypanothione, which maintains the "reducing environment" of the cell. (Kennedy, 2012) Unfortunately, frequent resistance to this substance has been reported, with several areas reporting that up to 30% of all treatments are unsuccessful . Currently, treatment involves 10 daily intravenous injections (Etet et Mahomoodally, 2011), which are considered very painful. The major drawback of this drug is that it is extreme toxic, which can lead to reactive encephalophathy. This complication occurs in 10% of all cases and leads to the death of half of these patients (Kennedy, 2012).

An alternative drug used for second stage HAT treatment is eflornethine. This is a derivative of the amino acid ornithine, which inhibits the ornithine decarboxylase. (Etet et Mahomoodally, 2011) It wasn’t widely used until 2001, even though its efficiency has been known since 1981. It is increasingly replacing melarsoprol wherever possible (Kennedy, 2012). Medication with eflornithine is quite complicated as it is administered via 56 intravenous infusions over a period of fourteen days. As this is a great logistic challenge in rural African areas, the combination therapy with the above mentioned nifurtimox was introduced. Now the treatment has been reduced to 14 eflornithine infusions over 7 days, which is supported by oral nifurtimox 3 times a day.(Etet et Mahomoodally, 2011) Unfortunately, eflornithine can cause serious side effects that include bone marrow toxicity, seizures and gastrointestinal issues (Kennedy, 2012).

Summing everything up, it can be said that general treatment methods are inconvenient. The available compounds are mostly very old, highly toxic and increasing instances of resistance are being reported. Additionally, applying the complex treatment procedures is very difficult under the prevalent infrastructure in the affected regions. Therefore, the research for novel treatment strategies that replace or support the existing ones is crucial.

1.3 The F_OF_1-ATP Synthase complex

1.3.1 Functions

The F_OF_1 -ATP Synthase is an enzymatic protein complex that is present in most organisms. Its predominant function is to synthesize ATP by harnessing the chemical potential of an electrochemical Na^+ or H^+ gradient across a membrane. This gradient is established by various mechanisms, including bacteriorhodopsin, a light fired proton pump that occurs in *Halobacterium salinarium,* the photosynthetic light reactions in plants and the respiratory chain of mitochondria. In the process of oxidative phosphorylation, the electrochemical energy stored in this gradient is released by the ions migrating along the gradient across the membrane through the ATP synthase. Thereby rotational energy is generated, which is used to produce ATP (van Ballmoos et al. 2008).

As in many other enzymes the ATP synthesizing function is reversible and an ATP consuming function that can pump protons against the gradient across a membrane is possible (van Ballmoos et al. 2008). This function is known for several anaerobic prokaryotes. Moreover, it also exists in eukaryotes such as special yeasts, when cultivated under anaerobic conditions. In mammals this doesn't usually happen. However, the FoF1 -ATP synthase will reverse its function during ischemia- when the cells are under anoxic conditions. This occurs mainly to maintain the surface potential of prokaryotic cells or the mitochondrial membrane potential, both of which enables the import of essential proteins. Interestingly, *T. brucei* demonstrates the same mechanism in their bloodstream stage, where this function is essential for the parasite. (Schnaufer et al. 2005) Since ATP hydrolysis by this protein complex doesn't occur in humans under normal conditions, it poses a putative drug target as the human physiology wouldn't be disturbed by a drug specially aimed at hindering the ATPase activity of the complex (Ziková, 2013).

1.3.2 Structure

From the structural point of view, the F_OF_1 -ATP Synthase can be divided into two moieties that combine to create a "rotary engine". The F_O moiety is hydrophobic and incorporated into the inner mitochondrial membrane. It is the one that converts the electrochemical energy into torque. The F_1 moiety is hydrophilic, extending form the inner membrane into the matrix and it is responsible for the actual ATP synthesis. These two sub-complexes are connected via perpheral stalk- like proteins (van Ballmoos et al. 2008).

In its most basic form, the prokaryotic F_o subcomplex consists of four subunits termed a, b, c and δ. Each complex has one a, one δ, two b, and several c units. The a and c subunits form the

membrane proton pore, whereas b and δ contribute to the peripheral stalk. The basic composition of the F_1 moiety is comprised of four subunits α, β, γ and ε, where three α and three β units alternate to form a hexamer ring on top of the central stalk that is comprised of the subunits γ and ε. The peripheral stalk attaches via δ to the ring. While the overall structure is very similar in eukaryotes, the nomenclature becomes quite confusing as the peripheral stalk is comprised mainly of four subunits named b, d, F_6 and OSCP. Furthermore, there are two subunits, δ and ε, that bind to γ to

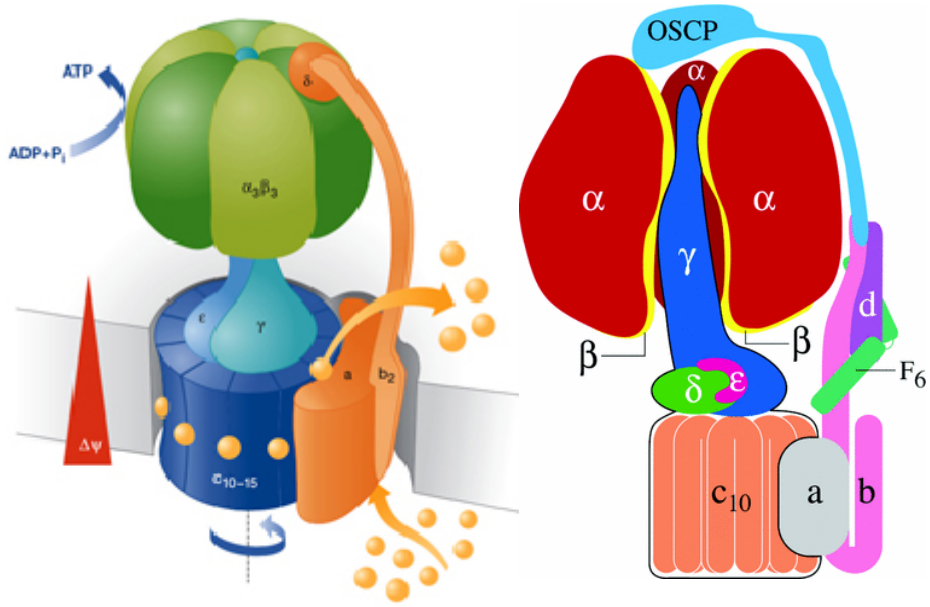

Figure 1.4: Comparing the procaryotic form of the FoF1 -ATP Synthase (left) (Dimroth et al. 2006) with the one of eucaryotes (right) (http://www.bio.davidson.edu/Courses/Molbio/MolStudents/spring2010/Sween/page2.html); accessed . 17.05.13

form the central stalk. To make matters worse, the eukaryotic δ is the homologue of the prokaryotic ε (van Ballmoos et al. 2008) (Stock et al. 1999).

In *T brucei,* only the core components of the ATP synthase are conserved. These include the F_1 subunits α, β, γ, $\delta_{Eucaryotic}$ and $\varepsilon_{Eucaryotic}$ and the F_o subunits OSCP, b and c. Moreover, 14 additional subunits have also been identified. Surprisingly, they have no homologues outside the kinetoplasts. (Ziková et al. 2009) These hypothetical proteins are therefore not present in the human cV or they are so divergent that their homologues are not recognized. Therefore, a drug aiming specifically at these *T. brucei* spefic subunits shouldn't affect human health. Considering this and the reversed functionality of the enzyme complex in the bloodstream *T. brucei,* the F_OF_1 -ATP synthase seems to be a promising putative target for future drugs. However, the functions of those hypothetical proteins need to be determined in order to pinpoint the exact mechanism with which a drug could successfully work.

1.4 Yeast two Hybrid

In order to gain an understanding of the role these hypothetical proteins play, it is a necessity to know how they are arranged within the structural assembly of the whole complex. Yeast two-hybrid, a technique to detect interactions of proteins is hence a valuable tool in this undertaking.

The principle underlying this technique is the regulation of the transcription of an essential *Sacheromyces cervisiae* reporter gene by the interaction of two interacting proteins. This is achieved by fusing one protein of interest (the bait) to the DNA binding domain (BD) of a transcription factor and one protein of interest (the prey) to the corresponding activation domain (AD) of this transcription factor. If the proteins do interact, the parts of the transcription factor domains are assembled and enable transcription. If no interactions occur, the gene can't be transcribed and therefore the yeast can't proliferate.

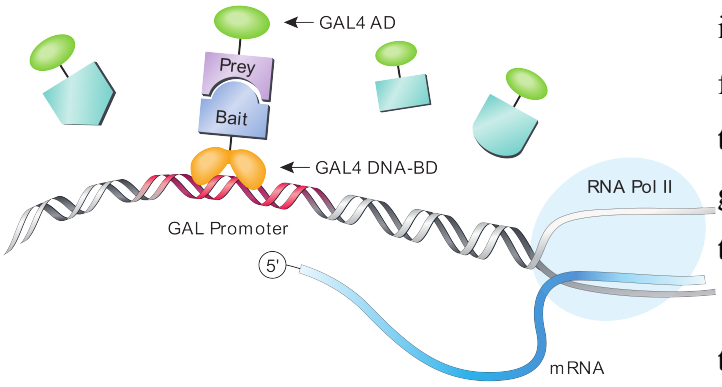

Figure 1.5: Principle of the Yeast two hybrid technique (MatchmakerTM Gold Yeast two Hybrid System; User Manual; Clontech)

In practice, the genes encoding the desired proteins are cloned into special plasmids that are designed to combine the gene with the activation or binding domain. Next, these plasmids are cloned into yeast - the BD vectors into one haploid mating type and the AD vectors into the complement mating type. Upon mating of those two haploids, diploid yeasts are formed, containing both plasmids, one coding for an AD protein and one coding for a BD protein. By growing the yeast on media where they are dependent on the expression of the reporter gene, the interactions can be observed due to growth of the different diploids (Clark, 2005).

Using subunits of the trypanosoman F_OF_1 -ATP Synthase or parts of them in yeast two-hybrid screens can result in valuable information about the composition of this protein complex, which in turn can yield knowledge about the function of the single hypothetical subunits. On the whole, it can reveal a possible target for a future drug to fight HAT exists.

In this Y2H screen the interactions of the F_OF_1 -ATP Synthase subunits β (Tb927.3.1380), γ(Tb927.10.180), δ(Tb927.6.4990), ε(Tb927.10.5050), OSCP(Tb927.10.8030) and ATP 11(Tb09.244.2700), an assembly factor of the F_OF_1 -ATP synthase binding initially to β and ensuring thereby the correct installation of the $\alpha_3\beta_3$ hexamer, (Wang et al. 2001) were examined.

2 Aims

- To clone genes encoding subunits of the *T. brucei* F_OF_1-ATP synthase complex (cV) into suitable yeast two hybrid (Y2H) plasmids.
- To verify protein expression levels of these Y2H plasmids in *Saccharomyces cervisiae.*
- To screen for possible interactions between these *T. brucei* cV subunits using the Y2H system and thereby proving the suitability of this method for *T. brucei* cV proteins.
- To gain insight into the possible function of some *T. brucei* cV subunits through their protein interactions with other known subunits.

3 Methods

3.1 Cloning

The initial step of the project was to insert the genes, which are coding for the individual proteins in the F_OF_1-ATP synthase complex, into the suitable vector plasmids and amplify those constructs. Using different techniques this was achieved. First, the insert DNA had to be amplified to a yield sufficient for assembling it to the plasmid. This was done by PCR (Polymerase Chain Reaction). After purifying the PCR products, they were inserted into the plasmids. To do so we used a special method, which is based on homologous recombination. The plasmids were subsequently transformed into bacteria and plated onto agar plates that had been treated with antibiotics for selection purposes. From some resulting colonies plasmid DNA was extracted and digested with restriction enzymes. By means of the restriction patterns it was determined whether the plasmids were correct or not. Those plasmids which showed the right restriction patterns were submitted for sequencing as a further step in controlling them.

3.1.1 Primer Design

In order to increase the efficiency of the cloning process involving two expression plasmids for six *T. brucei* F_oF_1-ATP synthase subunits, we employed a homologous recombination technique – simplified by a kit from Invitrogen called GENEART® Seamless Cloning and Assembly Kit. This kit is an alternative to the typical sub-cloning techniques involving restriction enzymes and ligases, which can be time-consuming for a high throughput cloning project that is needed for a comprehensive yeast two hybrid analysis. Instead, this molecular biology kit utilizes a commercial enzyme mix that will homologously recombine PCR fragments with the linearized expression

plasmid if both molecules contain an overlapping sequence at their terminus. Therefore, special attention was required when designing the primers for each gene, as the 5'end of the forward and reverse primers needed to contain the last 15nucleotide of the linearized expression plasmid. This 5' plasmid specific sequence was then followed by 18-25 nucleotides of the gene of interest, aiming for a GC content of ~50%. Furthermore, to ensure the proper folding of a mature protein, we eliminated the mitochondrial targeting sequence from each gene when it could be accurately predicted by MitoProtII freeware. This detailed planning enabled the PCR products containing plasmid specific sequences at both ends to be introduced into the expression plasmids in the correct orientation for eachF_OF_1-ATP synthase gene.

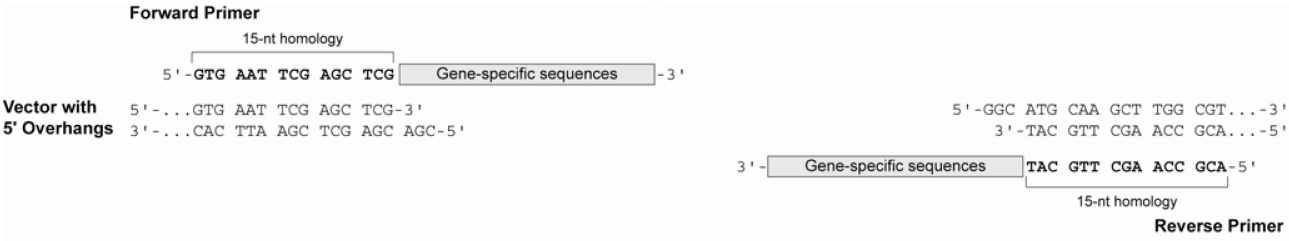

Figure 3.1: Scheme of how the primers are constructed (GENEART® Seamless Cloning and Assembly Kit, Users Manual, Invitrogen)

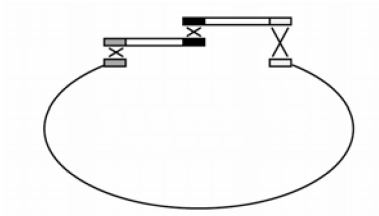

Figure 3.2: Scheme showing the working principle of the GENEART® Seamless Cloning and Assembly Kit ((GENEART® Seamless Cloning and Assembly Kit, Users Manual, Invitrogen)

Table 3.I: List of used primers; Underlined nucleotides belong to the vector

Subunit	Oligo	Forward Primer	GC%	bp	Oligo	Reverse Primer	GC%	bp
Atp11 -mls pGADT7 AD	AZ209	CCAGATTACGCTCATGGACGCAAATCCCTCGGTGAGATT	51	39	AZ210	CGATTCATCTGCAGCCTACTCTTTCCTATATCCATTGC	45	38
Atp11 -mls pGBKT7 BD	AZ211	GAGGAGGACCTGCATGGACGCAAATCCCTCGGTGAGATT	56	39	AZ212	TTATGCGGCCGCTGCCTACTCTTTCCTATATCC	51	33
β –mls pGADT7 AD	AZ213	CCAGATTACGCTCATGCCTCAACTGCTCCTGTT	52	33	AZ214	CGATTCATCTGCAGCTTAGCTACTGGCTTGAGCA	50	34
β –mls pGBKT7 BD	AZ215	GAGGAGGACCTGCATGCCTCAACTGCTCCTGTT	58	33	AZ216	TTATGCGGCCGCTGCTTAGCTACTGGCTTGAGCAA	54	35
δ –mls pGADT7 AD	AZ217	CCAGATTACGCTCATCTTCAATCGGCACCTCATG	50	34	AZ218	CGATTCATCTGCAGCCTAGTGGTGTTTTAGGGCAG	51	35
δ –mls pGBKT7 BD	AZ219	GAGGAGGACCTGCATCTTCAATCGGCACCTCATGA	54	35	AZ220	TTATGCGGCCGCTGCCTAGTGGTGTTTTAGGGCAGCAAT	54	39
ε –mls pGADT7 AD	AZ221	CCAGATTACGCTCATCATGGCATCAGTTATCTC	45	33	AZ222	CGATTCATCTGCAGCTCAGTAATCCTTGGTGTGG	50	34
ε –mls pGBKT7 BD	AZ223	GAGGAGGACCTGCATCATGGCATCAGTTATCTC	52	33	AZ224	TTATGCGGCCGCTGCTCAGTAATCCTTGGTGTGGAT	53	36
γ cds pGADT7 AD	AZ225	CCAGATTACGCTCATATGTCAGGTAAGCTTCGTC	47	34	AZ226	CGATTCATCTGCAGCCTACTTGGTTACTGCCCCTT	51	35
γ cds pGBKT7 BD	AZ227	GAGGAGGACCTGCATATGTCAGGTAAGCTTCGTCT	51	35	AZ228	TTATGCGGCCGCTGCCTACTTGGTTACTGCCCCTT	57	35
OSCP –mls pGADT7 AD	AZ229	CCAGATTACGCTCATGTGGCGGCCCGCTTCTA	59	32	AZ230	CGATTCATCTGCAGCTCAAACGCCATATTTCGCC	50	34
OSCP –mls pGBKT7 BD	AZ231	GAGGAGGACCTGCATGTGGCGGCCCGCTTCTA	66	32	AZ232	TTATGCGGCCGCTGCTCAAACGCCATATTTCGCCT	54	35

3.1.2 PCR (Polymerase Chain Reaction)

With the primers ready, the genes of our interest could be amplified. This was done using the common method of PCR, which harnesses DNA polymerases as copying devices for the production of well defined pieces of DNA.

The reaction mixture of a PCR typically contains special oligonucleotides called primers, the starting points for DNA synthesis on the reverse and forward strand of the template, an excess of desoxyribonucleotides (dNTPs), which will be the building blocks of the newly synthesized DNA strands, template DNA containing the regions to be amplified as well as the heat stable DNA polymerase. Further, buffers ensures the stability of the polymerase. The reaction itself is performed stepwise, where every step requires a different temperature to work. Firstly, in a denaturation step at high temperature the double helix structure of the template DNA is broken up to yield single stranded DNA. Secondly, the primers are annealed to the single stranded template. This happens at lower but still elevated temperature, depending on the primers. And thirdly, the actual synthesis occurs at a temperature suitable for the polymerase to work. Repeating these steps over and over (commonly 25- 30 times) leads to exponential amplification of the DNA of interest as each new DNA strand works as template in the next cycle. (Alberts et al. 2008)

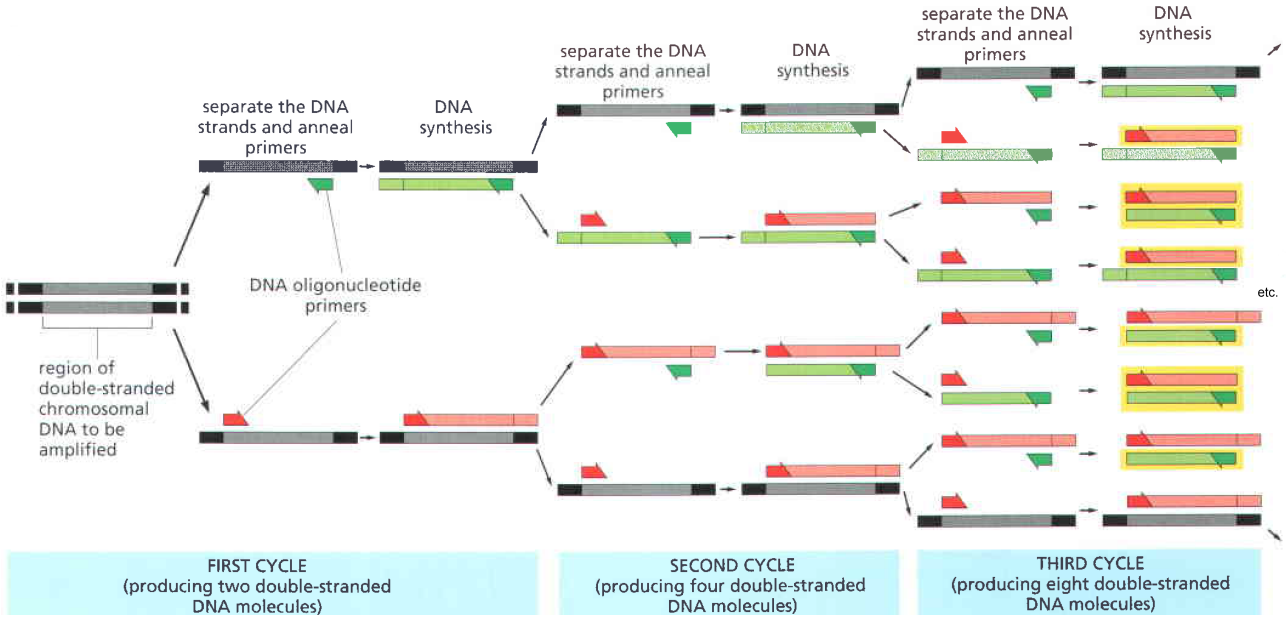

Figure 3.3: Illustration of a PCR reaction (Molecular Biology of The Cell; Fifth Edition; Alberts, Gohnson, Lewis, Raff, Roberts, Walter; page 545)

To enhance the quality of our PCR products the Phusion High Fidelity DNA Polymerase from Finnzymes was used. It produces blunt ends and has a proofreading activity (3'-5' exonuclease). Moreover, it shows a 50 times higher fidelity compared to the usual Taq polymerase.

Following parameters were used for the amplification of our inserts, employing the primers stated in the previous section:

Table 3.II: Composition of one PCR reaction mixture

Reagents	1 Reaction	Final Concentration
18 MΩ MilliQ water	31,5µl	
5x Phusion HF buffer w/$MgCl_2$	10µl	1x
10mM dNTP's	1µl	0,2mM
Forward Primer (10µM)	2,5µl	0,5µM
Reverse Primer (10µM)	2,5µl	0,5µM
BF SM gDNA (100ng/µl)	2µl	200ng
Phusion DNA Polymerase	0,5µl	1U
Total	50µl	

Table 3.III: Temperature program for the PCRs. It was repeated for 30 times (step 1-3).

Temp	Time / sec
98°C	30 sec
98°C	10 sec
72°C	75 sec
72°C	8 min
12°C	infinite

3.1.3 DNA Gel Electrophoresis & Gel Purification

To be able to use the PCR fragments for further experiments, it has to be checked if the reactions worked correctly. Moreover, the raw PCR products have to be cleaned from unwanted contaminants (unused dNTPs, wrong PCR products, the template DNA ...). To achieve this, two techniques were applied to the raw PCR products. Firstly, agarose gel electrophoresis, which separates the PCR fragments from other DNA and lets one check if the reaction had worked. Secondly gel purification using the GenElute™ Gel Extraction Kit from Sigma-Aldrich to free the DNA from further contaminants and the gel matrix.

Agarose gel electrophoresis is a widely used method and employes the fact that DNA molecules migrate along an electric field, due to their negatively charged phosphate backbone. Agarose is a polysaccharide, which forms a three dimensional network upon melting in boiling aqueous solution with subsequent cooling to room temperature. This gelatine-like substance is then used as "sieve", where smaller DNA molecules, driven by the electric field, can migrate through the pores of the agarose gel faster than bigger ones. (Buchberger, 2013)

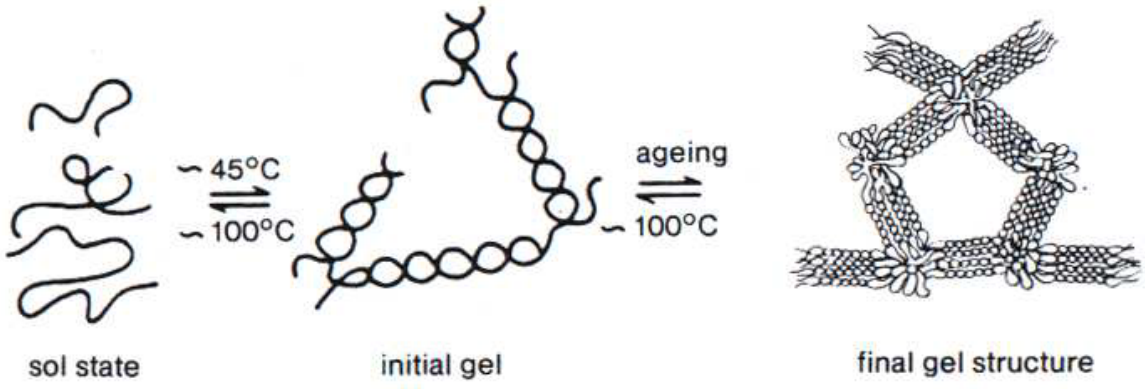

Fig. 25. Gel structure of agarose. (Låås, T. Doctoral thesis. Acta Universitatis Upsaliensis 1975. Reproduced by kind permission of the Author.)

Figure 3.4: Illustration of the formation of Agarose gels (http://students.washington.edu/uwfarm/2011/02/10/food-science-2-0-fragments-of-heredity/ accessed: 13.05.2013)

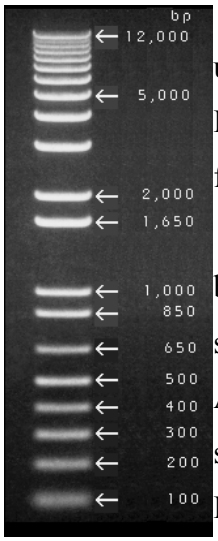

Figure 3.5: 1Kb Plus DNA Ladder; Users Manual; Invitrogen

To visualize the migrated DNA the fluorescent dye ethidium bromide was used. This is a DNA interchelator, accumulating at DNA molecules. The visualized DNA bands were compared to a DNA ladder to check the length of the PCR fragments and therefore the success of the reaction. (Šterba, 2011)

The gels used for our PCR fragments were made of 1% Agarose in 0,5x TAE buffer boiled for about 1,5 minutes in the microwave. 1µl of Ethidium Bromide solution was added to the still liquid gel after cooling it to approximately 60°C. After pouring the gel and inserting the comb, the gel was left for 20 minutes to solidify. Next, the gel was submerged into 0,5x TAE running buffer and ladder (1kb DNA Ladder Plus from Invitrogen see Figure 3.5) as well as the samples (mixed with 5x loading dye) were pipeted into the wells. The electrophoresis was run at 90V for approximately 45 minutes. The time was adjusted for each gel individually. The finished gels were examined under UV light and images were taken.

As next step the DNA had to be purified from the gel and other remaining contaminants.

This was done with the above mentioned kit, that works based on solubilization of the Agarose and subsequent binding of the DNA onto the silica membrane of a spin column tube. The DNA was eluted with 30µL 10mM Tris-HCl buffer of pH 9.
The DNA concentrations of the cleaned samples were determined using the NanoDrop™ from Thermo Scientific, a micro Volume Spectrophotometer.

3.1.4 Insertion of the amplified genes into Vectors and subsequent Transformation

Now that the inserts were amplified and purified they could be combined with the linearized plasmids and transformed into *Escherichia coli,* that would copy the constructs in a sufficient amount for transformation into yeasts.

The plasmids used are specially designed for the expression of a gene of interest fused to either the GAL4 activation (in pGADT7) or binding (in pGBKT7) domain in *S. cervisiae*. This is the main feature that enables us to perform the Y2H analysis (See "Actual Yeast Two Hybrid Analysis"), together with serveral other features. Some of those are resistance genes against antibiotics for selection purposes in *E. coli*, orgins of replication, restricition sites, epitope tags for the binding of commercial antibodies, the T7 promoter and genes for the nutritional selection in yeasts.

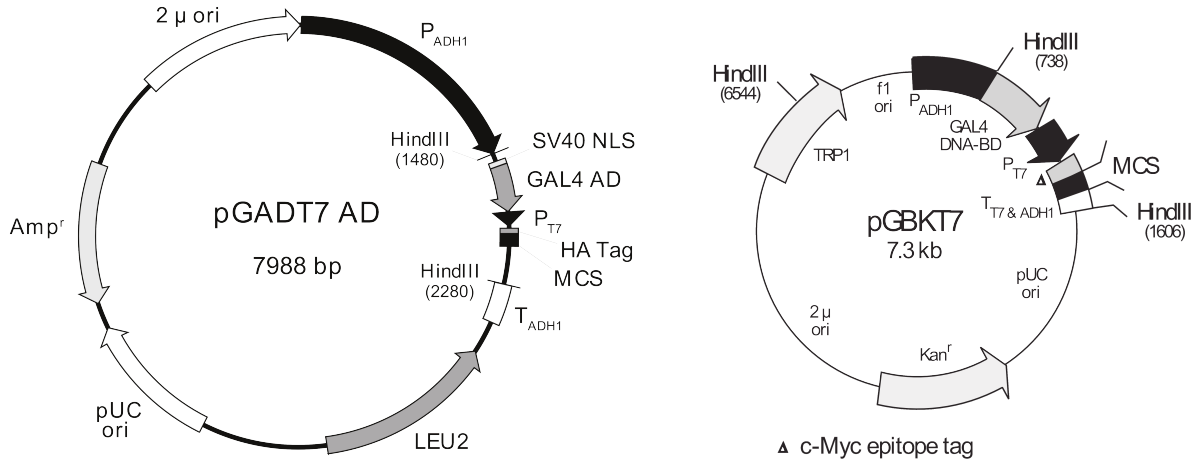

Figure 3.6: Maps of the used plasmids (pGADT7 AD Vector Information and pGBK Vector Information; both Clontech)

As previously stated the technique used for the assembly of the Y2H vectors is based on fusing together the homologous ends of the vector and the insert and was facilitated by the GENEART ® Seamless Cloning and Assembly Kit form Invitrogen, which provides a special enzyme mix and bacteria of high transformation efficiency for this purpose. It also unifies the

vector assembly and the transformation of the plasmids into *E. coli* into one work procedure.
As first step the required amounts of insert were calculated using this formula:

$$m_{insert}[ng] = \frac{2 \cdot bp_{instert} \cdot m_{\text{linearized Plasmid}}[ng]}{bp_{\text{linearized Plasmid}}}$$

Formulae 3.a: Equation used to calculate the required amount of insert DNA (GENEART® Seamless Cloning and Assembly Kit, Users Manual, Invitrogen)

Table 3.IV: Examples for the Amount of Vector and Insert DNA used in the assembly reaction filled up to 14 µl with MiliQ water

Sample	Conc	Size	Total	Vol
pGADT7 AD linearized vector	50ng/µl	7988bp	300ng	2µl
Atp11 –mls pGADT7 PCR GP	33,0ng/µl	732bp	55ng	1,7µl
MilliQ				10,3µl
pGBKT7 BD linearized vector	82,4ng/µl	7300bp	300ng	2µl
Atp11 –mls pGBKT7 PCR GP	10,3ng/µl	732bp	60ng	6µl
MilliQ				6,0µl

Then aqueous mixtures of 14µl volume containing the correct amounts of insert and linearized vector were prepared (See Table 3.IV). To those mixtures 4µl of 5x Reaction buffer and subsequently 2µl of the 10x Enzyme Mix were added. The reaction solutions were then homogenized by gentle tapping. After a 30 minute incubation period at room temperature, the solutions were put on ice and the transformation procedure was started. Of each of the assembly mixtures 8 µl (2,5µl for the positive control) were added to a separate aliquot of One Shot® TOP10 Chemically Competent *E. coli.* The mixtures were homogenized by tapping and subsequently incubated on ice for 30 minutes. Next, the cells were heat-shocked for exactly 30 seconds in a 42°C warm water bath. Immediately after that the cells were put onto ice for 2 minutes. Then 250µl of SOC medium were added and the mixtures were put into a shaking incubator for 1 hour at 37°C. Finally 250 µl of the mixture were plated on LB-plates, with the corresponding antibiotics (Ampicillin for the pGADT7 and Kanomycin for pGBKT7) and incubated over night at 37°C.

3.1.5 Isolating plasmid DNA

Due to the fact that many transformed bacteria incorporate damaged or wrong plasmids, colonies had to be found that contained the correct and intact plasmid, which ensures the success of a Y2H analysis. Therefore,3-5 mL LB cultures containing the correct antibiotics were inoculated with bacteria from the transformation plates. The cultures were incubated in a shaking incubator at 37°C over night. The cells of 3 mL of these cultures were spun down to a pellet and used for plasmid DNA isolation with the GenElute™ HP Plasmid Miniprep Kit. First the cells were lysed by

an SDS (Sodium Dodecyl Sulfate) containing solution. After neutralization of the mixture it was applied to a silica based spin column that bound the plasmid DNA. The column was washed and finally eluted with 50µl of 10mM Tris-HCl Elution Buffer at pH 8,5 (heated to 65°C). The DNA concentration of the Samples was determined with the NanoDrop™.

3.1.6 Restriction digest and subsequent gel electrophoresis to identify correct clones

In order to find out whether the construct was correct the isolated plasmid DNA was treated with restriction endonucleases, which have the ability to cleave DNA strands at specific DNA motifs called restriction sites (Bruce, 2011). Thus one can predict the lenghts of the fragments produced by the restriction digest of a DNA molecule of known sequence with a special restriction enzyme. Due to the unique restriction patterns of the constructs, their correctness could easily be veryfied.

The following table summarizes the expected restriction patterns for the clones:

Table 3.V: Expected restriction fragments of the clones

Construct	Restriction Enzyme	Expected Fragment Lengths / bp
pGADT7 AD	HindIII	800; 7188
Atp11-mls pGADT7 AD	HindIII	1439; 7188
β-mls pGADT7 AD	HindIII	874; 1360; 7188
δ-mls pGADT7 AD	HindIII	1238; 7188
ε-mls pGADT7 AD	HindIII	920; 7188
γ-cds pGADT7 AD	HindIII	502; 1153; 7188
OSCP-mls pGADT7 AD	HindIII	691; 778; 7188
pGBKT7 BD	HindIII	868; 1498; 4938
Atp11-mls pGBKT7 BD	HindIII	1498; 1531; 4938
	XbaI	988; 6979
β-mls pGBKT7 BD	HindIII	928; 1398; 1498; 4938
δ-mls pGBKT7 BD	HindIII	1330; 1498; 4938

ε-mls pGBKT7 BD	HindIII	1012; 1498; 4938
γ-cds pGBKT7 BD	HindIII	556; 1191; 1498; 4938
OSCP-mls pGBKT7 BD	HindIII	745; 816; 1498; 4938

The experimental procedure involved setting up the reaction mixtures, homogenizing and incubating them at 37°C for at least 1,5 hour.
The Reaction mixtures contained following reagents:

Table 3.VI: Composition of typical restriction reactions with HindIII or Xbal

Reagent	**Amount for 1 Reaction**	**Reagent**	**Amount for 1 Reaction**
Plasmid DNA	500-800ng	Plasmid DNA	500-800ng
10x Red Buffer	2µl	10x Tango Buffer	2µl
HindIII	0,2µl (~2U)	Xbal	0,2µl (~2U)
18 MΩ MiliQ water	up to 20µl	18 MΩ MiliQ water	up to 20µl

After incubation the restriction mixtures were mixed with 5x loading dye and loaded onto 1% agarose gels together with the DNA ladder. The gels were made and run according to the procedure stated above. The gels were then analyzed under UV light and images were taken.

3.1.7 Sequencing of correct constructs

To ensure the complete correctness of the clones, which had been selected by restriction analysis, DNA samples of those were submitted for sequencing. There the full insert (and tiny parts of the vector) was sequenced in both directions. The sequencer used for this task was an ABI PRISM 3130xl from Applied Biosystems, which works based on the Sanger sequencing method.
For one reaction following amounts of reagents were submitted:

Table 3.VII: Composition of a sequencing reaction mixture

Reagent	**Amount for 1 Reaction**
Plasmid DNA (75ng μL^{-1})	150ng (2µl)
T7 forward primer (1 µM) or; pGADT7 or pGBKT7 reverse primer (1µM)	2,5µl
18 MΩ MiliQ water	3µl

The sequencing data were then aligned to the corresponding in silico created constructs using the Geneiuos software. From those alignments error files were created, which contained all the deviations of the sequenced data from the virtual data.

3.2 Transformation into Yeast

The next crucial step in the project was to insert the finished plasmid constructs from *E. coli* into *S. cervisiae*. To do so, enough DNA from *E. coli* had to be isolated. The yeasts were made ready for transformation by treating the cells with Lithium acetate, which permeabilizes their cell walls. The transformation was then accomplished by heat shocking at 42°C.

3.2.1 Producing sufficient amounts of plasmid DNA for the transformation

Plasmid DNA isolation was achieved using the GenElute™ HP Plasmid Midiprep Kit, which is a scale up of the previously mentioned Miniprep Kit working in the same way. For those purposes 50 mL of LB over night culture (37°C shaking incubator) were harvested and treated according to the manual. In contrast to the minipreps, the final eluate (1 mL Elution Solution) was concentrated in a further step, which involved precipitation of the DNA with Sodium Acetate and Isopropanol. After that the DNA pellet was resuspended in 100 µL Elution buffer, which corresponds to a ten times concentration.

3.2.2 Handling yeasts

Now that enough DNA was available, the yeasts had to be prepared. Two yeast strains were used, PJ69-4A and PJ69-4α. Those haploid strains are identical except the mating type and the ade2 mutation in PJ69-4α,which results in the production of a red pigment. This is useful to disinguish the two strains as white colonies indicate the A mating type and red ones the α mating type. Generally, yeasts have to be treated a bit different from the common *E. coli*. They have to be incubated at 30°C and grow quite slowly compared to bacteria. An agar plate for instance has to grow at least 2-3 days to yield usable colonies.

They also require differently composed media. There is a rich medium, YPD (Yeast Peptone Dextrose) consisting of 1% Yeast Extract, 2% Tryptone and 2% Glucose (2% Bactoagar are added if plates are desired). It is used to grow up the untransformed yeast strains. Further there are synthetically defined (SD) media, which are used for growing yeasts under nutritional selection. They consist of 0,685% Yeast nitrogen base; 2 % Glucose; 1x Dropout media (composition in Table 3.7); 0,05 mg mL^{-1} Adenine and a combination of 0,1 mg mL^{-1} Histidine, 0,16 mg mL^{-1} Tryptophan and 0,2 mg mL^{-1} Leucine(2% Bactoagar are added if plates are desired). The combination (e.g.

Leucine and Histidine without Tryptophan) is dependent on which yeast strain with which plasmid should be selected.

Table 3.VIII: Composition of the 10x Dropout Media

10x Dropout Media	
Reagent	**Amount**
Arginine HCl	100mg
Isoleucine	150mg
Lysine HCl	150mg
Methionine	100mg
Phenylalanine	250mg
Threonine	1000mg
Tyrosine	150mg
Uracil	100mg
Valine	750mg
18 MΩ MiliQ water up to	500ml

In liquid cultures there is the possibility that the yeasts settle down at the bottom of the vessel, which accompanied in a slow down in growth. This can be avoided in most cases by tilting the culturing vessels during incubation.

3.2.3 The actual transformation procedure

For the transformation of the plasmids 1 mL of YPD liquid medium inoculated with one big yeast colony. The solution was vortexed for 1 minute to disperse the cells and then added to further 4 mL of YPD. This was done for each Yeast strain and the mixtures were then put into the shaking incubator at 225 rpm and 30°C over night.

On the next day the over night cultures each were added to 25 mL fresh YPD and incubated about 4 hours more till the optical density at 600 nm (OD_{600}) reached approximately 0,6. Subsequently, the yeast cells were pelleted at 2000g for 5 minutes with a centrifuge. After decanting the supernatant, the cells were washed once with 25 mL of autoclaved water (18 MΩ MiliQ) and pelleted again under the same conditions. The pellets were then resuspended in 0,75 mL 0,1M Lithium acetate/TE 7,5 buffer (0,1M Lithium acetate; 10mM Tris-HCl; 1mM EDTA; at pH 7,5) by pipetting up and down. The resuspended yeasts were then pelleted again in a micro centrifuge for 30 seconds at 7000 rpm (~3000g). After discarding the supernatant, the pellets were resuspended again in 1mL of the 0,1M Lithium acetate /TE 7,5 buffer.

To 50µL of this yeast suspension 1µg of the desired construct DNA was added. The clones with the pGADT7 palsmid were transformed into PJ69-4A and pGBKT7 clones were transformed into PJ69-4α. To these mixtures 250µL of 40%PEG 3350/ 0,1M Lithium acetate /TE 7,5 buffer (40%

Polyethylene Glycol 3350; 0,1M Lithium acetate; 10mM Tris-HCl; 1mM EDTA; at pH 7,5) were added. Next they were incubated for 30 minutes at 30°C and after that heat shocked for 25 minutes at 42°C in the heating block. The heat shocking was followed by pelleting all the different transformed cells at 7000 rpm (~3000g) for 30 seconds. The supernatant was dicarded and the pellet resuspended in 0,5 mL the autoclaved water. After another pelleting step at the same conditions the pellet was again resuspended in 0,1mL of auroclaved water. The whole suspension was then plated onto the corresponding SD (synthetic defined) selection plates, lacking one plasmid specific amino acid. The pGADT7 vector had a gene enabling the production of Leucine, so the yeasts with these plasmids grew on SD plates without Leucine (SD -Leu) and the pGBKT7 vector had a gene enabling the production of Tryptophan so the yeasts with this one grew on plates without this amino acid (SD -Trp). The plates were incubated at 30°C for 2 days.

3.3 Protein isolation

The following step was to verify the expression of the proteins, encoded by the plasmids, and therefore the success of the transformations. To do so we exploited the fact that in addition to the GAL4 activation or binding domain special epitope tags, recognized by commercial antibodies were fused to the proteins produced by the plasmids. Due to this the expressed proteins could be visualized by western blotting. Preliminarily, those proteins had to be grown and prepared for western blotting as described in the next two sections.

3.3.1 Growing and Harvesting the yeast cells

Initially, one yeast colony of each transformation was used to inoculate 5 mL of the corresponding SD media (SD -Leu for PJ69-4A yeasts containing pGADT7 and SD -Trp for PJ69-4α containing pGBKT7) in the same way as described above. These cultres were grown overnight at 30°C in the shaking incubator. Subsequently the cultures were vortexed to disperse any cell lumps and each of them was added to a separate 50 mL batch of YPD. Those cultures then were incubated further till their OD_{600} reached 0,4-0,6.

The following steps were all performed at low temperatures or on ice in order to inactivate proteases and therefore avoid loss of sample by proteolysis. As the cultures reached the desired optical density they were poured into centrifugation tubes half filled with ice and centrifuged at 1000g and 4°C for 5 minutes. The supernatatnt was decanted and the pellets were resuspended each in 50 mL deionized, ice cold water. Next, they were centrifuged again under the same conditions

and the supernatant was decanted so that as little water as possible stayed in the tube. The pellets were shock frozen in liquid nitrogen for 30 seconds and stored at -80°C for further use.

3.3.2 Preparing the yeast protein extracts

With the yeasts harvested the proteins had to be extracted. For this a method employing an urea cracking buffer and glass beads to break up the robust cell walls of yeasts was used. The composition of the cracking buffer is stated in following tables:

Table 3.IX: : Composition of the Cracking buffer stock solution

Cracking buffer Stock solution		
Reagent	**Final concentration**	**Amounts for 100 mL**
Urea	8M	48 g
SDS	5 % w V^{-1}	5 g
Tris-HCl (pH 6,8)	40 mM	4 mL of 1 M stock sol.
EDTA	0,1 mM	20 µL of 0,5M stock sol.
Bromophenol blue	0,4 mg mL^{-1}	40 mg
18 MΩ MiliQ water up to		100 mL

Table 3.X: Composition of the 10x protease Inhibitor solution.T he cOmplete ULTRA, Mini, EDTA-free protease inhibitor tablets from Roche were used. The protease inhibitors protected the expressed proteins from proteolysis by Aspartate Cysteine and Serine proteases. However, metalloproteases weren't inhibited by those tablets.

10x protease Inhibitors	
Reagent	**Amount**
Protease inhibitors	1 Tablet
40 mM Tris-HCl (pH 6,8)	1 mL

Table 3.XI: Composition of 1,5 mL of complete cracking buffer, which had to prepared just before the experiment

Complete Cracking buffer	
Reagent	**Amounts for 1,5 mL**
Cracking buffer Stock solution	1,335 mL
***β*-mercapto ethanol**	15µL
10x protease Inhibitos	150µL

After the preparation of the stock solutions, the amounts of final cracking buffer and glass beads for each individual cell pellet was calculated according to following formulas.

$$OD_{600}\,Units = V_{\text{final of culture}}[mL] \cdot OD_{600\text{ - final}}$$

$$V_{\text{Cracking buffer}}[\mu L] = \frac{OD_{600}\,Units}{7{,}5} \cdot 100\mu\text{L}$$

$$V_{\text{Glass beads}}[\mu L] = \frac{OD_{600}\,Units}{7{,}5} \cdot 80\mu\text{L}$$

100 µL Glass beads = 150 mg Glass beads

$$m_{\text{Glass beads}}[mg] = \frac{V_{\text{Glass beads}}[\mu L]}{100\,\mu L} \cdot 150\,mg$$

Formulae 3.b: Formulae and relations for the calculations of the amounts of complete cracking buffer and glass beads

According to the calculations the appropriate amount of complete cracking buffer was prepared and heated to 60°C. Next the frozen cell pellets were thawed with the corresponding volume of hot cracking buffer. Those cracking buffer cell suspensions were then transfered to 1,5 mL centrifuge tubes, that contained the correct amount of glass beads. These mixtures were then incubated at 70°C for ten minutes. After that the tubes were vortexed for 1 minute. Subsequently, they were centrifuged at 14000 rpm and 4°C for 5 minutes. The supernatants were pipeted into fresh 1,5 mL centrifuge tubes, which were kept on ice. The pellets were mixed with 100 µL of additional complete cracking buffer, and incubated in a boiling water bath for 5 minutes. After one minute of vortexing and subsequent pelleting at the above stated conditions the supernatants were combined with the corresponding previous ones. The so gained protein extracts were stored at -80°C for further use.

3.4 Western Blotting

The yeast protein extract contained not only the desired proteins, but also all kinds of proteins from yeast metabolism. As the interest was just upon the artificially produced proteins, those had to be sorted out and selectively visualized.

Firstly, the proteins were separated according to size using gel electrophoresis. Secondly the separated proteins were transferred to a PVDF (polyvinylidene difluoride) membrane with the technique of electroblotting. Thirdly the membrane was blocked for contamining proteins and treated with antibodies that specifically bound to epitope tags fused to the expressed proteins(HA tag fused to the pGADT7 proteins and c-Myc tag fused to the pGBKT7 proteins). With the help of a secondary antibody that was linked to the enzyme horseradish peroxidase and bound the first (the primary) antibody, the protein bands were visualized by a chemoluminescent reaction involving this enzyme. (Bruce, 2011)

3.4.1 SDS - PAGE (Polyacrylamide Gel electrophoresis) and Electroblotting

It is one of the most common techniques to separate proteins: SDS - PAGE. As agarose gel electrophoresis it is based on the migration of charged ions in an electric field through a gel matrix (In this case a polyacrylamid gel), which separates different molecules. Unlike DNA, protein molecules have no uniform charge distribution. This and the complex structural arrangements of proteins, would disturb the electrophoretic separation according to size. It is avoided by treating the samples with SDS (Sodium Dodecyl Sulphate), which destroys the tertiary and secondary structure of the proteins and creates a uniform charge distribution by binding to the protein molecules at a constant ratio (1,4g SDS per 1g of protein). Furthermore, β-mercaptoethanol reduces the disulfid bonds between cysteine residues because just treatment with SDS leaves them intact. Additional boiling of the sample facilitates denaturation of the proteins.
The gel in this kind of elecrtophoresis is made of a network of polymerized acrylamide and bisacrylamide molecules in a suitable buffer system. Depending on the concentration of the acrylamide in the gel the separation range varies due to the change of pore size at different concentrations.

A normal gel consists of two zones the resolving gel of a high concentration of acrylamide where the actual separation occurs and the stacking gel on top of the resolving gel, which is of lower concentration (5%) with a different buffer. (Šterba, 2011)

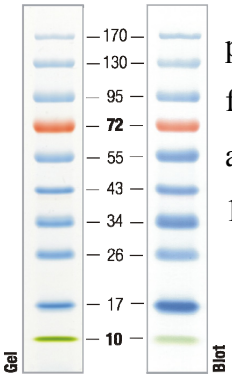

Figure 3.7: PageRulerTM Prestained Protein Ladder; Users Manual; Fermentas – Life Sciences

For the separation of the yeast protein extracts 12% polyacrylamide gels were poured. 5µL of prestained protein marker (PageRuler™ Prestained Protein Ladder from Fermentas – Life Sciences; 170 -10 kDa) and 40µL of each sample were applied to the wells of the gel. The electrophoresis was run for about 90 minutes at 120V.

After the electrophoresis the separated proteins were transferred to PVDF

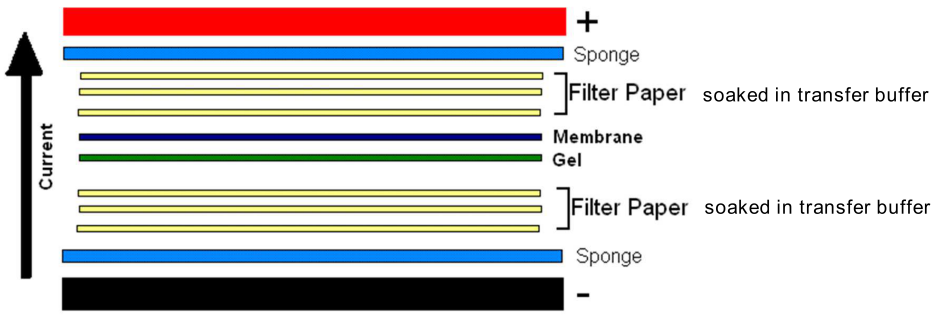

Figure 3.8: Scheme illustrating an electrobloting apparatus (Instrumental Analysis- Lecture Script: Electrophoresis; W. Buchberger; 2012)

membranes in a standard electroblotting procedure. Generally, electroblotting works based on the

same principles as SDS-PAGE. The main difference is, however, that the electric field is applied perpendicular to the direction of the electrophoresis. Due to this the proteins migrate out of the gel onto the membrane, which is placed directly next to the gel. They bind to the membrane and can then be used on the membranes for further experiments. (Šterba, 2011)
For the transfer of the yeast proteins, the PVDF membranes were equilibrated in Methanol washed with water and immersed into the transfer buffer. Next, the apparatus was assembled avoiding air bubbles and the blotting was run for 90 minutes at 90V.

3.4.2 Applying antibodies and probing the membranes

Immediately after electroblotting the membranes were blocked over night with 5% dry milk in PBS-T (Phosphate buffered saline with 0,05% tween). The proteins of the dry milk bind to all the membrane area, where no proteins were bound before. This is done to avoid contamination by the antibodies in further steps. Next, the primary antibodies, which bound the epitope tags of the expressed proteins were applied. To do so 1:1000 dilutions of the corresponding antibody solution (for pGADT7 proteins rabbit anti HA and for pGBKT7 proteins rabbit anti c-Myc) in 5 mL milk solution (5µL of 0,5µg μL^{-1} antibody solution) were poured to the membranes and the tubes containing the membranes were rolled over for one hour. Subsequently, the membranes were washed with 25 mL PBS-T; once for 15 minutes and twice for 5 minutes. As next step, the secondary antibody which was linked to horseradish peroxidase was applied analogously to the above stated procedure. The antibody was goat anti rabbit for both membranes.

Finally the protein bands were visualized by applying the 1:1 mixed Pierce ECL (Enhanced Chemoluminescence) reagents from Thermo Scientific, which consisted of a Peroxide buffer and a Luminol/Enhancer solution. Images of the chemoluminescence were taken with the Fuji Film LAS-3000 Imaging device.

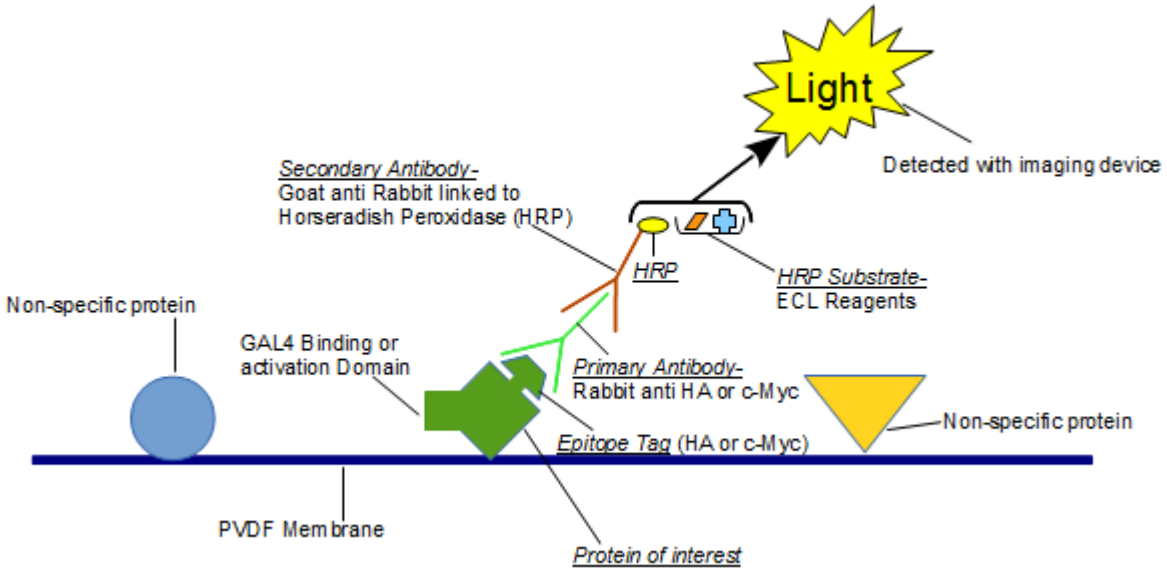

Figure 3.9: Scheme illustrating a western blot (Matthias Guggenberger; 2013 on the basis of Molecular Station 2006)

3.5 Actual Yeast Two Hybrid analysis

Now that the correct plasmid constructs were successfully transformed into the corresponding haploid Yeast strains, the actual Yeast two Hybrid screen for putative protein interactions in the selected subunits of the trypanosoman FoF1 ATP-Synthase complex could be performed. By mating the two yeast strains, PJ69-4A and PJ69-4α, the plasmids coding for the proteins fused to the GAL4 binding domain and the plasmids coding for the proteins fused to the GAL4 activation domain were both "transferred" into diploid Yeast cells. That means that a mated diploid Yeast cell carried one pGADT7 construct and one pGBK construct. Therefore those mated yeasts grew on SD-plates lacking leucine and tryptophan as they could transcribe both genes for producing those amino acids. Moreover, the proteins fused to the GAL4 activation and binding domain were produced. If now those proteins showed some interaction the Activation and Binding domain came into proximity and functioned as if the were one molecule activating the transcription of a reporter gene (HIS3). This transcription of the reporter gene allowed the yeasts to produce histidine so that they could grow on media also lacking this amino acid. If now growth was observed on such media (SD-Leu-Trp-His) one could assume binding interactions between the two proteins, the plasmids coded for.

3.5.1 Mating the Yeasts

The procedure for mating the haploid yeasts was following: In a 96 well microtiter plate 160µL YPDA (Normal YPD media with 0,003% Adenine Hemisulfate added) were pipeted to each

used well. Each PJ69-4A Yeast strain was mated with each PJ69-4α Yeast strain. Additionally, two strains containing the control constructs SV40 (in PJ69-4A) and p53 (in PJ69-4α) were mated. Dispersions of all the strains were prepared by vortexing a transformed colony (younger than 2 months) of the desired plasmid with 1 mL YPDA for 1 minute. 20µL of this solutions were pipetted to the corresponding wells, so that in the end one well contained 20 µL of a PJ69-4A dispersion and 20 µL of a PJ69-4α disprsion and the 160 µL of initial YPDA.
The plate was then incubated over night in a shaking incubator at 200 rpm. After that, the mating dispersions were checked under the microscope (40x objective) for diploid cells, which have a "Mickey Mouse" like shape. Next, the cultures were incubated for 4 more hours and then 100 µL of each culture was plated on SD -Leu-Trp plates. These plates were incubated at 30°C for 3 days.

Table 3.XII: Showing all the matings which were done. SV40 and pGADT7 in PJ69-4A Yeasts and p53 and pGBKT7 in PJ69-4α Yeasts

Samples	*pGBKT7*	*pGBKT7-ATP11*	*pGBKT7-β*	*pGBKT7-δ*	*pGBKT7-ε*	*pGBKT7-γ*	*pGBKT7-OSCP*
pGADT7	pGBKT7 + pGADT7	pGBKT7-ATP11 + pGADT7	pGBKT7-β + pGADT7	pGBKT7-δ + pGADT7	pGBKT7-ε + pGADT7	pGBKT7-γ + pGADT7	pGBKT7-OSCP + pGADT7
pGADT7-ATP11	pGBKT7 + pGADT7-ATP11	pGBKT7-ATP11 + pGADT7-ATP11	pGBKT7-β + pGADT7-ATP11	pGBKT7-δ + pGADT7-ATP11	pGBKT7-ε + pGADT7-ATP11	pGBKT7-γ + pGADT7-ATP11	pGBKT7-OSCP + pGADT7-ATP11
pGADT7-β	pGBKT7 + pGADT7-β	pGBKT7-ATP11 + pGADT7-β	pGBKT7-β + pGADT7-β	pGBKT7-δ + pGADT7-β	pGBKT7-ε + pGADT7-β	pGBKT7-γ + pGADT7-β	pGBKT7-OSCP + pGADT7-β
pGADT7-δ	pGBKT7 + pGADT7-δ	pGBKT7-ATP11 + pGADT7-δ	pGBKT7-β + pGADT7-δ	pGBKT7-δ + pGADT7-δ	pGBKT7-ε + pGADT7-δ	pGBKT7-γ + pGADT7-δ	pGBKT7-OSCP + pGADT7-δ
pGADT7-ε	pGBKT7 + pGADT7-ε	pGBKT7-ATP11 + pGADT7-ε	pGBKT7-β + pGADT7-ε	pGBKT7-δ + pGADT7-ε	pGBKT7-ε + pGADT7-ε	pGBKT7-γ + pGADT7-ε	pGBKT7-OSCP + pGADT7-ε
pGADT7-γ	pGBKT7 + pGADT7-γ	pGBKT7-ATP11 + pGADT7-γ	pGBKT7-β + pGADT7-γ	pGBKT7-δ + pGADT7-γ	pGBKT7-ε + pGADT7-γ	pGBKT7-γ + pGADT7-γ	pGBKT7-OSCP + pGADT7-γ
pGADT7-OSCP	pGBKT7 + pGADT7-OSCP	pGBKT7-ATP11 + pGADT7-OSCP	pGBKT7-β + pGADT7-OSCP	pGBKT7-δ + pGADT7-OSCP	pGBKT7-ε + pGADT7-OSCP	pGBKT7-γ + pGADT7-OSCP	pGBKT7-OSCP + pGADT7-OSCP
SV40	pGBKT7 + SV40	SV40 + p53					
Controls		*p53*					

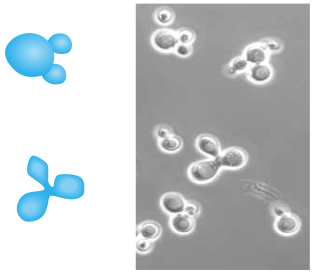

Figure 3.10: Diploid Yeast cells in theory and under the microscope (MatchmakerTM Gold Yeast two Hybrid System; User Manual; Clontech)

3.5.2 Growth on triple dropout media

As soon as the mated Diploids were grown to nicely visible colonies, four of them per mating were restreacked onto SD-Leu-Trp-His plates. On each quarter of a plate one colony was streaked. The plates were incubated for three days at 30°C. After that, the growth of the yeasts, indicating the interactions of the expressed trypanosoman proteins was analyzed by rating the growth empirically on a scale from 0 to 4.

4 Results

4.1 Cloning

Since the T. brucei ATP synthase contains up to 22 subunits, the method of yeast two-hybrid analysis will require a significant amount of cloning. Therefore, we decided to use the GENEART® Seamless Cloning and Assembly system that allows the direct homologous recombination of the PCR products with the vectors of interest. This requires that the primers include 15 nucleotides from the 5'and 3'ends of the vector for homologous recombination to be efficient. While this added some cost into the synthesis of these longer primers, it didn't affect the efficiency of the PCR reaction to any noticeable effect as we were able to generate the desired PCR products without too much troubleshooting of the proper annealing temperature. The PCR products for each subunit analyzed by agarose gel electrophoresis. The following gels were obtained, showing bands of the amplified DNA fragments at their expected size and in sufficient amounts for downstream cloning:

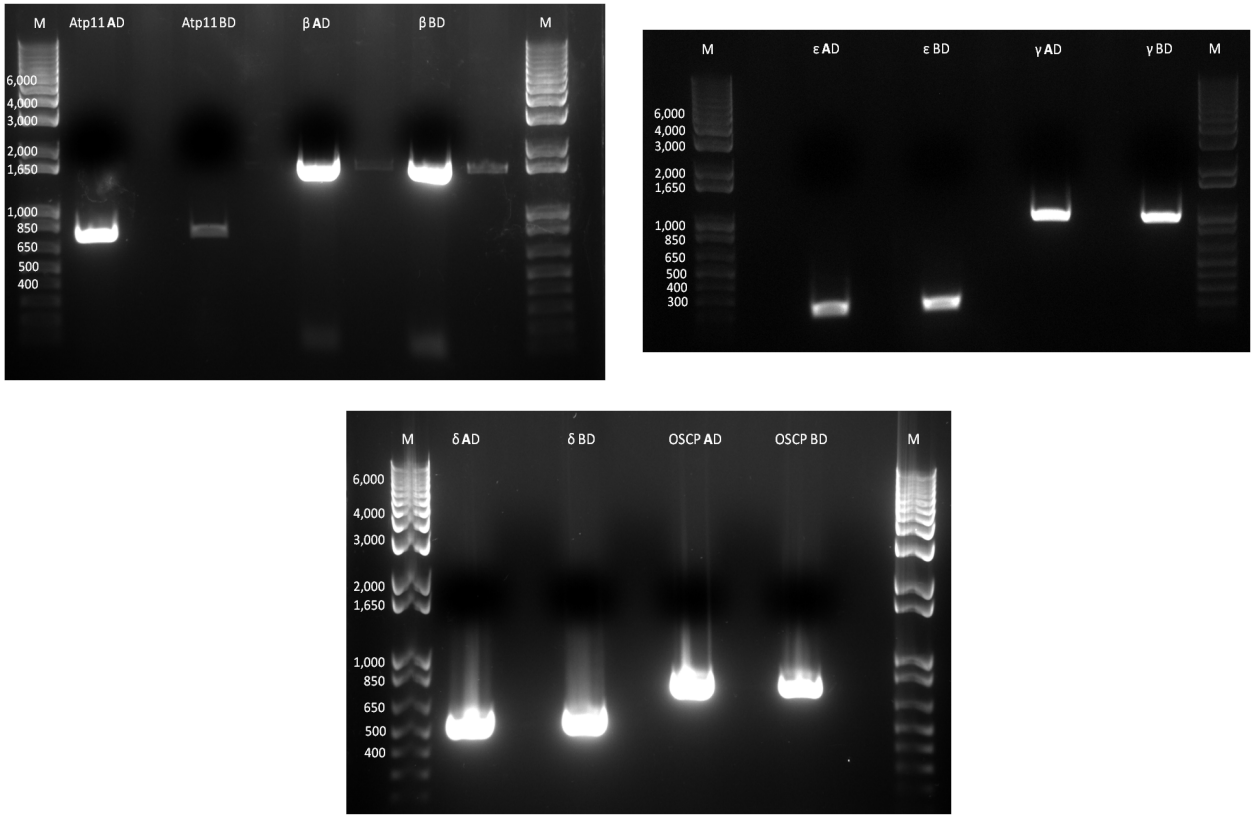

Figure 4.1: Gels showing the inserts of all used subunits, amplified with the primers for both plasmids

The amplified DNA bands were gel purified and ligaated into the corresponding vectors before being transformed into *E. coli* as stated in the Methods. In order to check which transformed colonies carried the desired plasmid with the correct insertion fragment, the plasmid DNA was extracted from a small culture of each colony and analyzed by restriction digest with subsequent agarose gel electrophoresis. The following gels were obtained showing the different restriction digests. The lanes highlighted in green refer to plasmids that produced the expected banding pattern, indicating that the bacterial clones contained the correct plasmid. The gels indicate that the cloning method from GENEART® Seamless Cloning and Assembly kit was fairly efficient as most pcr fragments were integrated at a high frequency.

pGADT7 constructs

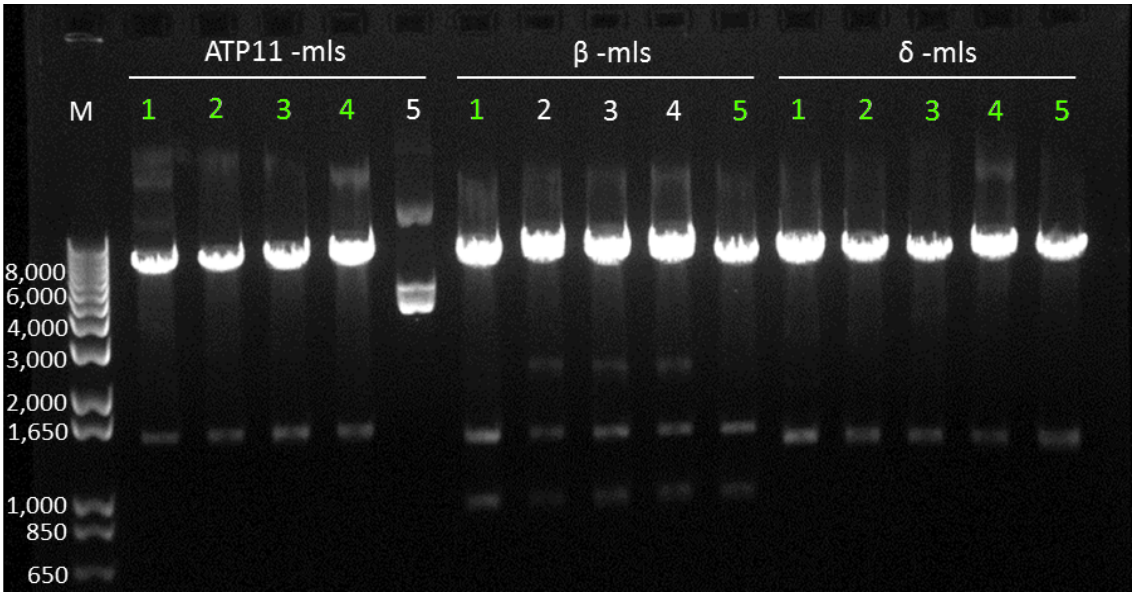

Figure 4.2: Agarose gel of the restriction digest of the ATP 11, β and δ pGADT7 constructs

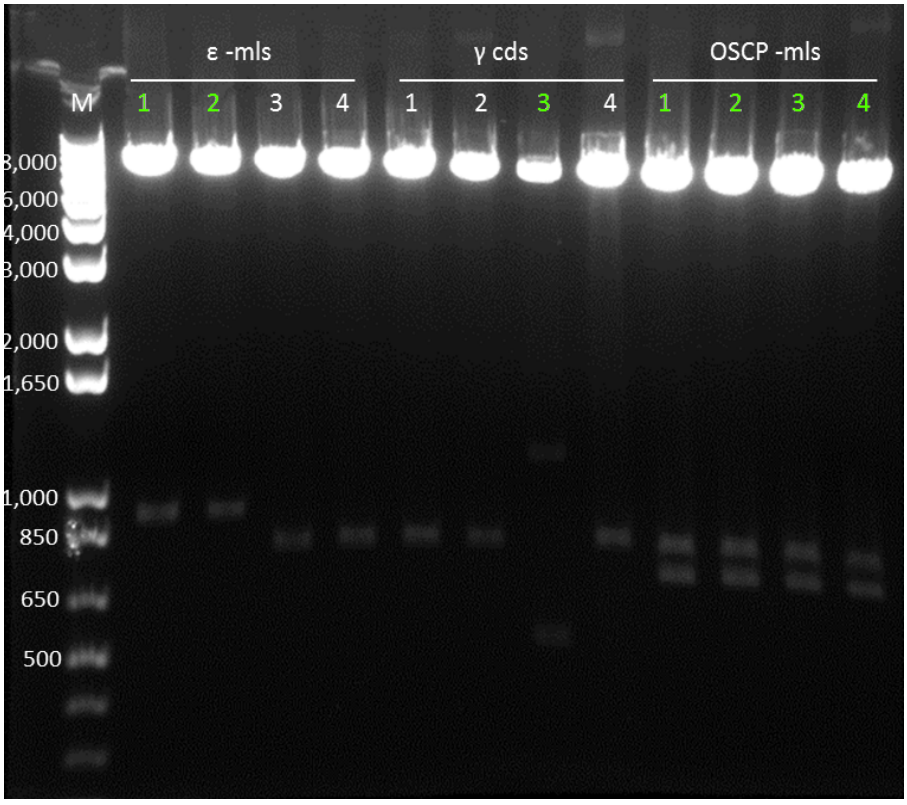

Figure 4.3: Agarose gel of the restriction digest of the ε, γ and OSCP pGADT7 constructs

pGBKT7 constructs

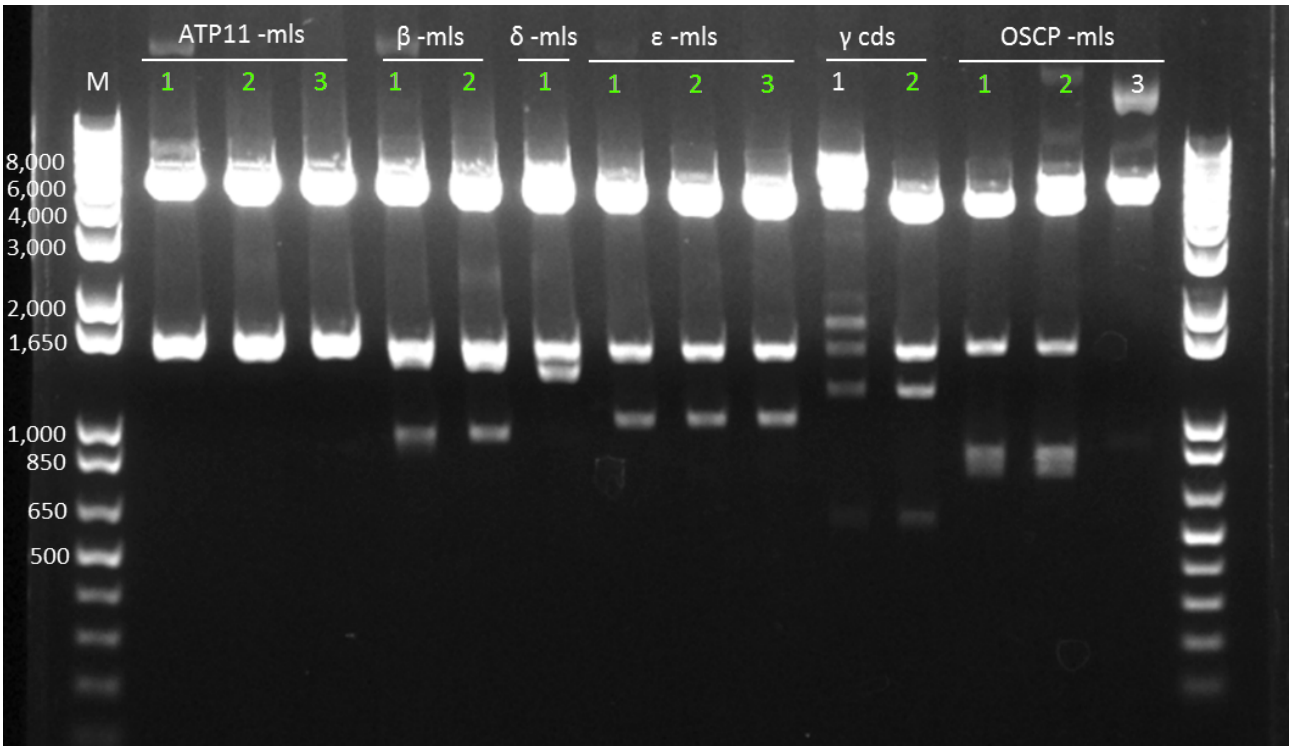

Figure 4.4: Agarose gel of the restriction digest of all pGBKT7 constructs

From the correct bacterial clones one plasmid was submitted for sequencing using vector and insert specific primers to make sure the amplified DNA fragment didn't contain any errors that would disrupt the predicted protein. The sequencing files were then aligned to the *in silico* generated constructs in Geneious. The alignments were then examined for discrepancies and every possible error was noted. This yielded an error report for each construct. An example of those reports is displayed in following table. It shows the discrepancies that were identified in the sequence of the ATP11-mls pGBKT7 BD construct:

Table 4.1: The error report of the ATP11-mls pGBKT7 BD construct. Red script indicates strain specificties.

Nt#	Nt Change	Codon	AA Change	Comments
5396 rev.	T→-	TTT→TT-	-	The software couldn't read this part of the chromatogram and left it blank. It is one nucleotide in front of the T7 RNA Polymerase promotor
5903 rev. + fwd.	T→A	CCT→CCA	conserved	The codon change in the ATP11-mls cds has no effects on translation→ may be strain specific.
5946 rev. + fwd.	A→G	ACC→GCC	Thr→Ala	Change from a polar to a hydrophobic AA in the ATP11-mls cds → may be strain specific
5975 rev. + fwd.	G→T	CGG→CGT	conserved	The codon change in the ATP11-mls cds has no effects on translation→ may be strain specific.
6343 fwd.	G→C	-	-	The chromatogram shows a part of an A peak and a part of a G peak (no C peak at all) in the ADH1 terminator→ software confused.
6352 fwd.	A→-	-	-	The software couldn't read this part of the chromatogram and left it blank. Location: ADH1 terminator
6358 fwd.	A →-	-	-	The software couldn't read this part of the chromatogram and left it blank. Location: ADH1 terminator
6381 fwd.	T→C	-	-	This Change is one nt before the trimmed region and it seems that the peaks on this site are kind of shifted.

Each of these discrepencies were then cross-checked by returning to the original abi chromatogram files to determine if the software had made a mistake during the assignment of a peak to a specific nucleotide. After this extensive analysis, we determined that most of the discrepencies were mostly due to software errors in regions of poor sequencing. Most of the remaining nucleotide changes occurred in the third wobble position, resulting in conserved changes in the coded amino acids. This is mostly likely due to the fact that the *in silico* DNA sequences were generated from a different *T. brucei* strain than the one used in the lab. In the rare instances where this change in the wobble position resulted in an amino acid with different properties, it was confirmed in the sequencing of both yeast two-hybrid plasmids, meaning that the same change occurred in two independent PCR

reactions and that this is also likely due to strain differences. Most importantly, there were no nonsense mutations created from any of these discrepencies.

4.2 Western Blotting

In order to verify expression of the proteins from the plasmid constructs in the transformed yeast strains, a western blot was carried out with the protein extract of those strains. The following blots probed with rabbit anti c-Myc antibody for the pGBKT7 vector proteins and rabbit anti HA antibody for the pGADT7 proteins were obtained:

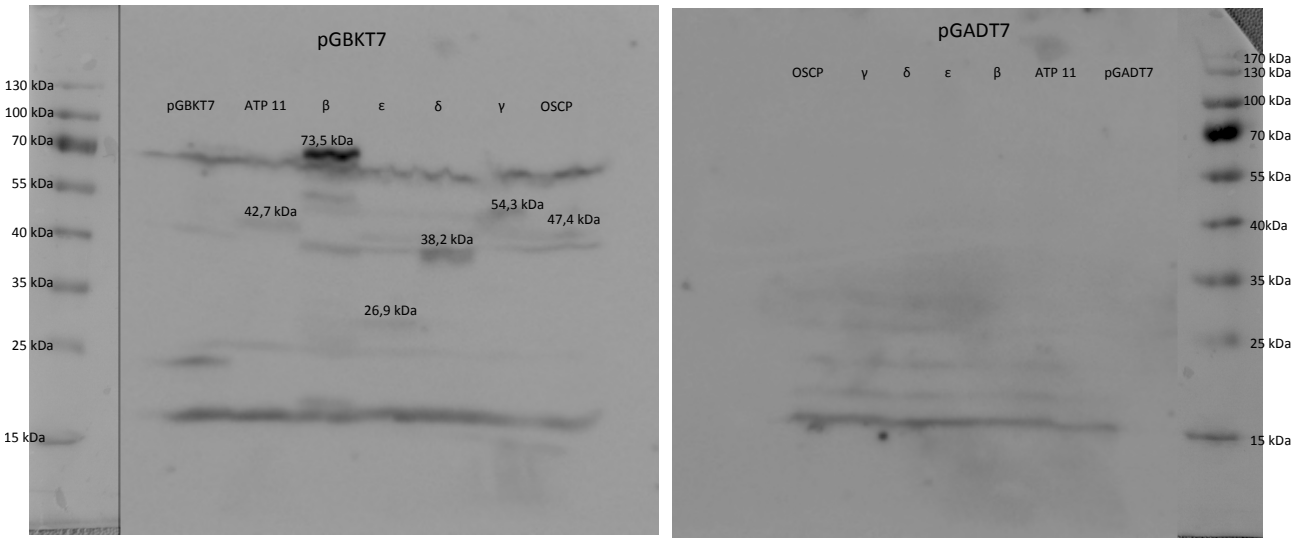

Figure 4.5: Western blots of the proteins from the yeast protein extract

While the blot of the proteins expressed in the pGBKT7 vector has some faint background issues, the bands migrating at the expected size are detected for each sample(See Table 4.I). However, the blot of the pGADT7 proteins was much less successful as no bands are detectable at the expected sizes. This blot was repeated with the same prepared yeast lysates, but it showed no different outcome and further investigation wasn't done due to time restrictions.

Table 4.II: Predicted masses of the expressed proteins

Fusion Protein	Predicted Mass
ATP-11 –mls pGAD	44.8 kDa
β –mls pGAD	71.2 kDa
δ –mls pGAD	35.9 kDa
ε –mls pGAD	24.5 kDa
γ cds pGAD	52.0 kDa
OSCP –mls pGAD	45.1 kDa
ATP-11 –mls pGBK	47.2 kDa
β –mls pGBK	73.5 kDa
δ –mls pGBK	38.2 kDa
ε –mls pGBK	26.9 kDa
γ cds pGBK	54.3 kDa
OSCP –mls pGBK	47.4 kDa

4.3 Yeast two Hybrid

After the mating process, the diploid yeast strains were finally streaked onto the triple knockout plates which lacked leucine tryptophane and histidine. The latter amino acid can only be synthesized by *S. cervisiae* if the Y2H reporter gene is transcribed. It is the actual selector for protein protein interactions. The cells were then incubated for three days at 30°C. After this incubation period photos of the plates were taken for record keeping and the growth of the cells were empirically scored.

Here are two sample photos of the plates.

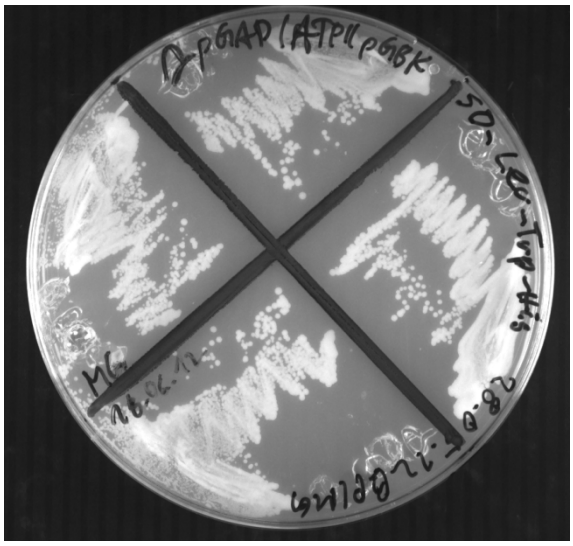

Figure 4.7: Sample picture of a plate with growth scoring of 4

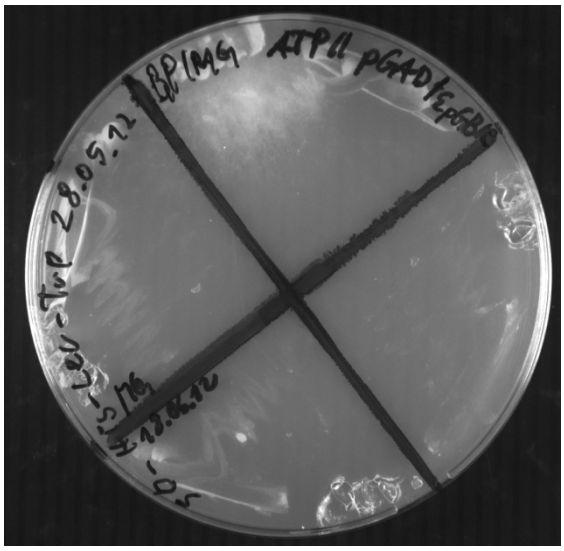

Figure 4.6: Sample picture of a plate with growth scoring of 0

The assessment of growth of the diploid yeast cells resulted in following scoring table, whereby a scale from zero to four was used. If no growth was observed, it received a score of zero while maximal growth was scored a 4. The results were then accentuated by adding a color coded heat map that indicated the highest levels of protein-protein interaction with a darker blue color.

Table 4.III: Scoring of the growth of the diploid yeasts on the triple knockout media

Samples	*pGBKT7*	*pGBKT7-ATP11*	*pGBKT7-β*	*pGBKT7-δ*	*pGBKT7-ε*	*pGBKT7-γ*	*pGBKT7-OSCP*
pGADT7	0	1	1	2	0	0	3
pGADT7-ATP11	1	0	4	2	0	0	4
pGADT7-β	1	4	3	4	4	2	4
pGADT7-δ	2	2	3	4	3	1	4
pGADT7-ε	1	1	1	4	1	1	4
pGADT7-γ	0	1	0	1	0	0	3
pGADT7-OSCP	1	3	1	1	1	0	4
SV40	0	4					
Controls		*p53*					

The positive controls of SV40 and p53 resulted in maximal growth, indicating that the assay was working. Each subunit was used as both a prey (pGADT7) and a bait (pGBKT7) and paired with every possible subunit to create this matrix. The empty vector combinations are used to test for a

subunits ability to auto-induce the yeast reporter gene, histidine. In our preliminary screen of the *T. brucei* ATP synthase subunits, only OSCP seems capable to auto-induce, possibly because it has some intrinsic properties to recruit the yeast transciprion machinery. The screen revealed several strong interactions between subunits beta and ATP11 and also between epsilon and delta as expected from previous studies in higher eukaryotes. Interestingly, delta was also shown to substantially interact with beta, suggesting that possibly some structural aspects of the complex *T. brucei* enzyme retain some prokaryotic features as delta is capable of binding beta and inhibiting ATP hydrolysis only in bacteria. Another positive interaction that will need to be further analyzed is the apparent binding of OSCP and ATP11, suggesting yet another function of ATP11 in the assembly process of the alpha beta ring. Finally, there are some false negatives in our screen as we did not identify any strong interactions between gamma, the core component of the central stalk, and the subunits epsilon or delta.

5 Discussion

5.1 Cloning

We implemented a new cloning strategy from Invitrogen into the lab to help speed up the process of creating the large number of constructs needed to perform a pairwise yeast two-hybrid analysis of the six subunits comprising the *T. brucei* F_oF_1-ATP synthase. Instead of having to subclone each PCR product into a T/A vector like pGEM-Tez before inserting it into its destination vector, we chose to take advantage of the unique homologous recombination properties of the GeneArt seamless cloning kit. To enable this recombination event, the PCR primers for each F_oF_1-ATP synthase subunit included 15 nucleotides from either the 5'or 3'ends of the linearized vector. While this incurs additional costs into the synthesis of the oligonucleotide primers, it saves significant time by skipping the subcloning step. Furthermore, because the integration of the PCR amplicon into the yeast expression vectors is not based on a ligase reaction, the whole cloning process from PCR to transformation can be done in one day. Our results from using this ligase free system suggest that the savings in time and manpower significantly outweigh the costs of the commercially available kit, especially for projects that involve high levels of cloning.

5.2 Expression verification via Western blotting

In order to validate the transformation procedure as well as the plasmid protein expression levels a western blot with the yeast protein extracts was carried out. The blot from the with pGBKT7 proteins was probed with a rabbit anti c-Myc antibody and bands corresponding to the

expected mass of each subunit can be detected. So for the yeast strains with the pGBKT7 plasmids it can be said with a high probability that the transformations have worked and the corresponding proteins are expressed correctly. The second blot of the pGADT7 proteins, however, shows nothing but background noise even after the blot was repeated with the same yeast lysates. There are three main problems that might have occurred. First and most likely, the protein extraction process may not have worked since the yeast wall is difficult to lyse efficiently. We used a cracking buffer containing SDS and then vortexed the cells with glass beads. This method involves using a certain ratio of cells to cracking buffer, perhaps this needs to be optimized. Alternatively, we can use other methods to lyse yeast cell walls. These include freezing the cells with liquid nitrogen and then grinding the cells with a mortar and pestle, treating the cells with enzymes like zymolase and lyticase or using a French press (EMBL, 2013). Secondly, there might be problems with the α-HA tag antibody as this western blot didn't contain a known positive HA fused protein sample. Moreover, the HA-tag could be folded into the expressed protein making it inaccessible to the antibody. Yet, this seems unlikely as the c-Myc tags of the pGADT7 protein samples were readily accessible for its antibody and the polyacrylamide gel contained enough SDS to theoretically denature the protein samples efficiently. Finally, there could be problems with the actual expression levels of these proteins. This is probably not the case because several diploid strains were able to grow well on the SD-Leu-Trp-His plates, which indicates successful expression and interaction of at least some of the subunits..

5.3 Motivation for the Yeast two Hybrid screen

As it was found by Ziková et al. the F_OF_1 ATP-Synthase of *T. brucei* consists of 22 subunits of which 14 have no homologues in other taxa besides kinetoplastids. One way to decipher on the functions of those hypothetical proteins is to dtermine their interactions using the yeast two hybrid method. Thereby, the single hypothetical proteins can be located within the complex, and depending on this location (either the F_O miety, the F_1 moiety or the peripheral stalk) their possible role can be proposed. As a result the unique structure and function of this enzyme in the parasite could be exploited for drug discovery, helping to effectively treat a still devastating disease.

5.4 Conclusions from the actual Yeast two Hybrid screen

In order to verify if the yeast two hybrid assay was working in the lab two control experiments were performed. These controls showed the expected behavior as the negative control (SV40 with empty pGBKT7) shows practically no growth (Score 0) and the positive control (SV40 with p53) shows

maximum growth (Score 4). Another negative control involved pairing the empty DNA binding pGKBT7 vector with the empty transcription activator domain pGADT7 vector. This resulted in no growth of the diploid cells since there were no proteins fused to these domains to mediate a protein interaction. Additionally, we tested for the capability of each subunit to auto-induce expression of the reporter gene by pairing each subunit in each expression vector with its matching empty vector (Matchmaker™Gold Yeast Two-Hybrid System User Manual, 2010). From these results we can conclude that the OSCP subunit has some inherent potential to recruit the transcription machinery all by itself, which explains why it produced positive interactions with every subunit it was paired with.

One of the purposes of this screen was to find out if the yeast two hybrid could successfully be applied to the *T. brucei* subunits of cV. Therefore six hydrophilic subunits were chosen, whose interactions are already known in other eukaryotes.For instance, one such interaction is between ε and δ both components that interact gamma and form the central stalk of the enzyme. Not only do they interact according to literature (Stock et al. 1999), but also in our screen. The interaction results in maximal growth of the diploid cells in one pairwise matching and the other combination (with bait and prey switched) results in a high level of growth (3).

However, it is dangerous to over interpret the results from a yeast two-hybrid screen as these assays are quite susceptible to false negatives and false positives for a variety of reasons. (MacDonald ed. 2001). For example, we predicted that the subunit gamma should interact with beta, delta and epsilon in our screen, as it is the core of the central stalk of the F1 moiety (Stock et al. 1999). However, the analysis of all the pairwise combinations with gamma never exceeds a growth score of 1, except when used as the bait for beta. Even then it only scores a 2, suggesting that the gamma subunit is not folded properly in yeast or the levels of protein expression are not high enough for this subunit. This most likely indicates a false negative in the screen.

An example of a false positive in our screen is probably the interaction depicted between beta and epsilon, which is not shown to be able to interact in other eukaryotic enzymes. Interestingly, this interaction is only positive when epsilon is used as a bait (growth score of 4) compared to when it is used as a prey (growth score of 1). Therefore, this interaction will have to be examined further under harsher conditions for interaction (Caufield et al. 2012).

One of the important applications of the yeast two hybrid system is to identify F_OF_1-ATP synthase assembly factors. Two of the best studied assembly factors in eukaryotes are ATP 11 and ATP 12. These are involved in the correct installation of the F1 hexamer composed of alternating α

and β subunits. Thereby, ATP 11 binds β and ATP 12 binds α, both hindering the subunits from forming non functional poly-α or poly-β aggregates (Wang et al. 2001). The binding of β with ATP 11 was confirmed by our screen showing high growth for both plasmid combinations. Moreover, the β subunit shows a strong self-interaction, which also fits with the presumption that if left uncoupled with ATP 11, beta will form aggregates with itself. Furthermore, our screen indicates that when OSCP is used as a prey, when it is not capable of auto-induction, that it also interacts with ATP 11. This would be an interesting result if further proven to be true because OSCP sits atop the beta subunit and connects the alpha beta ring with the peripheral stalk. This could be a new function for the *T. brucei* ATP 11 subunit. These finding gives further evidence to the utility of these experiments in attempting to assign location and therefore function to the unknown subunits of the T. brucei ATP synthase.

Although all these results are still just preliminary verifications of the method for *T. brucei* ATP-synthase subunits one quite unexpected interaction has been observed. The interaction between δ and the β subunit, showed strong growth. In prokaryotes this subunit is termed ε (Stock et al. 1999) and has the unique ability to inhibit ATP -hydrolysis. This occurs when the ε switches its conformation and gets into contact with the α β ring (Hausrath et al. 2001). In eukaryotes this function is normally performed by the ubiquitous IF1 peptide, which specifically inhibits the ATP hydrolysis function but doesn't interfere with ATP synthesis. (Robinson et al. 2013) The finding of the δ, β interaction suggests a second way of regulating ATP-hydrolysis in *T. brucei* and seems quite remarkable as the protist relies on ATP-hydrolysis in its bloodstream form to maintain its mitochondrial membrane potential (Schnaufer et al. 2005). Generally, *T. brucei* IF1 is strictly regulated and not expressed in bloodstream cells, otherwise it would be fatal for the parasite. In general, it could be possible that in our evolutionary ancient (Ziková et al. 2009) protist the δ ,β does actually occur, but most likely its expected prokaryotic function to inhibit ATP hydrolysis function has been lost.

5.5 Future tasks

In order to verify these outcomes several steps have to be undertaken in order to verify them. First of all the current screening has to be repeated several times to show if it is reproducible. As the yeast two hybrid technique is quite susceptible to false positive results (MacDonald ed. 2001) we should attempt eliminate as many as those outcomes as possible. One approach is to use media containing 3-amino triazole (3AT). This molecule hinders the histidine production in yeast so that a higher rate in transcription of the reporter gene is required for the yeast to prosper. Therefore,

false positives which often have very weak transcription levels of the reporter will not survive under these more stringent conditions (Caufield et al. 2012).
In a final step of verification the plasmid DNA of positive interacting strains has to be extracted from the yeast and analyzed via sequencing in order to verify if the plasmids contain the correct inserted subunit sequence..
After these final verification steps, new Y2H screens can be set up that include the hypothetical proteins in the subunit matrix that analyzes all the possible pairwise protein interactions. If there are some interesting new interactions that materialize from this screen, then we can further map the positive interactions of two proteins by expressing just portions of each subunit to determine more specifically the regions involved in protein binding. All of which will be important to better understand the unique organization of this complex enzyme and how that affects its function.

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