Identifying protein interaction partners for DEXI

Encoded by the multiple sclerosis associated *dexamethasone-induced gene, DEXI*

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Thesis presented for the degree of
Master of Science

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October 2017

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http://www.duo.uio.no/

Print: Reprosentralen, Universitetet i Oslo

Abstract

Multiple sclerosis (MS) is a chronic demyelinating disorder of the central nervous system. The pathology of the disease is thought to be inflammation and demyelination, presumably caused by autoimmune T cells. The disease has a complex aetiology, affected by environmental factors as well as genetics. 200 MS-associated single nucleotide polymorphisms (SNPs) has been discovered through genome-wide association studies (GWAS). One interesting gene region, 16p13, was among the first to be discovered to contain MS associated SNPs. The gene region contains three genes associated with immune regulation: *SOCS1*, *CIITA* and *CLEC16A*, and one gene encoding a protein with unknown function, *DEXI*. Expression of *CLEC16A* has been shown to correlate with expression of *DEXI* in thymic tissue and an intron of *CLEC16A* that contains several MS-associated SNPs has been shown to interact with the promoter region of *DEXI*. These discoveries has led to the suggestion that *DEXI* may be an MS risk factor.

We wanted to study the function of DEXI by investigating its protein interaction partners, by using a yeast two-hybrid system, in addition to investigating the protein subcellular localization in Jurkat cells.

Through the yeast two-hybrid screening and subsequent *in silico* analysis of results we discovered five potential interaction partners for DEXI; CAMLG, MARCH2, MDK, SLTM, and SNAPIN. Subcellular localization of DEXI was investigated by overexpressing tagged DEXI in Jurkat cells, followed by immunostaining and confocal microscopy. From our experiment it appears as though DEXI is located in cytosol or plasma membrane.

Acknowledgements

The work presented in this thesis was carried out at Oslo University Hospital, at the Neuroscience Research Unit, from January 2016 to October 2017.

I would like to take this opportunity to thank my supervisor Tone Berge for allowing me to undertake my Master's degree at the Neuroscience Research Unit, and for providing me with such an exciting project. Her guidance and encouragement, and not least her active presence has been invaluable. Thank you for always providing solutions so enthusiastically!

I am grateful for the collaboration with Odd Stokke Gabrielsen and his group at UiO, and in particular for the help of Marit Ledsaak. In addition to providing me with protocols, reagents, and equipment, she has aided me with tips, tricks, and troubleshooting without which I would surely have become lost.

Likewise, I have received help and support from other members of our lab. Especially Anna Eriksson, Ingvild Leikfoss, Ina Brorson and Margrete Langmyhr. Drawing from your experience has been invaluable for me. Thank you for always taking time to explain any and every question I might have had.

I would also very much like to thank professor Hanne Harbo for allowing me to be a part of the MS research group. I have gained a lot of insight from participating in conferences, seminars, and group meetings, and for these experiences I am truly grateful. I am also thankful to the other members of the research group for making me feel included. You have created an open and supportive working environment of which you should be proud.

I am also indebted to my peers at DM4. Especially to Fernanda and Elisabeth, for sharing office, jokes and stress levels. Working alongside you has been delightful. A great thanks is also owed my wonderful family for unwavering support, and especially to Hugo, whose inexhaustible patience and daily encouragement has been quintessential.

October 2017

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1 - Introduction

Multiple sclerosis (MS) is a presumed autoimmune disease with complex aetiology affected by gene susceptibility and environmental factors. This thesis explores the protein interaction partners of a protein encoded by a suggested MS susceptibility gene. In this chapter an overview of MS is given before immunology and the immunopathology of MS is described. Then follows a section on genetics in general, and genetics of MS, focusing on a particular chromosome region. Finally, a section describing protein-protein interaction is included, before the aim of this study is given.

1.1 Multiple sclerosis

MS is an inflammatory, neurodegenerative disorder of the central nervous system (CNS), affecting over 2.3 million people worldwide [1]. The disorder can cause mild to severe physical impairment, cognitive disabilities, depression and fatigue [2]. The average age of onset is 30, and the median survival from disease onset in Norway is 41 years [3], so many patients will live with physical disabilities for a long time. Approximately 50% of patients will require permanent use of wheelchair within 25 years after diagnosis [4]. Although no definite cure has been found, and no single diagnostic tool or biomarker has been identified, increased knowledge of the disease and design of drugs able to slow down disease progression has contributed to the great increase in survival for MS patients, with afflicted patients today living more than twice as long as patients did in 1969 [3].

1.1.1 Clinical manifestation

Due to the complexity and heterogeneity of the disorder, no single parameter has been identified from which to run diagnostic tests. Consequently, diagnosis of MS is determined symptomatically, and several criteria must be met in order for the diagnosis to be given. Initially, patients may experience what is termed a clinically isolated syndrome (CIS), a

neurological incident that share characteristics with MS [5]. Alternatively, though less commonly, a radiologically isolated syndrome (RIS) may be revealed, if inflammatory lesions of CNS are discovered by magnetic resonance imaging (MRI) [6]. These isolated syndromes may contribute to raise suspicion of MS, but neither is sufficient to set the diagnosis. CIS is not sufficient, as two neurological incidents separated by at least 30 days is required to meet the criteria of dissemination in time (DIT), whereas RIS is not sufficient as neurological damage at two separate structures of the CNS is required to meet the criteria of dissemination in space (DIS) [5]. Clinical evidence, preferably supported by MRI, must be evident to establish both DIT and DIS [5] in order for the diagnosis to be given.

A classification system of MS has been developed based on clinical manifestation of the disease, dividing MS into three subtypes: relapsing-remitting MS (RRMS), primary progressive MS (PPMS) and secondary progressive MS (SPMS) [6] (figure 1.1). RRMS is a state where the patient experiences periods of acute increases in disability, called relapses, followed by remission and a period of time with little or no signs of increase in disease activity. The attacks are usually unpredictable, and may leave permanent deficits in the patient. In progressive MS, patients experience gradual progression of disease without relapses. If a patient is initially diagnosed with progressive MS, it is referred to as primary progressive MS, however the most common disease course is initial RRMS that develops into secondary progressive MS [6].

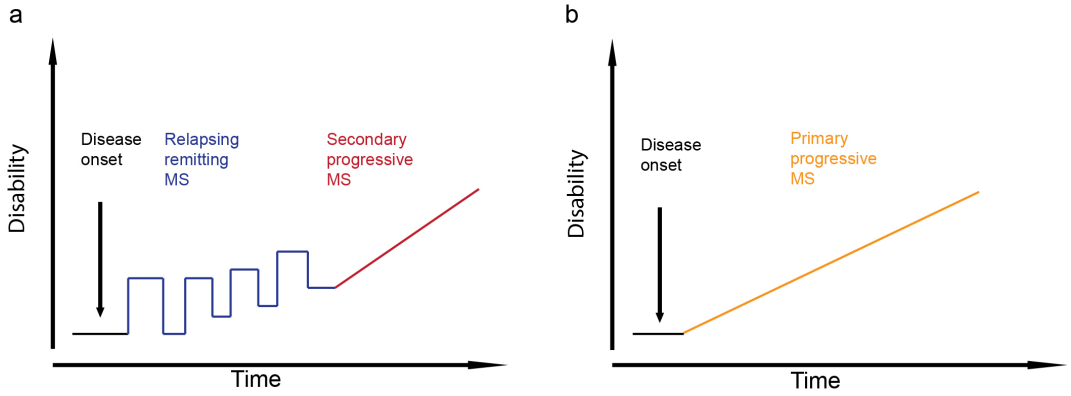


Figure 1.1: Schematic illustration of the clinical manifestation of MS
Illustration shows the development of MS over time. a) The most common disease course involves initial relapsing-remitting MS, characterized by intervals of acute worsening, followed by improvement. RRMS slowly develops into secondary progressive MS, where disability gradually decreases over time. b) Alternatively, patient does not experience acute incidents or remitting periods, but rather gradual clinical worsening as disease progresses, starting from onset. Figure by Leikfoss

1.1.2 Causes

MS has an uneven geographical distribution, as both prevalence and age of onset is significantly correlated with latitudes [7, 8]. Norway has one of the highest prevalences of MS in the world, with an estimated 203 people affected per 100 000 inhabitants [9], contributing greatly to neurological disorders being responsible for 5-6% of the total disease burden in Norway [10]. It has been shown that immigrants coming to Norway from areas with a lower risk for MS, display a prevalence similar to the one in the general Norwegian population, indicating that environmental factors contribute to the risk of developing MS in Norway [11]. However, deviations from the latitudinal gradient can be found, for example in the Saami population of northern Scandinavia, who display a markedly lower prevalence than the general Norwegian population [7, 12]. Genetic studies of the Norwegian Saami population has revealed a significantly reduced frequency of certain gene variants known to be associated with increased MS risk [13], and this has been suggested as a contributing factor to the relatively low prevalence of MS in the Norwegian Saami population.

These examples clearly demonstrate that MS is a multifactorial disorder whose development is affected by genetic make-up as well as environmental factors. Among the established environmental factors of MS are smoking, early Epstein-Barr virus (EBV) infection, low levels of vitamin D, and obesity at a young age [14]. The genetics of MS are not fully understood, but the first genetic factor that was recognized, and still established to have the largest effect, is a genetic variant of human leukocyte antigen (HLA) complex, accounting for $\sim$20% of the heritability of MS [15]. A significant number of the other identified genetic risk factors for MS are also related to immune responses, and so it has been suggested that genes of the immune system play an important role in the disease progression of MS [14, 16]. In fact, the driver of pathogenesis in MS is considered to be autoreactive $CD4^+$ T cells causing inflammation in the CNS [17, 18].

1.2 The immune system

The immune system is our body's main defence against invading pathogens such as bacteria, virus and fungi. Most of the pathogens we are exposed to are unable to penetrate our skin or epithelial tissues, and cause no harm. If a pathogen manages to cross epithelial cells and enter the body, the first line of defence is collectively called the innate immune system. It consist of cells and proteins that recognize and bind to pathogens or pathogen products. Pathogens are recognized by so-called pattern-recognition receptors (PRRs) that recognize what is commonly referred to as pathogen-associated molecular patterns (PAMPs). These types of molecular patterns are commonly found in several species or classes of pathogens, for example lipopolysaccharides (LPSs) from Gram-negative bacteria or flagellin from flagellated bacteria [19]. In this way, the innate system can recognize a great many pathogens without needing to be very specific. Pathogens that have been bound by components of the immune system are targeted for phagocytosis by effector cells, such as macrophages, that are natural residents of most healthy tissue. Once a pathogen has been identified, and immune system is activated, immune cells and plasma proteins are recruited to the site of infection. A part of the innate response is to make endothelial tissue permeable to these plasma proteins and cells. This causes inflammation - a state of oedema caused by plasma influx to connective tissue. In many cases, the innate immune response is sufficient to drive off infection, but sometimes the innate immune system will be overwhelmed by the number of pathogens, and must call on the adaptive immune system. The initial step is to activate small lymphocytes called B and T cells. These cells express surface-bound receptors, called immunoglobulins and T cell receptors, respectively, are highly specific against foreign particles. Any molecule recognized by one of these receptors is referred to as its antigen. B and T cells normally circulate the body in an inactive state, looking for their antigens. If interaction between the receptors and their antigens occur, the cells become activated. B cells develop into plasma cells and start secreting soluble versions of their immunoglobulins, called antibodies. T cells differentiate into different categories of effector cells called cytotoxic T cells, helper T cells and regulatory T cells. Unlike the PRRs of the innate immune system, which are encoded completely by germline DNA, the receptors of B and T cells undergo somatic recombination,

enabling cells to generate millions of unique receptors. This gives B and T cells the ability to maintain high specificities against innumerable pathogens and pathogen products that constantly change and develop.

1.2.1 Immunoglobulin and TCR diversity

Immunoglobulins consist of two heavy chains and two light chains, and a TCR consist of two chains, most often an α-chain and a β-chain (figure 1.2). The loci encoding these antigen-binding sites of these proteins contain several versions of variable regions (V), joining regions (J), and constant (C) regions. The α-, β-, and heavy chain locus also contains diversity (D) regions. During development of B and T cells, these regions are rearranged through a complex set of reactions called somatic recombination. The random rearrangement of these different regions could generate millions of different receptors [20]. Additional recombination enzymes facilitate the introduction of random nucleotides, even further increasing diversity of antigen binding sites [21].

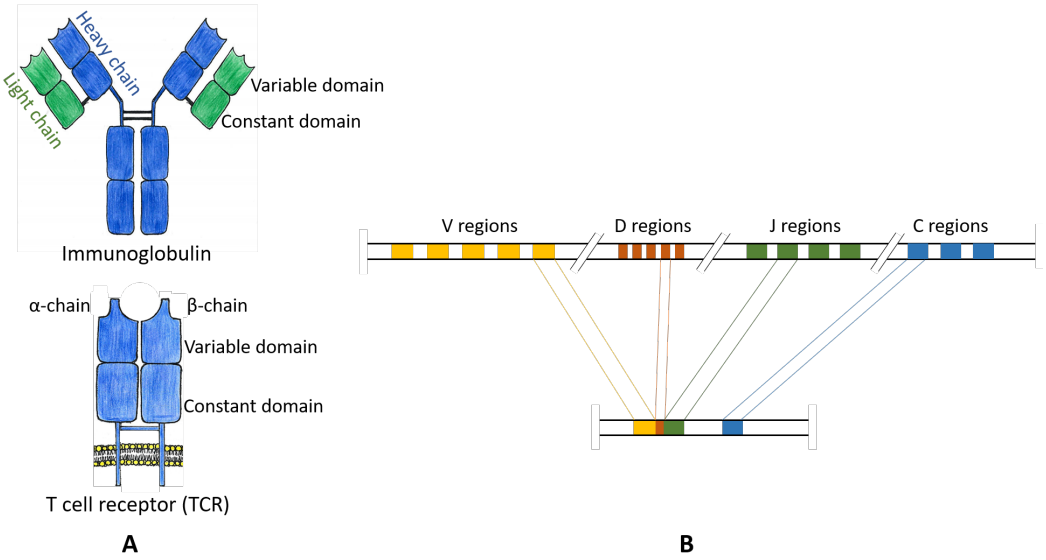


Figure 1.2: Illustration showing the structure of immunoglobulins and T cell receptors, and somatic recombination of immunoglobulins
Illustration shows a simplified overview somatic recombination exemplified by the immunoglobulin heavy chain. On chromosome 14, there are 38-46 V regions, 23 D regions, 6 J regions and 9 C regions. Random combinations of these can generate 1.6 million unique immunoglobulin heavy chain exons [21]. The same principle applies to somatic recombination in light chains of immunoglobulins as well as TCRs

Consequently, through somatic recombination and insertion of random nucleotides, B and T cells generate receptors that are highly variable and will bind random antigens. Since B and T are instrumental for immune response, it is of great importance to prevent them from expressing receptors that recognize proteins that are not pathogenic, or indeed foreign. Receptors that are able to bind to antigens that are naturally present in a healthy individual, so-called self recognition, can be highly detrimental. To prevent this from happening, B and T cells, developing in bone marrow and thymus, respectively, undergo a strict set of selection steps before they are allowed to enter the bloodstream [22].

1.2.2 T cell education and activation

T cell precursors are developed from lymphoid precursors in the bone marrow and migrate to the thymus. In the thymus they undergo somatic recombination and begin expressing TCRs as well as two surface co-receptors, cluster of differentiation 8 (CD8) and CD4. From here the cells must pass two selection steps. First, the cell must demonstrate that it recognizes major histocompatibility complex (MHC). MHC is a protein cells use to present antigens by loading them onto their MHC, and then expressing MHC and antigen together on their surface. By convention, human MHC is called HLA. There are two classes of MHC: class I is expressed in all nucleated cells and class II is expressed in antigen-presenting cells (APCs). In thymus, developing T cells are exposed to both types. If the TCR recognizes MHC class I, the cell stops expressing CD4 and becomes a naïve $CD8^+$ T cell. If it recognizes MHC class II, it stops expressing CD8, and becomes a naïve $CD4^+$ T cell. If it recognizes neither MHC class, the cell undergoes apoptosis. This is the positive selection of naïve T cells. In the second selection step, the cell is exposed to specializes thymic anitgen-presenting cells (APCs) expressing MHC loaded with self antigens, or autoantigens. To avoid autoimmunity, any T cell that binds tightly to a loaded MHC will undergo apoptosis. Once a T cell has passed both selection steps, it is released to the circulation system [21, 22].

If a naïve $CD4^+$ T cell encounters an APC presenting its antigen, it assembles what is called the TCR complex (fig 1.3). This complex consist of TCR, coreceptor CD4, two CD3 molecules and two ζ-chains. To be activated and induce an immune response, the cell must

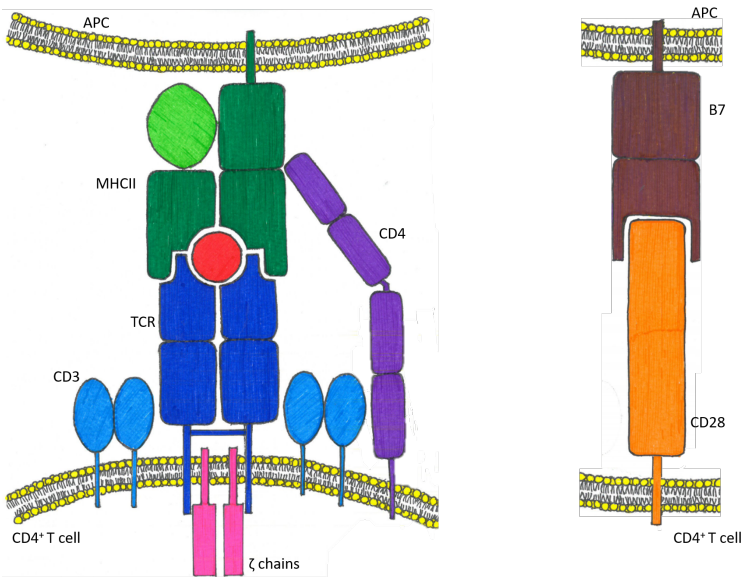


Figure 1.3: Signals required for activation of T cells
TCR is a part of a signalling complex in T cells, consisting of TCR, ζ-chains, two CD3 molecules and one CD4 coreceptor. For a T cell to be activated, the TCR complex must associate with an antigen-loaded MHC class II on an antigen presenting cell (APC). Coreceptor CD4 on the T cell must bind to MHC as well. In addition, interaction must occur between receptor B7 on the APC and its ligand CD28 on the T cell. B7 is only expressed in the presence of an infection, and acts as a secondary signal required for T cell activation. Downstream effects of activation are mediated through the cytosolic tails of CD3 [23]

be exposed to two signals. First, it must recognize an antigen presented on the MHC class II of an APC, through both the TCR and its coreceptor, CD4. Second, there must be an interaction between receptor B7 on the APC and its ligand CD28 on the naïve T cell [23].

1.2.3 MS as an autoimmune disease

During the negative selection of naïve T cell, only the cells that express TCRs who bind tightly to MHC presenting autoantigens are directed to undergo apoptosis. Consequently, most humans have circulating T cells that recognize self-antigens, but who display only moderate binding to MHC. B cells undergo a selection step similar to T cells, but in bone marrow. However, since not all self-antigens are available in bone marrow, B cell selection is slightly leaky, and self-reactive B cells are sometimes released to the circulatory system. In most, but not all, cases, B cells that are activated by self-antigens will undergo apoptosis or become anergic. When circulating, self-recognizing B and T cells are activated, and induce

an immune response against self-tissue, the result is what we call an autoimmune disease (AID) [22].

Three proteins have been proposed as autoantigen candidates in MS: myelin basic protein (MBP), myelin proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). T cells that are autoreactive to these proteins have been found in peripheral blood of MS patients as well as healthy controls [24, 25, 26]. In addition to defects in negative selection during T cell development, two mechanisms have been proposed to explain activation of myelin-reactive T cells in the periphery. Through one of the mechanisms, molecular mimicry, self-reactive T cells are activated by invading pathogen through an antigen that shares similarities with the self-antigen [27]. Alternatively, in bystander activation, T cells may be activated by the pro-inflammatory cytokines and chemokines that are secreted by other immune cells upon infection from a pathogen [28]. Under normal circumstances, movement of substances from blood to CNS is tightly regulated by the blood-brain barrier (BBB), and trafficking of circulating lymphocytes is kept at a low level [29]. Activated $CD4^+$ T cells, however, secrete proinflammatory cytokines that leads to an increased expression of endothelial adhesion molecules, making it easier for activated $CD4^+$ cells to cross the BBB and enter CNS [17]. Once myelin-reactive T cells have entered CNS, they may be reactivated by local APCs carrying their antigen and initiate an inflammatory state [17]. During inflammation, the BBB becomes less restrictive, and more immune cells are able to enter the CNS. Patients with MS have been shown to contain higher levels of autoantibodies against MOG within the CNS [30]. The chronic inflammation in the CNS, and the targeting of myelin proteins from B and T cells drives the characteristic demyelination of MS. Although no cures have been found for the disease, drugs are available to halt the progression, and these are mainly immunosuppressants or immunomodulatory drugs [17]. One example is Natalizumab, which is an antibody that targets the α4-integrin (VLA4). By inhibiting the funciton of VLA4, Natalizumab inhibits lymphocyte migration into CNS, preventing autoreactive lymphocytes from entering CNS and initiaing inflammation in CNS. However, it also prevents regular immunosurveillance of CNS, thus making subjects more susceptible to pathogens of the CNS [17]. Both immuosuppressants and immunomodulatory drugs can have severe side-effects, and it is difficult to predict how individual patients will respond. MS patient are often pre-

scribed drugs on a trial-and-error basis, which can be very exhaustive for the patient. It is hoped that greater knowledge of molecular pathways in MS will enable us to design better drugs, as well as finding biomarkers that can help doctors prescribe drugs more precisely. Studying the genetics of patients could provide us with more knowledge of the molecular pathways through which MS works.

1.3 Genetics

1.3.1 Mutations and genetic variation

Random mutations may occur every time a cell duplicates its DNA in preparation for cell division, or they may be induced by mutagenic factors. It is estimated that approximately three nucleotides change for each time human DNA is replicated [31]. Most mutations occur in somatic cells, and will not affect the germline DNA. Only mutations that occur in germ cells will be passed on to offspring. Any mutation that arises in germline DNA could potentially have an advantageous or detrimental phenotypic effect, affecting the fitness of the individual. As a gene with a mutation is passed on through generations, it generates populations of what is called gene variants.

The most common form of genetic mutations are changes in solitary base pairs, termed single nucleotide polymorphisms (SNPs). Due to the way DNA is read in triplets, a SNP in the coding region of a gene may not affect sequence of the protein product, and is called a silent, or synonymous, mutation. Conversely, a mutation may be non-synonymous, and lead to an amino acid change in the sequence of the protein product, and thus may confer a range of downstream effects.

More commonly however, SNPs arise in the vast non-coding areas of DNA. Exons of encoded protein-coding genes make up less than 3% of the human genome [32] and the rest was previously thought to be mostly artefacts and duplications - so-called "junk DNA". The Encyclopedia of DNA Elements (ENCODE) project, however, has been able to assign biochemical function to 80% of the genome [32], indicating that non-coding DNA play im-

portant roles, including forming stable RNA transcripts (for example transfer RNA (tRNA) and micro RNA (miRNA)) and serving as regions for histone modification, DNA methylation and chromosome-interaction. These latter examples are of vital importance when it comes to regulation of gene expression.

1.3.2 Gene regulation

The transcription of DNA to mRNA is mediated by proteins called transcription factors, who are affected by a group of proteins collectively known as regulators (see fig 1.4). Regulators can activate or repress transcription by direct or indirect interaction with transcription factors, or by altering conformation of DNA, for example to remodel nucleosomes.

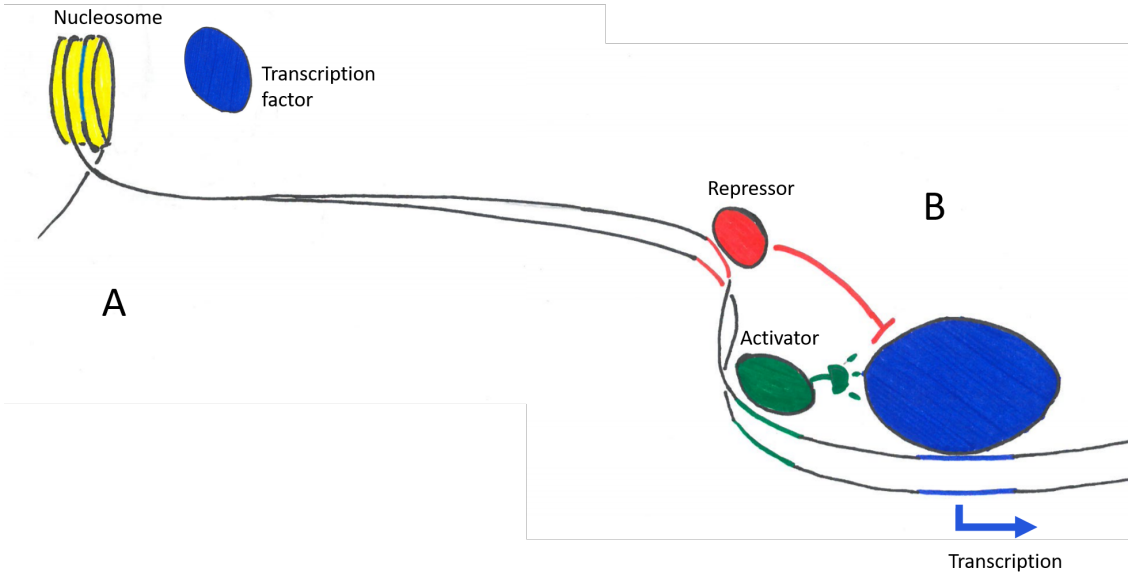


Figure 1.4: Effect of regulators on transcription factors
Regulators may exert their effect on activity of transcription factors by modifying accessibility of binding domains as in A), where transcription factor is unable to bind DNA bound to a histone complex, or B) regulators may bind to specific DNA segments, and act directly on transcription factors by inhibiting or promoting transcription.

None of these mechanisms for regulating gene transcription would be possible without binding between proteins and specific regulatory sequences of DNA called enhancers. Although not strictly part of the coding region of genes, these enhancers are essential for regulation of gene expression. Enhancers may affect transcription of genes located at a substantial

distance, forming DNA loops. These interactions are not stable, and cannot be seen *in vitro*, unless a covalent interaction has been induced, for example by treating intact cells with formaldehyde. This technique, called chromosome confirmation capture (3C) enables the study of the cross-linked DNA to reveal the identity of interacting segments [33]. If a genomic region is found to have an effect on the expression of other genes, they are referred to as expression quantitative trait loci (eQTL).

1.3.3 Genetics of multiple sclerosis

The strongest genetic association with MS has been shown to be gene variant HLA-DRB1*1501 [16], but that is only estimated to account for 20.2% of the observed heritability of MS [15]. Links between SNPs and disease are unveiled through genome-wide association studies (GWAS), where a large number of SNPs spanning the genome is mapped for patients of complex diseases and healthy controls. The data is analysed to discover SNPs that are more prevalent in patients than controls [34]. Several GWASs have been performed on MS to identify potential non-HLA, disease-associated SNPs, and now more than 200 gene variants have been identified [15]. One of the first MS-associated SNPs to be identified, was rs6498169 located within chromosome region 16p13 [35]. This finding has been replicated in a range of studies [36, 37, 38, 39, 40, 41, 42]. The region 16p13, and especially the *CLEC16A* gene within the region, has later been found to harbour many SNPs associated with various autoimmune diseases [43], and *CLEC16A* is considered a strong MS susceptibility gene (reviewed in [44]).

Interestingly, the 16p13 harbours two other genes that have been shown to contain MS associtated SNPs, *class II MHC transactivator, CIITA* and *suppressor of cytokine signalling 1, SOCS1* [45, 46]. Both are involved in immunoregulation, and are consequently of great interest in an autoimmune context. The function of the protein encoded by *C-type lectin-like domain family 16A* (*CLEC16A*), is not yet fully understood, but its association with many autoimmune diseases highlights its expected function in immune regulation. Among other functions, it has been found that CLEC16A has an effect on autophagy in human epithelial thymic tissue, causing decreased response to TCR stimulation [47].

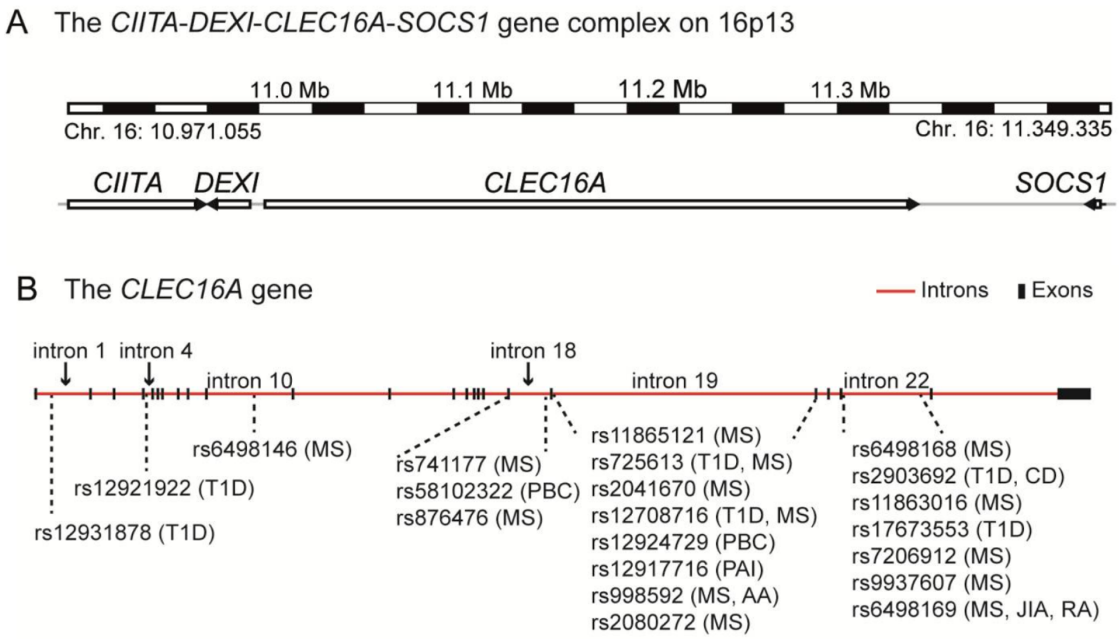


Figure 1.5: Schematic overview of chromosome region 16p13, and the *CLEC16A* gene A) The chromosome region covers approximately 179 kb on chromosome 16, and contains the four genes *CIITA*, *DEXI*, *CLEC16A*, and *SOCS1*. B) The dominating gene contains numerous SNPs associated with disease, including MS, type 1 diabetes (T1D), primary biliary cirrhosis (PBC), primary adrenal insuffiency (PAI), alopecia areata (AA), and juvenile idiopathic arthritis (JIA). Figure reprinted from [44]

Like more than 90% of disease-associated SNPs [48], the disease-associated SNPs in *CLEC16A* are located in non-coding regions, most notably in introns 19 and 22 [36], so it is difficult to establish whether it is the function of *CLEC16A* that is important for disease development. It has been shown that the genotype of SNPs in *CLEC16A* correlate with expression of its neighbouring genes *SOCS1* and *DEXI* in thymic tissue [49] (see figure 1.6).

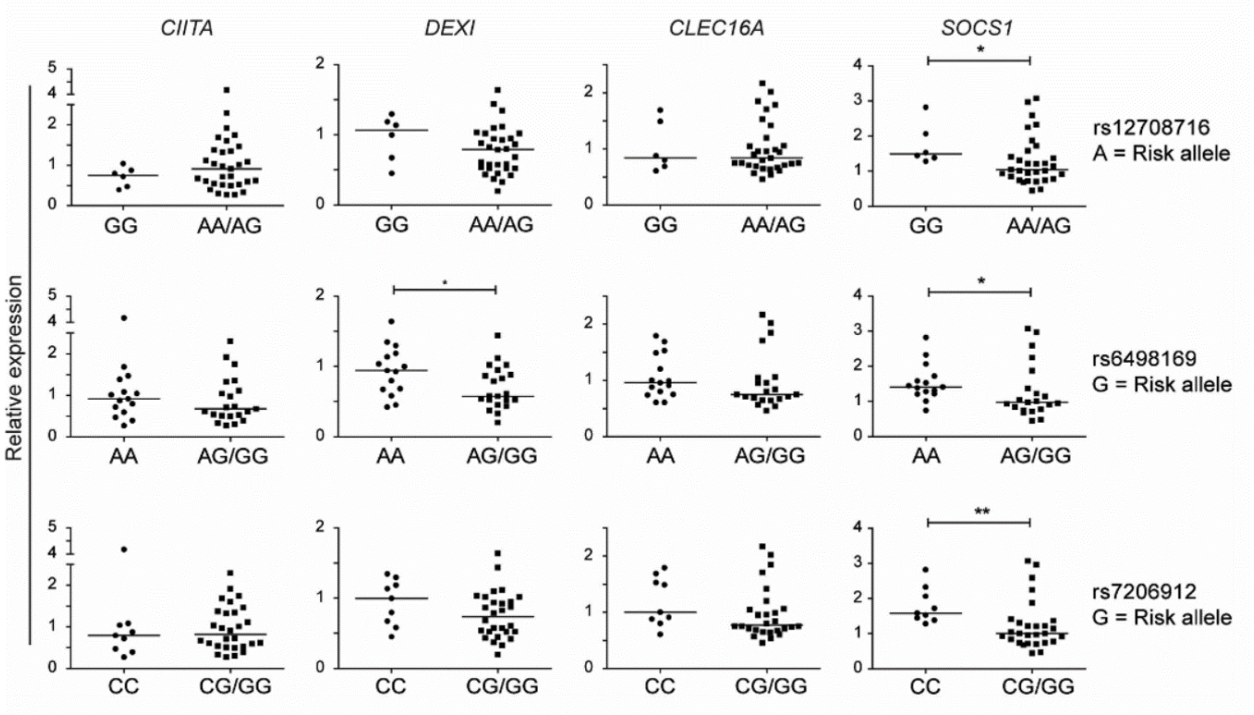


Figure 1.6: The genotype of MS associated SNPs correlates with expression of *DEXI* and *SOCS1* in thymic tissue
The figure shows relative expression of the 16p13 genes *CIITA*, *DEXI*, *CLEC16A* and *SOCS1* in individuals homozygotic for risk SNPs vs carrier/heterozygous individuals. *p-value 0.05-0.01, **p-value 0.01-0.001. Figure reprinted from [49].

In addition, evidence has been found for an interaction between *CLEC16A* intron 19 and the promoter region of *DEXI*. Together with gene expression analyses, this indicates that a region within intron 19 acts as an eQTL for *DEXI* [50].

1.3.4 DEXI

DEXI is suggested to be a susceptibility gene for MS because of the correlation between expression of *DEXI* and SNPs in intronic regions of the disease-associated *CLEC16A* gene. Similar conclusions have been drawn in studies investigating SNPs and eQTLs in other disorders. One study found that the promoter region of *DEXI* interacts with a region in the vicinity of the rheumatoid arthritis (RA) associated *ZC3H7A* gene, and with a region in the proximity of the T1D- and JIA-associated *RMI2* gene [51]. There has also been shown a correlation between expression of *DEXI* and SNPs associated with elevated risk for T1D

[52]. Similarly, *DEXI* has been suggested to be a susceptibility gene for asthma [53, 54], and to play a role in selective immunoglobulin A deficiency [55].

DEXI, or *dexamethasone induced gene*, was named in a paper describing its upregulation in the cell line A549 upon treatment with dexamethasone [56]. DEXI is 95 amino acids long, and is predicted to contain a transmembrane domain and a leucine zipper motif with a predicted casein kinase II phosphorylation site. Due to its transmembrane domain and the leucine zipper motif, it is predicted to be localized in plasma membrane of cells [56]. No functional studies of DEXI has yet been published, and so no hypotheses have been proposed as to how DEXI might contribute to the pathology of the various diseases.

1.4 Protein-protein interactions

When studying the function of a protein, it is interesting to look at its interaction partners. Proteins are often part of signalling cascades and pathways, and by establishing interaction partners, the type of function of a protein may be narrowed down. Many different methods are available to identify protein-protein interactions. Some methods are based on the physiochemical properties of the proteins, such as affinity chromatographies or immunoprecipitations, and takes place *in vitro*. In chromatography, a protein of interest is immobilized in a column. The immobilization usually occurs through modifications of the protein of interest. A protein solution, for example a lysate, is run through the column, and any proteins that binds to your protein of interest are retained in the column. They may later be eluted and analysed. In immunoprecipitation, your protein of interest is mixed with a protein solution, and an antibody against the protein of interest. Beads that bind to the antibody are added to the mix and the antibody-protein-complex can be pulled down. The pulled-down proteins can then be dissociated and analysed. These methods can be used to confirm suggested protein-protein interactions, in which case an immunoblotting will reveal whether the anticipated protein partner is present in the eluate. The methods can also be used when there is no hypothesis, in which case the proteins must be analysed using more advanced methods like mass spectrometry [57].

Another type of method that can be used to detect interaction partners involves the use DNA libraries. This library can be cloned to produce protein libraries with desirable properties that can be used in various systems. The major advantage of using these methods is the availability of DNA sequences, which are more easily analysed and identified than proteins. One example of a library method is the phage display, where DNA libraries are used to generate a library of fusion proteins within a bacteriophage. The fusion protein is displayed on the surface of the phages, and can be screened against your protein of interest. Another commonly used library method is the yeast two-hybrid assay, in which your protein of interest and a protein library are connected in a system that gives rise to expression of reporter genes in yeast cells [57].

1.4.1 The yeast two-hybrid assay

As implied by its name, the yeast two-hybrid (Y2H) assay involves the interaction between two hybrid proteins often referred to as "bait" and "prey", in a yeast (*Saccharomyces cerevisiae*) model system. The bait protein consist of your protein of interest fused to the DNA binding domain (DB) of transcription factor, most commonly the fungal Gal4 [58]. The prey protein is a hybrid between the activation domain (AD) of the same transcription factor, and proteins encoded by a cDNA library. The proteins are encoded by two plasmids that can be used to transform yeast cells of opposite mating types (figure 1.7). The yeast cells can then be mated, giving rise to diploid yeast cells that express both plasmids simultaneously. When expressed in yeast cells, both bait and prey proteins migrate to the nucleus, where the DNA binding domain of the bait protein will find its binding site in DNA, and attach itself to it. If there is interaction between the bait and prey protein, this will bring the activation domain in proximity to the DNA binding domain, initiating transcription of downstream reporter genes [58, 59].

The yeast strains used in Y2H screens are genetically modified to facilitate reporter gene assays. Most notably, the strains do not express transcription factor Gal4, and they are auxotrophic for amino acids adenine, histidine, leucine and tryptophan, meaning these amino acids must be provided in the yeast growth medium in order for cell cultures to survive. The

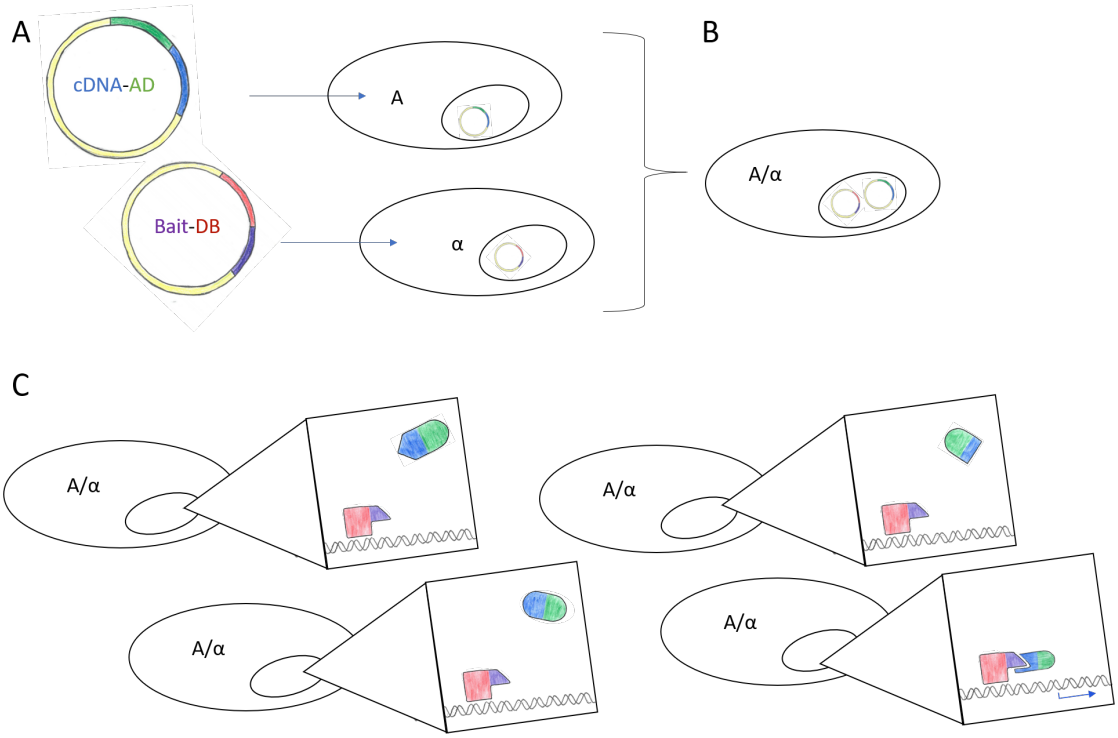


Figure 1.7: Schematic overview of the yeast two-hybrid system
A) Two plasmids encoding the bait protein fused to the DNA binding domain(BD) of transcription factor and a cDNA library fused to the activation domain (AD) of the same transcription factor. The plasmids are used to transform yeast cells of two mating types, A and α. B) The yeast cells are mated to form diploid A/α cells, containing both plasmids. C) The mated culture consist of many cells expressing two hybrid proteins, some of which may interact, and cause transcription of reporter genes, indicated by arrow

ability to synthesize these amino acids can be rescued by introducing expression of single genes; *ADE2*, *HIS3*, *LEU2*, and *TRP1*. The genes *LEU2* and *TRP1* are found in the plasmids used to introduce bait and prey, and are used as reporters to confirm expression of these proteins. *ADE2* and *HIS3* are found in the genome of the modified yeast cells, under the regulation of Gal4 promoter elements. When Gal4 is re-introduced to the yeast cells via the bait and prey proteins, expression of these genes resume [58, 59]. A third reporter gene is also placed under the regulation of Gal4, namely *MEL1*. It encodes α-galactosidase, an enzyme that catalyse the cleaving of the sugar 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (x-α-gal) into galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter is immediately hydrolysed to 5,5-dibromo-4,4-dichloro-indigo. This product, when found in yeast cells, will colour the colonies blue [60].

1.5 Aim of the study

DEXI has been suggested as an MS risk gene candidate, due to the correlation in expression of *DEXI* and *CLEC16A* in thymus and the proposed eQTL-effect *CLEC16A* has on *DEXI*. We wanted to investigate the function of the protein product of *DEXI* by identifying its protein interaction partners. We also wanted to investigate the subcellular localization of DEXI, in order to assess the physiological relevance of identified interaction partners.

The aim of this study is to investigate the protein interaction partners for DEXI using a yeast two-hybrid assay and to study the subcellular localization of DEXI in Jurkat cells using immunostaining and confocal microscopy.

2 - Methods

This chapter describes the laboratory methods and protocols used in the work leading to this thesis. Materials, equipment and recipes used are detailed in appendices B and C.

2.1 DNA and bacterial techniques

2.1.1 Growth and maintenance of bacteria

In this thesis, two different strains of the classical model bacteria *Escherichia coli* were used. XL1-Blue was used for subcloning and recombinant DNA experiments, and BL21(DE3) was used to express recombinant proteins

XL1-Blue is a commonly used strain that has been optimized for working with recombinant DNA techniques. The cells are deficient for endonuclease, which increases efficiency of miniprep procedures, and they are recombination deficient, which improves insert stability. The XL1-Blue strain was made electrocompetent, enabling quick and efficient transformation by electroporation.

BL21(DE3) is a strain specifically modified to enhance expression of heterologous proteins. The strain is deficient for OmpT and Lon proteases, making it easier to isolate intact recombinant proteins, and it is enhanced with additional copies of genes containing tRNAs that are originally rare in *E. coli*, helping to overcome the problem of codon bias. The BL21(DE3) strain is resistant to the antibiotic chloramphenicol.

E. coli cells are grown on agar plates or in liquid culture. Commonly used growth media are lysogeny broth (LB), and derivatives of it, such as super optimal broth (SOB) or SOB with catabolite repression (SOC). For optimal growth, pH of media should be adjusted to $\sim$ 7.0. A standard overnight (ON) incubation lasts for 12-17 h at 37°C. For liquid cultures, the incubation should be carried out while shaking at 225-250 rpm to ensure sufficient oxygen

supply.

Antibiotics are commonly used as a selection marker in cloning. In this thesis all plasmids used facilitate resistance against ampicillin (see appendix D for plasmid maps), whereas resistance to chloramphenicol is found in all BL21(DE3) cells. Chloramphenicol is therefore always added to growth media when cultivating BL21 cells. Unless stated otherwise, the following concentrations of antibiotics are used:
Ampicillin: 100 µg/mL
Chloramphenicol: 25 µg/mL

When preparing stock solutions of bacteria, cultures are mixed 50/50 with a 30% solution of glycerol and stored at -80 °C. Frozen bacteria should be thawed on ice.

All work with bacteria is performed using aseptic methods with sterilized equipment. Any genetically modified waste is autoclaved prior to disposal.

2.1.2 Inducing competence in *E. coli*

Bacteria have several mechanisms to obtain genetic material: conjugation, transduction and transformation. In conjugation, DNA can be transferred laterally between two bacterial cells through direct contact and in transduction, a bacteriophage may act as a vector, transporting DNA from one host to the next. Bacteria that are competent are also able to take up DNA molecules directly from their environment[61]. Competence is a natural property of some bacteria, but it may also be induced in bacterial strains with desirable properties, such as strains of the common model bacterium *E. coli*. In this thesis, *E. coli* strains XL1-Blue and BL21(DE3) were used. Two different methods of inducing competence were used for the different strains.

In subcloning techniques, resistance to antibiotics is commonly used as a reporter for successful transformation. When preparing bacterial stocks it is therefore necessary to check that no such resistance can be found in the stock. To do this, bacterial colonies are cultivated on complete LB medium, and restrictive medium LB + ampicillin (amp) and LB + kanamycin

(kan). Only clones that do not grow on restrictive plates should be used to prepare stock.

Inducing competence in XL1-Blue - Procedure

Day 1

1. Thaw bacteria on ice
2. Plate out 1, 1:100 and 1:1000 dilutions onto LB plates and incubate at 37°C overnight

Day 2

3. Pick a few colonies and streak each colony onto three different plates: LB, LB + 50 µg/mL ampicillin and LB + 25 µg/mL kanamycin
4. Incubate the plates overnight at 37°C

Day 3

5. Select a colony that grows only on LB without antibiotics
6. Use the colony to inoculate 20 mL of liquid LB medium. Incubate overnight at 37°C while shaking at 200-250 rpm

Day 4

7. Use the pre-culture to inoculate 1 L LB medium, pre-warmed to 37°C
8. Grow while shaking at 200-250 rpm at 37°C
9. Check optical density (OD) at 600 nm every ten minutes. At OD_{600}=0.94, stop the culture growing by placing the flask into an ice-water bath and cool for about 15 min. From now on, always keep the cells on ice
10. Centrifuge at 4000g for 5 min at 4°C
11. Wash the cells twice with 200 mL cold dH_2O, and once with cold 10% glycerol
12. Centrifuge at 4000g for 5 min at 4°C
13. Resuspend the pellet in 4 mL cold glycerol/yeast extract/tryptone (GYT) medium
14. Aliquot to 50 µL and shock freeze in liquid nitrogen or an ethanol-dry ice bath
15. Store the aliquots at -80°C
16. Compare transformation abilities between the new bacteria and an old batch to confirm that the cells are sufficiently competent

Inducing competence in BL21(DE3) - Procedure

Day 1

1. Streak out BL21(DE3) LysS cells onto LB plates with 25 µg/mL chloramphenicol. Incubate overnight at 37°C

Day 2

2. Pick a few colonies and streak part of each colony onto three different LB plates:
   1) 50 µg/mL ampicillin and 25 µg/mL chloramphenicol
   2) 25 µg/mL kanamycin and 25 µg/mL chloramphenicol
   3) 25 µg/mL chloramphenicol
   Incubate overnight at 37°C

Day 3

3. Check that no colonies are found on plates with antibiotics. Resuspend cells from the LB plate without antibiotics in 4 mL SOB- or LB-medium using a bent glass rod. Transfer resuspension to a sterile tube. Remove 100 µL, dilute to 1 mL and measure OD_{600}
4. Calculate how much resuspension is needed for an inoculation of 100 mL LB + chloramphenicol to reach OD_{600} of 0.05-0.1. Incubate cells at 37°C until OD_{600} is approximately 0.3
5. Put 100 mM $CaCl_2$ on ice
6. When OD is sufficient, put cells on ice for 10 minutes
7. Transfer the cells to two 50 mL sterile tubes. Centrifuge at 5000g for 10 minutes at 4°C
8. Remove supernatant and resuspend cells in 10 mL chilled 100 mM $CaCl_2$ pr tube
9. Leave on ice for 5 minutes
10. Centrifuge as before
11. Remove supernatant and resuspend cells in 2 mL chilled 100 mM $CaCl_2$ pr tube
12. Leave on ice for at least 30 minutes. Competence increases if cells are left longer on ice

13. Make 200 µL aliquots and store at -80°C
14. Compare growth of transformants with the old batch of bacteria to confirm that cells are competent

2.1.3 Transformation of bacteria

The process of modifying competent bacteria to express selected DNA through recombinant DNA technology, is a practice that has immense value, both for research and industry. Heterologous DNA can be expressed by *E. coli* in two ways: it may be inserted into the host genome, constituting a stable transformation, or the DNA may be introduced to the cell via a self-replicating cloning vector, such as a plasmid. When using this latter, so-called transient, transformation, the inserted DNA will be contained in the plasmid within the cell and may be lost or diminished through mitosis. Although stably transformed cell lines may be more reliable in the long run, transiently transformed cells are more than adequate when performing short-term experiments.

To insert a DNA sequence of interest, for example a gene, into bacteria, the DNA sequence must first be introduced to the plasmid vector. In order to verify the success of a transformation, these plasmids often contain selection markers. The selection markers are genes that affect the transformed bacteria with an easily distinguishable phenotype, such as resistance to antibiotics. If the recombinant plasmid contains a gene encoding resistance to an antibiotic, only successful transformants will be able to survive if the bacteria are cultivated on media containing the antibiotic in question.

When competent bacteria are transformed, they are exposed to environments that temporarily increases cell permeability by weakening cell walls and membranes. Examples of such treatments are heat shock and electroporation. In this thesis, competent *E. coli* XL1-Blue were electroporated using ECM® Electroporation System, BTX™ from Harvard Apparatus, and competent *E. coli* BL21(DE3) were transformed using heat shock.

Transformation by electroporation procedure

1. Put 2 mm electroporation cuvettes on ice, pre-warm SOB medium to 37°C and thaw electrocompetent bacteria on ice
2. Add approximately 1 ng DNA template to 50 µL bacteria. Transfer the mix to cuvettes
3. Pulse the cuvettes at 1800 V
4. Immediately after the pulse, add 500 µL pre-warmed SOB medium to the bacteria mix.
5. Transfer the bacteria-mix to microcentrifuge tubes and incubate the tube on a shaking heating block at 37°C, 240 rpm for 1 h
6. Transfer 150 µL of the bacteria-mix to an LB agar plate with appropriate selection markers and incubate at 37°C overnight
7. The rest of the bacteria-mix can be stored at 4°C overnight and be used the day after if no colonies appear. In that case, spin the bacteria down and resuspend in a smaller volume before adding to a new agar plate

Transformation by heat shock procedure

1. Thaw competent cells on ice
2. Mix 200 µL cells with 1 µL plasmid (∼50-100 ng/µL) in microcentrifuge tubes
3. Incubate on ice for 20 minutes
4. Incubate cells in a 42°C waterbath for exactly 45 seconds. Immediately put cells back on ice
5. Incubate cells on ice for 2 minutes
6. Add 800 µL LB medium
7. Incubate at 37°C, while shaking at 240 rpm for minimum 30 minutes
8. Centrifuge cells at 11 000g for 30 seconds. Remove 800 µL supernatant and resuspend cell pellet in remaining media
9. Streak out bacteria onto LB or SOB agar plates with appropriate selection markers.
10. Incubate overnight at 37°C

2.1.4 Isolation of plasmids from bacterial cultures

Isolating DNA from bacteria is a useful technique, especially in unison with recombinant DNA techniques, as it allows cheap and rapid amplification of recombinant plasmids. The isolation involves lysing of the cells and cleaning of the lysate in several successive steps. The simplest way is to use pre-made kits, which include all the materials and buffers needed. QIAGEN provides several types of kits that differ in throughput, called miniprep, midiprep and maxiprep.

The cells are harvested and resuspended in a provided buffer before they are exposed to an alkaline lysis buffer containing sodium dodecyl sulphate (SDS) that break the cell walls. After incubation, a neutralizing buffer is added. For the miniprep kit, the lysate is then transferred to a cartridge containing a silica filter. Under the appropriate pH conditions and in the presence of certain salts from the buffers, the silica filter selectively binds plasmid DNA. Wash buffer is used to rinse the DNA bound to the filter. Then the DNA can be eluted by adding buffers that breaks the interaction between plasmid DNA and the silica filter. The maxiprep procedure greatly resembles the miniprep procedure, but in place of a silica-gel membrane, plasmid DNA is selectively bound by an anion-exchange column.

Procedure maxiprep and miniprep

In this thesis, the QIAprep® Spin Miniprep Kit and EndoFree® Plasmid MAXI Kit from QIAGEN were used. The isolation of plasmid DNA from bacterial cultures were executed according to the Quick-Start protocols included in the kits [62, 63]. Miniprep was performed using centrifuge processing, and washing with buffer PB was omitted.

2.1.5 Concentration and purity measurement of nucleic acids

The concentration and purity of DNA can be checked using a spectrophotometer. Nucleic acids absorb light at wavelength 260 nm, and the aromatic amino acids tryptophane, tyrosine and, to a lesser degree, phenylalanine, absorb light at 280 nm. A spectrophotometer can send light of either wavelength through the sample, and measure how much of it passes through

to the photodetector on the other side. The amount of light of each wavelength that has been absorbed by the sample will depend on the concentration of nucleic acids or aromatic amino acids in the sample. The ratio of absorbance at the two wavelengths is used as an indicator of sample purity. Generally, DNA is said to be sufficiently pure if the 260/280 ratio is ∼1.8, and RNA is sufficiently pure at 260/280 ratio ∼2.0 [64].

In this thesis, a NanoDrop 2000 spectrophotometer from Thermo Scientific was used to measure absorbance of nucleic acids, and its software used to calculate absorbance ratios and concentrations.

2.1.6 Restriction enzyme digestion

To create recombinant DNA, one must be able to excise targeted pieces of DNA and adhering them to other DNA sequences. The excising is carried out by restriction enzymes, proteins that recognize and bind to specific DNA sites and cut the DNA strands at these particular sites. Originally a part of a cells defence against viral infections, restriction enzymes will cleave any recognized sequence of DNA not protected by methylation. Many restriction enzymes with different recognition sites have been studied, and hundreds are available commercially. Restriction enzymes will produce either "blunt ends" or "sticky ends" of DNA (see figure 2.1). In blunt-end restriction, the two sugar-phosphate backbones of DNA are nicked at the same site, whereas enzymes that produce sticky ends nick the backbones at different sites, creating a small overhang of nucleotides

```
5'...CCC|GGG...3'     →     5'...CCC    GGG...3'
5'...GGG|CCC...3'           3'...CCC    GGG...5'


5'...G|AATTC...3'     →     5'...G    AATTC...3'
5'...CTTAA|G...3'           3'...CTTAA    G...5'
```

Figure 2.1: Blunt and sticky end restriction digestion
Digesting DNA with SmaI will result in blunt end DNA (top), and digesting with EcoRI leads to sticky end DNA (bottom)

In this way, any two DNA strands cleaved with the same restriction enzyme will be compatible, and can be adhered together through a process known as ligation.

In this thesis, restriction analysis was performed using Thermo Scientific FastDigest™ restriction enzymes. The protocols are written based on recommendations from Thermo Scientific [65].

Procedure

1. Make a reaction mixture per sample as indicated in table 2.1. Mix reagents at room temperature in the order indicated

Table 2.1: Restriction reaction components
The table shows components included in a reaction mixture for restriction analysis.

Component	Volume	
	Plasmid DNA	**PCR product**
dH_2O	to a total of 20 µL	to a total of 30 µL
10x reaction buffer	2 µL	3 µL
DNA	1 µg (0.5-2 µL)	∼0.2 µg
Restriction enzyme	1 µL (10 U/µL)	1 µL (10 U/µL)

2. Incubate the samples on a heating block at 37°C. Incubation times depend on the enzymes used. In this thesis, FastDigest™ enzymes and 10x FastDigest™ reaction buffer from Thermo Scientific was used. These enzymes are effective in 15 minutes, but incubation time may be extended if desirable. Make sure incubation time does not exceed time of star activity of the enzymes in question
3. If performing successive reactions using several enzymes, inactivate by heating to 80°C for 5 minutes before new enzyme is added. Alternatively, several enzymes can be used simultaneously
4. To prevent re-ligation of DNA, add 1 µL alkaline phosphatase and incubate at 37°for 30 minutes (optional)
5. The reaction can be stopped by adding 6x DNA loading dye or by heat inactivation
6. Check the restriction fragments by agarose gel electrophoresis (see section 2.1.8)
7. Store samples at 4°C

2.1.7 Ligation

To adhere two nicked DNA sequences together, one makes use of DNA ligase. Ligases are a family of proteins that bind to DNA and repair double-stranded breaks in DNA. When setting up a ligase reaction you need to calculate the amounts of each fragment needed and the ratio (R) between them, taking the sizes of the two fragments into consideration. Equation 2.1 shows the relationship between these values.

$$\frac{ng\ insert}{bp\ insert} / \frac{ng\ vector}{bp\ vector} = R \tag{2.1}$$

It may be necessary to optimize the ratio between the two fragments you want to adhere. The reaction is most efficient when the total amount of DNA in a ligation reaction does not exceed 100 ng. In this thesis, optimization of ligation reaction involved setting up three parallel reactions, containing 75 ng vector DNA each, and using vector/insert ratios of 1:2, 1:5 and 1:10.

Procedure

1. Set up ligation reactions in microcentrifuge tubes on ice, following table 2.2

Table 2.2: Components of a ligation reaction
The table shows components necessary for a ligation reaction. Amount of insert DNA is calculated using equation 2.1

Component	Volume
T4 DNA ligase buffer, 10x	2 µL
Vector DNA	Variable (∼75 ng)
Insert DNA	Variable
T4 DNA ligase	1 µL
Nuclease-free water	To a total of 20 µL

2. Incubate at 16°C overnight
3. Inactivate enzymes by heating to 65°C for 10 minutes
4. Chill on ice

2.1.8 Agarose gel electrophoresis

Gel electrophoresis is a widely used technique that enables separation of DNA fragments based on size. The method utilizes the inherent negative charge of DNA molecules by introducing an electrical current through a closed system containing the DNA samples. The samples are loaded onto an agarose gel, a meshwork of polysaccharide polymers, which will retain DNA molecules as they are carried towards the anode of the system. The size of the pores in the gel determines how fast a DNA molecule can move, because small molecules will be able to travel through the pores of the gel with less retention than larger molecules. The size of the pores in the gel can be modified to accommodate sufficient separation of DNA fragments of various sizes, by simply changing the agarose percentage. A small percentage of agarose ($\sim 0.5\%$) is used to separate large fragments, and a large percentage ($\sim 2.0\%$) is used to separate very small fragments.

One way to visualize DNA in the gel is to add ethidium bromide (EtBr) to the gel. EtBr is a fluorescent chemical, visible under UV light. The intensity of fluorescence intensifies ∼20-30 fold upon intercalation with DNA [66]. When looking at the gel under UV light, EtBr will therefore reveal the presence of even very small amounts of DNA.

To determine the sizes of DNA fragments, one may load a sample containing fragments of known sizes, called DNA ladders or DNA size standards, to the gel. The position of the fragments of known sizes may then be compared to the position of the unknown samples, to roughly determine their size. In this thesis, GeneRuler™ 1 kb and 100 bp DNA ladders from Thermo Scientific were used.

Procedure

1. Mix agarose powder with 1x Tris-acetate-EDTA (TAE) buffer. The amount of agarose powder used depends on the size of the DNA fragments to be separated. For the experiments performed in this thesis, 1% [w/v] gels were used.
2. Microwave the mix until the agarose is dissolved
3. Allow the agarose TAE to cool to about 50°C

4. Add EtBr to a final concentration of 0.5 µg/mL
5. Prepare a gel casting tray with combs, and pour the agarose gel into the tray. Allow to solidify for 20 minutes
6. Place the solidified gel into the electrophoresis chamber, remove the comb, and fill the chamber with 1x TAE buffer until the entire gel is submerged
7. Load the DNA ladder and all the samples into separate wells
8. Run the gel at 80 V, 240 mA for 40-60 minutes
9. Take a picture of the gel under UV light

2.1.9 Polymerase chain reaction

The polymerase chain reaction (PCR) is another essential method in cloning. The method is used to amplify specific DNA molecules, utilizing DNA polymerases. Polymerases are a family of enzymes able to bind to a DNA-primer pair and elongate the primer, using the DNA strands as template (figure 2.2)

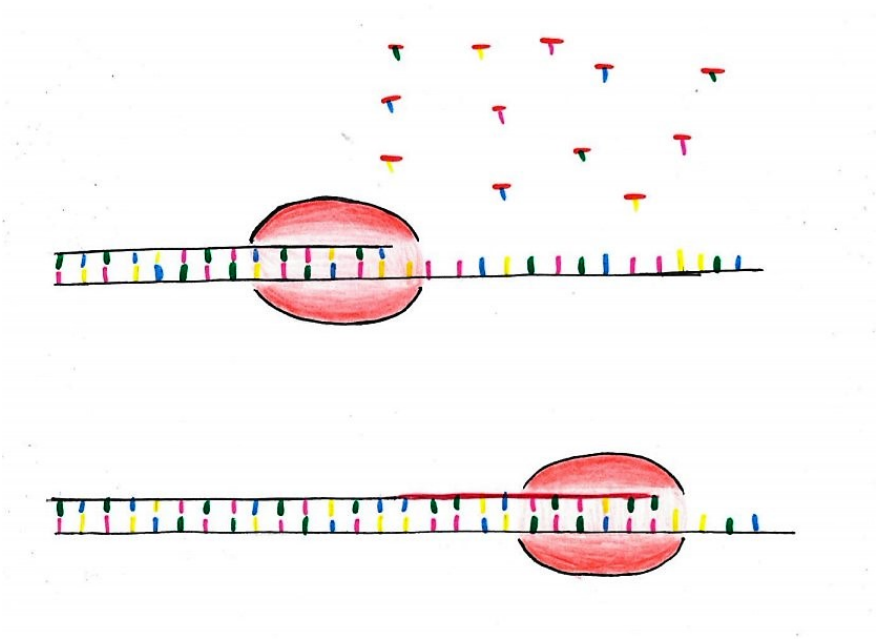


Figure 2.2: Primer elongation by DNA polymerase
DNA polymerase bind to a DNA-primer pair and uses the DNA sequence to elongate the primer, so a double-stranded DNA molecule is formed.

The reactions takes place in a pre-programmable thermal cycler, and requires only a few components. The principle behind the method is to heat DNA until it denaturates (∼94°C), then cool it to a temperature where primers may anneal to the single strands of DNA. After that the temperature is increased to the optimal temperature of the polymerase, which will use free deoxynucleotide (dNTPs) added to the reaction mixture, to elongate the primer and form a second strand of DNA, complementary to the initial single strand. The temperature of the reaction mixture is increased to $\sim$ 94°C again, and the cycle is repeated. In this way, for each cycle, the number of DNA molecules is doubled, and so large amounts of DNA molecules can be generated in a short time.

PCR reactions can be optimized by adjusting annealing time and/or temperature. For example, if increased specificity is desired, it is possible to run a touchdown-PCR, where annealing temperature is gradually decreased over the course of the PCR run. Higher annealing temperature requires high specificity between DNA and primer to anneal. The first cycles of PCR will therefore allow for replication of only DNA strands with high specificity for the primer. In later cycles, annealing temperature is lowered to reach the optimal annealing temperature, allowing for less specific binding, increasing chance of successful replication of DNA.

In this thesis, PCR is performed on purified DNA and on complex solutions, such as cell lysate. PCR on unpurified cell lysates, known as colony PCR, may have a lower success rate due to the level of noise in the sample. In these circumstances a touch-down PCR program was used, using Colony PCR Buffer.

Several polymerases suitable for PCR are commercially available. In this thesis Taq and Pfu polymerases were used, along with their respective pre-made buffers. Taq polymerase is efficient, but more prone to make small errors than Pfu polymerase. Consequently, Pfu polymerase is often used when it is especially important to amplify DNA with as few errors as possible. PCR cycles was performed using 2720 Thermal Cycler from Applied Biosystems.

Procedure

1. If performing colony PCR, prepare template by transferring a small amount of cells to a PCR tube containing 25 µL 0.25% SDS. Incubate at 95°C for 5 minutes, then put tube on ice. Use 1 µL of the cell lysate as template in the PCR reaction
2. Make reaction mixtures containing reagents shown in table 2.3. Prepare reactions on ice in 0.2 mL PCR tubes
3. Run the reaction mixtures on a pre-programmed thermal cycler. Use program showed in table 2.4 for touchdown PCR and colony PCR, and program showed in table 2.5 for PCR using Pfu polymerase
4. Store the PCR products at 4°C

Table 2.3: PCR reaction mixes
The table shows reagents and volumes used for three different PCR setups used in this thesis. PCR using Taq polymerase is used with Taq polymerase buffer, unless cell lysates are used as template, in which case colony PCR buffer is used. PCR using Pfu buffer is used with Pfu polymerase buffer.

Reagent	**Colony PCR**	**Taq PCR**	**Pfu PCR**	**Final concentration**
Template	1-4 µL	1-4 µL	1-4 µL	
Colony PCR buffer [10x]	2.50 µL			1x
GoTaq buffer [5x]		5.00 µL		1x
Pfu reaction buffer [10x]			2.50 µL	1x
Forward primer [5.0 µM]	2.00 µL	2.00 µL	2.00 µL	0.40 µM
Reverse primer [5.0 µM]	2.00 µL	2.00 µL	2.00 µL	0.40 µM
dNTP [5.0 mM]	1.00 µL	1.00 µL	1.00 µL	0.20 mM
$MgCl_2$ [25.0 mM]	1.50 µL			1.50 mM
Triton™ X-100 [20%]	1.25 µL			1.00 %
Taq polymerase [5.0 U/µL]	0.25 µL	0.25 µL		0.05 U/µL
Pfu polymerase [2.5 U/µL]			0.50 µL	0.05 U/µL
dH_2O	To 25 µL	To 25 µL	To 25 µL	

Table 2.4: Touchdown/Taq PCR program
The table shows the touch-down program used in PCR with Taq polymerase

Temperature	Process	Time	Cycles
94°C	Denaturation	4 min	
94°C	Denaturation	30 s	5
62°C	Annealing	30 s	
72°C	Elongation	2 min	
94°C	Denaturation	30 s	25
60°C	Annealing	20 s	
72°C	Elongation	2 min	
72°C	Elongation	7 min	
4°C	Cooling down	∞	

Table 2.5: Pfu PCR program
The table shows the program used for PCR performed with Pfu polymerase

Temperature	Process	Time	Cycles
94°C	Denaturation	4 min	
94°C	Denaturation	30 s	35
62°C	Annealing	30 s	
72°C	Elongation	2 min	
72°C	Elongation	7 min	
4°C	Cooling down	∞	

2.1.10 Isolation of DNA from agarose gels and cleaning of PCR products

If one wishes to continue using DNA separated on an agarose gel or produced using PCR, the products must first be cleaned. A kit from Promega called Wizard® was used for this purpose. When extracting from gel, the process involves cutting out a gel slice containing the DNA fragment to be isolated and melting the gel together with a membrane binding solution. For PCR, the end product of the reaction is simply mixed with membrane binding solution. For either procedure, the mixture is then transferred to a cartridge containing a filter that binds DNA. When bound to the filter, DNA can be washed with ethanol and later eluted from the filter with water.

Procedure

In this thesis, the Wizard® SV Gel and PCR Clean-Up System from Promega was used. The experiments were conducted according to their protocol for gel slice preparation and DNA purification by centrifugation [67].

2.1.11 Sequencing

All sequencing in this thesis was performed using Lightrun barcodes from GATC Biotech (www.gatc-biotech.com). 5 µL DNA solution with a concentration of 20-80 ng/µL for PCR product and 80-100 ng/µL for plasmid were mixed with 5 µL 5 µM primer. The tubes were labelled with barcodes and sent to GATC. In this thesis, only one primer was used when sequencing.

2.1.12 Subcloning

Subcloning is the name given to the process of moving a specific DNA sequence into a vector. Subcloning can be performed by using classical methods described in this chapter - PCR, restriction enzyme digestion, ligation, bacterial transformation, miniprep, and agarose gel electrophoresis (see figure 2.3). This process is tedious, and consists of many steps that may go wrong. Alternatively, a streamlined protocol for subcloning is available from Invitrogen, called Gateway® technology.

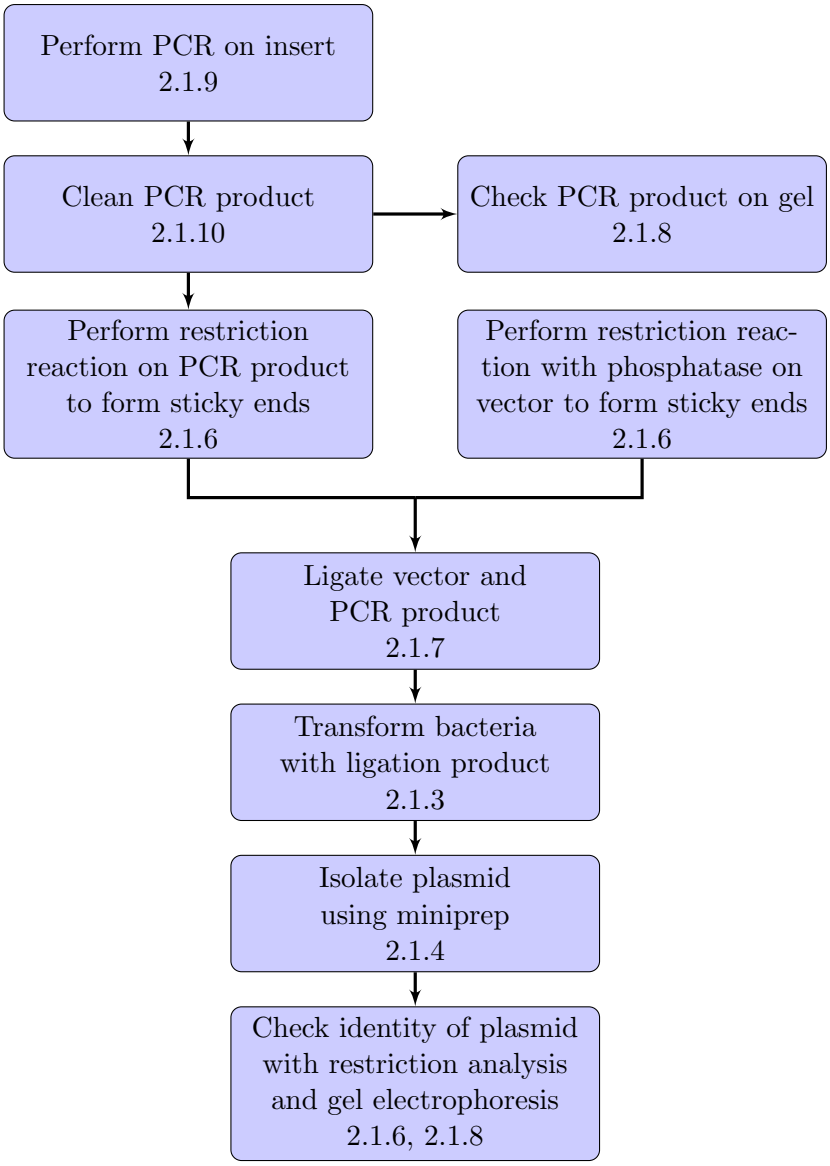


Figure 2.3: Pipeline of procedures used in conventional subcloning
The overview shows the procedures used to subclone a DNA sequence called "insert" into a vector

The Gateway® system of bacterial cloning is based on the ability of bacteriophage λ to integrate its genome to, and later excise it from, its bacterial host. To perform a Gateway® cloning, you need a PCR product flanked by *attB*-sites, and a donor vector containing a negative marker gene flanked by *attP* sites (figure 2.4). One example of a negative marker gene is *ccdB*, which kills *E.coli* if it is present in a plasmid in the cell.

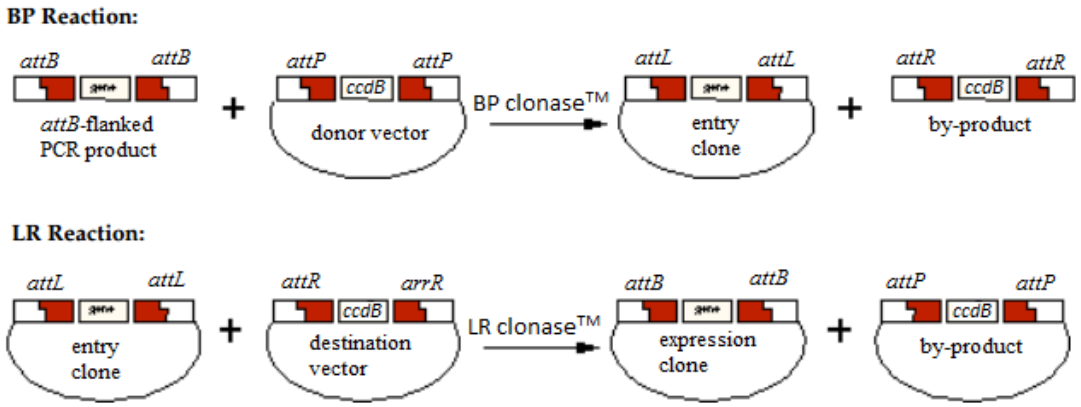


Figure 2.4: Schematic illustration of Gateway® cloning
Figure adapted from [68]

The *attB*-PCR product and the donor vector are mixed and exposed to an enzyme mixture called BP clonase™. This mixture contains the enzymes bacteriophage λ Integrase (Int) and *E.coli* integration host factor (IHF). The products of the reaction are "entry clones", containing the PCR gene flanked by *attL*-sites, and a byproduct consisting of the *ccdB* gene flanked by *attR*-sites. In the follow-up reaction, the entry clone is mixed with a destination vector, containing the same marker gene, *ccdB*, flanked by yet another pair of attachment sites, namely *attR*. LR clonase™, containing containing Int, IHF and bacteriophage λ Excisionase (Xis) is added to the reaction. The enzyme mix catalyzes the recombination of entry clone and destination vector to form an expression clone containing the PCR product flanked by *attB* sites, and a by-product containing the marker gene *ccdB* flanked by *attP* sites.

In this thesis the entry and destination vector used were pENTR-3C-DEXI, prepared by Pankaj Keshari in our lab by conventional subcloning, and pDEST-3x-FLAG, which was provided by Anne Simonsen (UiO).

Gateway® LR recombination procedure

1. Add the following components to a 1.5 mL tube at room temperature and mix.

   50-150 ng entry vector

   150 ng destination vector

   TE-buffer, pH 8.0 to a total of 8.0 µL

2. Thaw LR clonase™ II enzyme mix on ice for about 2 minutes. Vortex the mix briefly twice (2-4 seconds each time)
3. Add 2 µL LR clonase™ II enzyme mix to each sample and mix well by vortexing briefly twice
4. Incubate the reactions at 25°C for 1h
   Note that the time can be extended up to 18 h. For large plasmids (≥ 10 kb) longer incubation time is required
5. Add 1 µL Proteinase K solution to each sample to terminate the reaction. Vortex briefly and incubate for 10 min in 37°C

2.2 Protein techniques

2.2.1 Protein expression

When studying or performing experiments with a specific protein, it may be helpful to overexpress the protein in bacterial cells. One method to accomplish this is to place a gene under the regulation of a lac operator. Transcription of the lac operone is originally induced by allolactase, but may also be induced by adding a chemically similar molecule, isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells that are transformed with plasmids containing a gene of interest in a lac operon can be cultivated with IPTG in the medium to overexpress the gene product of interest. In this thesis, protein expression is performed in BL21(DE3) cells.

Procedure

Day 1

1. Transform BL21(DE3) cells with a plasmid containing a gene of interest under the regulation of a lac operon using the heat-shock protocol (section 2.1.3)

Day 2

2. Add 4 mL liquid SOB medium to the plate and use a bent glass rod to resuspend

colonies. Transfer to a 15 mL tube

3. Remove 100 µL and dilute it with 900 µL SOB medium. Measure OD_{600}
4. Calculate how much cell resuspension needed for a 100 mL SOB + ampicillin dilution to have $OD_{600} \approx 0.05$

$$\frac{100\ mL \cdot 0.05}{measured\ OD_{600}} = mL\ of\ original\ resuspension \tag{2.2}$$

5. Transfer the diluted cell resuspension to a 250 mL Erlenmayer flask. Incubate the culture at 37°C, shaking at 200 rpm, until $OD_{600} \approx 0.7$
6. Transfer 100 µL of the cell culture to a microcentrifuge tube labelled "-IPTG". Centrifuge at $4000g$ for 3 minutes. Discard supernatant and freeze pellet at -80°C
7. Add IPTG to the flask to a total concentration of 0.4 mM and incubate at 37°C, 200 rpm for 2 h
8. Remove 200 µL culture and dilute it with 800 µL SOB medium. Measure OD_{600} and calculate how much volume will contain the same amount of cells as removed in step 6. Transfer calculated amount to a microcentrifuge tube labelled "+IPTG". Centrifuge at $4000g$ for 3 minutes. Discard supernatant and freeze pellet at -80°C
9. Transfer cell culture to 50 mL tubes and chill on ice for 10-15 minutes
10. Centrifuge at $6000g$ for 10 minutes at 4°C
11. Remove supernatant and resuspend in a total of 10 mL cold Tris-HCl, EDTA, NaCl (TEN)-buffer. Pool the suspension and centrifuge at $6000g$ for 10 minutes at 4°C
12. Remove supernatant and resuspend in 2 mL Tris-HCl, EDTA, glycerol $(TGE)_{500}$ with protease inhibitor cocktail (PIC) and freeze tubes at -80°C overnight.
13. Lyse cells as described in section 2.2.2
14. Run SDS-PAGE (section 2.2.5) with the following samples:
    -IPTG: Cell pellet from step 6, resuspended in 30 µL 3x SDS loading dye (LD)
    +IPTG: Cell pellet from step 8, resuspended in 30 µL 3x SDS LD
    Lysate: 3 µL cell lysate from step 13 + 30 µL 3x SDS LD
15. Stain gel with Coomassie Blue, as described in section 2.2.6

2.2.2 Cell lysis

Cell lysis is the process of breaking cell walls and/or membranes in order to extract proteins from the cell. Cell membranes can be ruptured by physical disruption, like sonication, and/or by exposing cells to chemicals such as detergents. Many cells contain proteases, enzymes whose function is to break down proteins. To avoid this, a protease inhibitor cocktail (PIC) is added to lysis reactions to prevent degradation of proteins in the lysate.

In this thesis RIPA lysing was performed on Jurkat cells, whereas lysis with Triton™ X-100 or BugBuster®, with or without sonication was used to lyse *E. coli* cells.

Procedures

RIPA lysis of Jurkat cells

1. Add 20 µL RIPA lysis buffer with 1x PIC per $1 \cdot 10^6$ Jurkat cells
2. Vortex tubes and put them on a rotator machine at 4°C for 30-60 min
3. Centrifuge at 12,000g, or maximum speed, at 4°C for 15 minutes
4. Transfer supernatants to new, chilled microcentrifuge tubes. Store the lysate at -20°C

Lysis of *E. coli* with detergents

1. Thaw 2 mL *E. coli* cells resuspended in TGE_{500} (see section 2.2.1) on ice. Add
   2 µL 1 M DTT, 40 µL 50 mM PMSF and 10 µL 20% Triton™ X-100
   Or
   200 µL BugBuster® 10x protein extract reagent
   Mix until solution is viscous
2. *Optional:* sonicate cells 3 x 10 seconds, with 10 s pause
3. Ultracentrifuge tubes at 20 000g for 2 h at 4°C
4. Transfer supernatant to new microcentrifuge tubes and store at -80°C
5. *Optional:* Resuspend cell pellets in 300 µL dH_2O each, and sonicate as described above

2.2.3 GST pull-down assay

The Glutathione S transferase (GST) pull-down assay is a method used to determine whether interaction occurs between two specific proteins. One of the proteins, the bait, is fused with GST, and mixed with glutathione sepharose® (GS) beads, which have a high affinity with GST (figure 2.5). The other interacting protein, the prey, is overexpressed in cells, and a lysate is prepared. Mixing the GST-fusion protein, bound to GS beads with the lysate enables the two proteins to interact. The complex formed can then easily be pulled out of the lysate with centrifugation, due to the weight of the GS beads. After pull-down, a Western blot (section 2.2.7) against the prey will reveal whether the prey was a part of the interaction complex, and by that, reveal whether there was interaction between the bait and the prey. It is necessary to run a parallel pull-down using only GST without bait protein as a control.

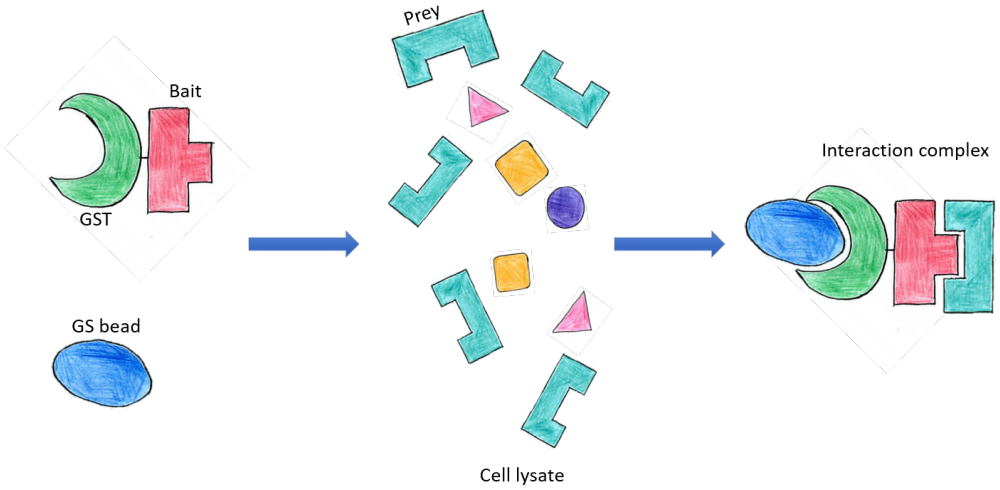


Figure 2.5: Schematic illustration of GST pull-down
A bait protein is fused with glutathione S transferase (GST), a protein with a high affinity to GS beads. When beads and GST-bait fusion protein is run through cell lysate containing the proposed interaction partner, a complex will form between the bait and its interaction partner. The weight of the GS beads will facilitate easy separation of the complex from lysate with centrifugation

The entire GST pull-down assay requires many procedures (figure 2.6). First, the bait protein must be fused with GST. In this thesis, the GST expression plasmid pGEX-6p-1 was used as a vector, and this plasmid was used to overexpress GST-fusion protein in *E. coli* BL21(DE3) cells. A cleaning protocol was then used to separate the GST-fusion protein

from the rest of the *E. coli* lysate. SDS-PAGE (section 2.2.5) was run on these samples to confirm the presence of GST-fusion protein in the solution. In parallel, the prey protein is overexpressed in Jurkat cells (section 2.4.2), and a RIPA lysate is prepared from these cells (section 2.2.2). Western blot (section 2.2.7) is performed on the Jurkat lysate to confirm presence of prey protein. When bait protein is succesfully cleaned from *E. coli* lysate, and prey protein detected in Jurkat cell lysate, the pull-down can be performed.

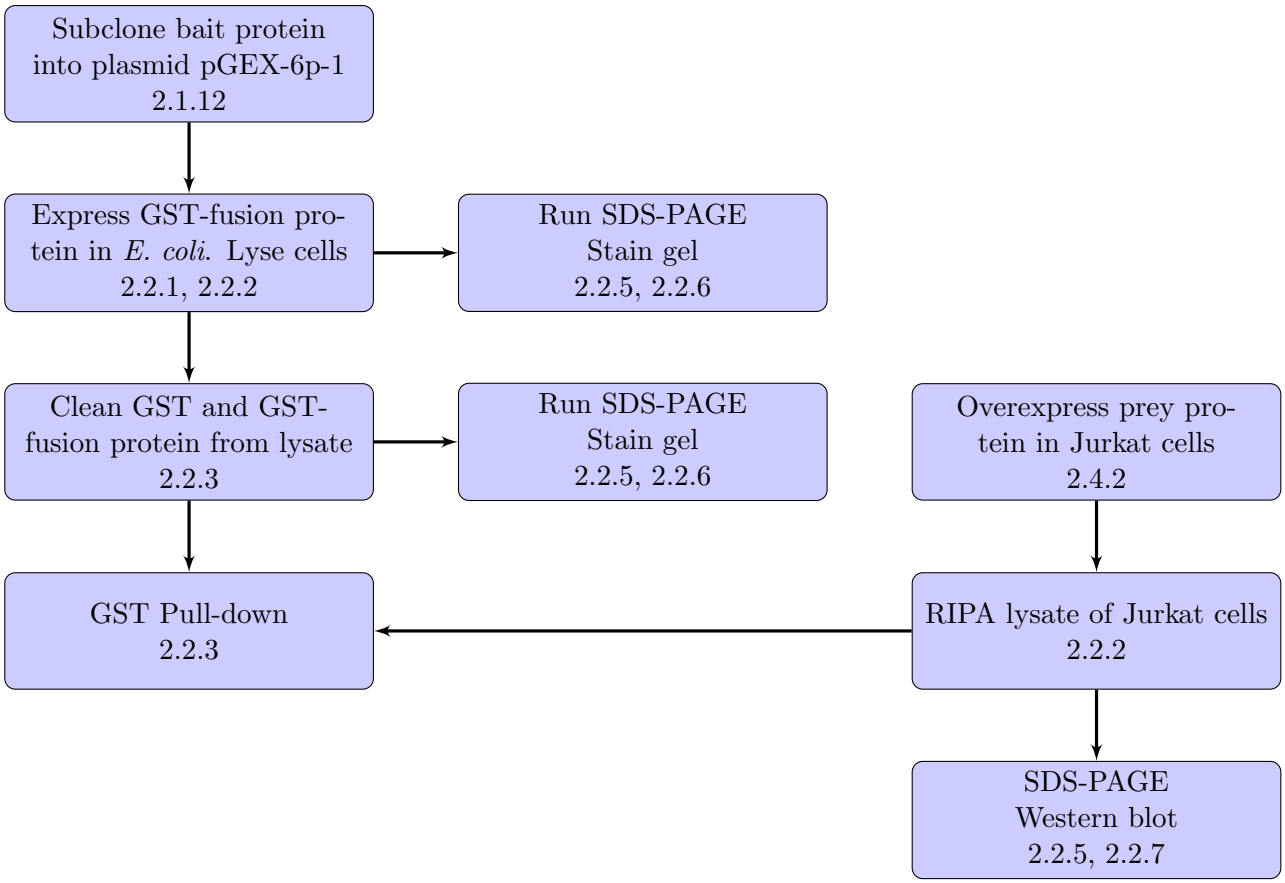


Figure 2.6: Overview of GST pull-down assay
Figure shows methods involved in a GST pull-down assay with reference to subsections where procedures can be found

Procedure

Cleaning GST fusion protein

1. 40 µL GS beads in ethanol is required to perform one GST pull-down. To ensure that there is sufficient beads, 33% additional GS/ethanol volume is added to the reaction

mixture ($40\ \mu L \cdot 1.33\% = 53.2\ \mu L$)

2. For each sample, transfer 53.2 µL GS solution into a microcentrifuge tube. Add 3 mL cold binding buffer without DTT or protease inhibitor (PI) pr 400 µL start volume
3. Centrifuge at $500g$ for 5 minutes at 4°C and carefully remove the supernatant
4. Resuspend pellet in binding buffer without DTT or PI. Use 300 µL buffer pr 400 µL start volume
5. For each sample, mix the following into a microcentrifuge tube
   - 500 µL binding buffer with DTT and PI
   - 20/50/100/200 µL cell lysate containing GST-fusion protein or 10 µL cell lysate containing GST (see section 2.2.1).
   - 40 µL resuspended GS beads from step 4
6. Incubate on a rotator at 4°C for 1 h, or overnight
7. Centrifuge for 1 min, 4°C, $3000g$
8. Carefully remove supernatant and wash pellet three times with 500 µL binding buffer with DTT and PI. Centrifuge for 1 min, 4°C, $3000g$ between each wash. Discard the final supernatant
9. Add 20 µL 3x SDS LD to the pellet, and boil the samples at 95°C for 2 minutes
10. Run SDS-PAGE (section 2.2.5) with cleaned GST protein and stain gel with Coomassie Blue (section 2.2.6). Use gel to determine optimal volume of GST-fusion protein lysate to use in the pull-down

GST pull-down

11. Repeat steps 1-4
12. Mix 500 µL binding buffer with DTT and PIC, 40 µL GS-solution and GST-fusion protein cell lysate (volume determined in step 10)
13. Incubate on rotator for 1 h at 4°C
14. Centrifuge at $3000g$ for 1 minute at 4°C
15. Remove supernatant carefully and wash three times with 500 µL binding buffer. Centrifuge at $3000g$ for 1 minutes at 4°C between washing
16. Add 300 µL Jurkat lysate and incubate for 1 h on a rotator at 4°C
17. Wash twice with 500 µL interaction buffer. Centrifuge at $3000g$, 1 minute, 4°C between

washing

18. Remove supernatant and resuspend pellet in 20 µL 3x SDS LD. Heat to 95°C for 2 minutes and perform SDS-PAGE (section 2.2.5)and Western blot (section 2.2.7)

2.2.4 Bradford assay

Concentration of proteins in a cell lysate can be highly variable. One way to determine protein concentrations is the so-called Bradford assay. In the assay, different concentrations of bovine serum albumin (BSA) are stained with Coomassie Blue, an unspecific protein staining solution. OD_{595} is measured for each dilution, and a standard curve is made based on the OD values. A protein solution, like a cell lysate, can then be stained with Coomassie, and its OD can be used to estimate concentration based on the standard curve.

Procedure

1. Prepare two spectrophotometer cuvettes per protein solution + ten cuvettes for the protein standard curve
2. Thaw frozen protein solutions on ice
3. Add 1 mL Coomassie Blue to each cuvette. Keep cuvettes in the dark as much as possible
4. Add BSA to standard cuvettes according to the table below. Two parallels should be made of each dilution

Table 2.6: Bradford assay standard curve setup
Table shows volumes and final concentration of BSA in Coomassie Blue. The OD values of each sample is used to generate a protein concentration and absorbance standard curve

Volume BSA	0.5 µL	1.0 µL	2.0 µL	4.0 µL	8.0 µL
Final conc. BSA	1.0 µg/mL	2.0 µg/mL	4.0 µg/mL	8.0 µg/mL	16.0 µg/mL

5. Add 2 µL of sample to cuvettes with Coomassie Blue. Check that the samples are within colour range of the standard
6. Incubate at RT in the dark for 10-15 minutes
7. Measure absorbance at 595 nm, using 1 mL Coomassie Blue + 2 µL RIPA buffer as blank

2.2.5 SDS-PAGE

SDS polyacrylamide gel electrophoresis (SDS-PAGE) is a method used to separate proteins based on size, similar to an agarose gel electrophoresis. Because proteins consist of many amino acids with different physiochemical properties, one cannot use a single parameter directly to separate proteins by size, as charge is used when separating DNA molecules. Proteins may, however, be treated in a way to induce such a parameter. SDS is a detergent that denatures proteins, and introduces a negative charge relative to the number of amino acids. Reducing agents such as β-mercaptoethanol or DTT may be added to further denature the tertiary structures of proteins. After treatment with SDS, proteins may be separated by size on a gel by electrophoresis. For SDS-treated proteins, the gel is made up of polyacrylamide, and is often purchased pre-made. As in agarose gels, the separation resolution depends on the percentage of polyacrylamid in the gel. Protein- or molecular standards are added and used to determine sizes of proteins in the gel.

Neither the gel nor the samples contain any visualizing agent, so the gel must be treated in order to display the proteins within. This is done either by staining the gel with an unspecific colour that binds to all proteins in the gel (see section 2.2.6), or by blotting the gel to a membrane and immunostain it to visualize a specific protein, a process known as Western blotting (section 2.2.7).

In this thesis, 4-15% Criterion™ TGX™ and 10% mini-PROTEAN® TGX™ gels, as well as Precision Plus Protein™ colour standard from BioRad were used.

Procedure

1. Carefully pull out the comb from the gel. Wash the wells with dH_2O to remove any excess gel residues or air bubbles
2. Place the gel into the Electrode Assembly
3. Fill the inner chamber with running buffer so that the wells in the gel are completely covered with buffer. Fill the lower chamber with running buffer to cover the bottom of the gel

4. Add 50 µL β-mercaptoethanol to 1 mL 6x LD
5. Add LD to samples and heat to 95°C for 2 minutes
6. Load samples onto the gel
7. Apply the molecular standards
8. Run the gel at 100 V for 10 minutes, then at 120 V for 20-30 minutes
9. Disassemble the gel electrophoresis equipment

2.2.6 Coomassie staining of SDS-PAGE gels

For unspecific visualization of proteins on an SDS-PAGE gel, stain the gel using an all-round staining solution. In this thesis, Coomassie Brilliant Blue R-250 staining solution from BioRad was used along with a de-staining solution prepared in the lab.

Procedure

1. Carefully remove gel from the electrophoresis equipment and transfer to a small tray or box. Cover the gel in destaining solution. Incubate while gently shaking or tilting for 15 minutes
2. Pour off solution and cover the gel in Coomassie Brilliant Blue staining solution. Incubate on shaker for 30-60 minutes
3. Remove staining solution and rinse the gel in destaining solution and pour it off
4. Submerge gel in de-staining solution and incubate on shaker for 1 h or overnight at 4°C
5. Change destaining solution and rinse until excessive staining is removed

2.2.7 Western blot

After running a protein sample on an SDS-PAGE (section 2.2.5), a specific protein can be visualized from the gel by immunostaining. First the gel must be blotted onto a polyvinylidene difluoride (PVDF) membrane, or similar. The blotting is performed by another electrophoretic transfer, this time applying the current vertically on the surface of the gel, making

proteins move from the gel to the membrane without changing their position on the surface. Once blotting is complete, the membrane is first treated with a blocking agent to prevent unspecific binding of antibodies. Then the membrane is incubated with a primary antibody specific for the protein of interest, and finally with the secondary antibody, conjugated to the enzyme horseradish peroxidase (HRP). When adding the substrate of HRP to the solution, the enzyme catalyzes a reaction resulting in the emission of light. This reaction, known as enhanced chemiluminescence (ECL), may be observed using an exposure chamber, and the intensity of light can be used to quantify the amount of proteins in the gel.

Procedure

1. Wash pre-cast polyacrylamide gel in water
2. Put the gel in transfer buffer for 1-2 minutes
3. Cut out a piece of PVDF membrane and activate it in methanol for 15 seconds.
4. Wash the membrane in dH_2O and put it in transfer buffer
5. Soak two 3 mm blot filter papers in transfer buffer
6. Place one of the filters in the blotting apparatus. Place a sheet of plastic film around the filter in the blotting apparatus
7. Place the membrane on top of the filter
8. Place the gel on the membrane, then place the last filter on top of the gel
9. Press out potential air bubbles. Place the lid on the apparatus and run the blot at 50 V, 1.12 mA pr cm^2 for 1 hour
10. Block the membrane with 3% skimmed milk powder in TBS-T in room temperature for 1 h
11. Add the primary antibody in 3% skimmed milk/TBS-T. The dilution of antibody depends on the antibody in question
12. Incubate for 1 h at RT or at 4°C overnight
13. Wash the membrane 3 x 20 min in TBS-T
14. Add the secondary antibody in 3% skimmed milk/TBS-T. Incubate for 1 h at RT
15. Wash the membrane 3 x 20 min in TBS-T
16. Mix the two components A and B of Clarity™ Western ECL containing HRP substrate, in equal parts

17. Lift the membrane with a set of tweezers and allow the excess washing solution to run off on a piece of paper. Place the membrane on a plastic sheet
18. Add Clarity™ evenly to the membrane. Incubate for 5 min at RT
19. Lift the membrane and place it carefully, signal side up, on a piece of paper to dry it
20. Place the membrane between two plastic sheets and place in the exposure chamber, making sure that the signal side faces up. Take pictures of the membrane

2.2.8 Western blot loading control

After immunostaining a Western blot membrane, the antibodies can be stripped off the membrane, and the membrane may be re-probed using different antibodies. This is a useful technique to use when performing loading control. The intensity of a target protein on a Western blot will depend on the amount of sample loaded to the gel. By comparing the intensity of target protein bands with intensity of a uniformly expressed protein, such as actin, one should be able to determine whether some samples contain more of the desired protein than others. If the target protein is substantially separated from actin by size, the membrane may simply be cut in two, and the two halves may be probed with different antibodies. If target protein and actin are too close in size, the membrane may be probed with one set of antibodies, stripped, and then reprobed with a different set of antibodies. In this thesis, stripping of Western membrane was facilitated by Restore™ PLUS buffer from Thermo Scientific.

Procedure

1. Reactivate membrane in methanol for 5 seconds
2. Rinse the membrane in dH_2O
3. Soak the membrane in stripping buffer and incubate for 15 minutes
4. Wash the membrane 3 x 20 minutes in TBS-T
5. Repeat steps 10 to 20 from section 2.2.7, using primary antibody against actin

2.3 Yeast cell techniques

Budding yeast, *Saccharomyces cerevisiae*, is a common and well studied model organism used in molecular biology. It has a relatively short generation time, it can easily be transformed with exogenous DNA, and is unchallenging to keep in the lab. The life cycle of yeast cells involve a haploid and a diploid stage. Haploid cells show mild sexual differentiation, and can be divided into two categories: the a and the α mating type, depending on a single locus named *MAT*. Haploid cells of opposite mating types may mate and form diploid cells, that can continue to grow and reproduce by mitosis. In stressful environment, diploid cells may undergo sporulation and meiosis to revert to the haploid stage.

2.3.1 Growth and maintenance of yeast

In this thesis, the PJ69-4A and PJ69-4α strains of *S. cerevisisae* were used. The strains are auxotrophic for amino acids adenine (ade), histidine (his), leucine (leu) and tryptophane (trp). Both strains contain three genes placed under the control of GAL4 inducible promoters. Two of these reporter genes, *ADE2* and *HIS3* encode products that enable the cells to synthesize adenine and histidine, respectively. Expression of the third reporter gene, *MEL1*, enables the cells to produce the enzyme α-galactosidase. This enzyme cleaves substrate x-α-gal, resulting in a product that will colour yeast colonies blue.

Yeast cells can grow in liquid medium or on agar plates that provide a minimal of dextrose, nitrogen, phosphorous and trace metals. Strains that are auxotrophic for any amino acid will require the addition of these amino acids in their medium. Rich medium, containing additional reagents, will allow for more rapid growth of cells, but will not allow for selection based on reporter genes. In this thesis, yeast peptone dextrose adenine (YDPA) was used as a non-selective rich medium, whereas synthetic complete (SC) drop out medium lacking various amino acids were used as selective medium. The base recipe of SC lacks adenine, histidine, leucine, and tryptophan, but could easily be enriched with solutions of any of these amino acids according to necessity. Growth on YPDA rich medium allows for a doubling

time of approximately 90 minutes, and generally 48h of incubation is needed when cultivating cells on YPDA plates. Growth on drop out media such as SC requires twice as long (3-4 days). All cultivation is carried out at 30°C. When growing in liquid media, use $\leq$20% of the total volume of the flask and incubate while shaking 250-300 rpm to ensure proper aeration.

Stock solution of yeast cells can be prepared by mixing an overnight liquid culture with glycerol to a final concentration of 15%. Stock solutions are stored at -80°C. Cells can be revived by scraping cells off the frozen stock and streak out onto YPDA plates.

When preparing media, the following concentrations were used:
adenine, histidine, leucine and tryptohan - from a 50x solution (see appendix C) to a final concentration of 1x.
3AT - as determined by the autoactivation test
x-α-gal - from a 20 mg/mL stock to a final concentration of 0.04 mg/mL

All work with yeast cells is performed using aseptic methods with sterilized equipment. Any genetically modified waste is autoclaved prior to disposal. Yeast colonies cultivated on plates will quickly grow stale, so all experiments should be performed on fresh yeast colonies.

2.3.2 Transformation of yeast cells

Transformation of yeast can be achieved using various methods, including electroporation, enzymatic removal of cell wall (the Spheroplast method) or by chemical treatment with alkali cations [69]. All of these treatments leads to weakening of cell walls in order to facilitate movement of DNA across the plasma membrane and into the cells. A fast and reliable method for alkali cation transformation is using One-Step buffer [70] containing lithium acetate (LiAc).

Procedure:

1. Mix 100 µL One-step buffer with 10 µL 1 M DTT in a microcentrifuge tube
2. Inoculate each tube with a yeast colony
3. Add $\sim$ 1 µg plasmid. Vortex

4. Incubate on heating block set to 45°C for 30 minutes
5. Spread out 50 µL of each sample to a plate with suitable selection medium
6. Incubate at 30°C for 2-5 days

2.3.3 Autoactivation and toxicity test

When using yeast as a model system in studies involving mammalian proteins, there is a possibility that the protein will have an effect on the viability of the yeast. The potential toxicity may give false negative results in a yeast two-hybrid screening, and so the baseline effect of expressing the protein in yeast cells must be surveyed before a screening can take place. A toxicity test involves comparing growth of yeast cells transformed with a plasmid containing your bait gene with growth of yeast cells transformed with the same plasmid containing a gene whose product is known to be non-toxic in yeast. In this thesis, plasmid pDBT containing the bait, DEXI, was compared to pDBT-c-Myb.

In Y2H screens, there is also a possibility that the protein may have unforeseen effects on the reporter system used. It is therefore necessary to check for expression of reporter genes in absence of an interaction partner. This form of activation of reporter genes is called autoactivation, and it may lead to false positive results in a Y2H screening. In this thesis, the Y2H screen involves expression of several reporter genes, including *HIS3*. If autoactivation is detected, it can be inhibited by adding a competitive inhibitor of *HIS3*, 3-amino-1,2,4-triazole (3AT) to the medium. Optimization is required to find the lowest possible concentration of 3AT that still inhibits autoactivation. c-Myb is known to be able to autoactivate the reporter system used in this thesis, and was used as a positive control for autoactivation.

Procedure:

1. Prepare plates with SC/-trp, SC/-trp/-his, and SC/-trp/-his with different concentrations of 3AT (for example 1 mM, 2 mM, 5 mM, 10 mM, 25 mM and 50 mM)
2. Pick colonies of yeast strain PJ69-4α transformed with pDBT-DEXI using a sterile loop and resuspend them in 3 mL dH_2O.
3. Do the same for colonies transformed with the empty pDBT vector (negative control

for autoactivation) and colonies transformed with pDBT-c-Myb (positive control for autoactivation)

4. Pipette 1 mL of each yeast solution into a cuvette and measure OD_{600} using a UV-spectrophotometer.
5. Dilute suspensions to an OD_{600} of 0.1 (equals to approximately 1000 cells/µL).
6. Make 10x dilutions so that you have 1000, 100, 10, and 1 cells/µL
7. Drip 2 µL of each dilution onto SC/-trp, SC/-his/-trp and SC/-his/-trp/+3AT plates.
8. Allow the cells to grow in a heating cabinet at 30°C for for 1-3 days.

2.3.4 Yeast two-hybrid mating

The yeast two-hybrid system is a useful method to find protein interaction partners for a protein, where no established interaction partners are identified. The method is described in section 1.4.1. In this thesis, a Matchmaker Gal4 Y2H screening was performed, using DEXI as a bait to look for interaction partners in a library generated from cDNA from human thymic tissue. DEXI was fused to Gal4 DB by subcloning DEXI into vector pDBT.The subcloning was performed by someone in our lab. The resulting plasmid pDBT-DEXI, containing reporter gene *TRP1* was used to transform PJ69-4α cells. Yeast cell strain PJ69-4A containing plasmid pACT2 with a cDNA library from human thymic tissue was provided by Marit Ledsaak (UiO). Vector pACT2 contains reporter gene *LEU2*. PJ69-4α and PJ69-4A are genetically identical strains, except for the mating locus. Mated cells were screened for expression of *HIS3*, *ADE2* and *MEL1* successively.

Procedure

Day1

1. Cast approximately 60 14 cm plates of SC/-his/-leu/-trp/+3AT, and set to dry at RT covered in plastic. Use ∼40 mL medium pr plate. This must be done in advance to ensure that they are sufficiently dry before screening begins.

Day 2

2. Pick a large colony (2-3 mm) of PJ69-4α cells containing the bait vector and resuspend

in a microcentrifuge tube with 1 mL SC/-trp medium. Vortex and transfer to 250 mL flasks containing 50 mL SC/-trp. Incubate overnight (16-24 hours) at 30°C with 250-270 rpm shaking

Day 3

3. Check OD_{600}; it should be ≥ 0.8. If the OD is lower, the bait vector may be toxic for the cells.
4. If OD is acceptable; transfer to sterile 50 mL centrifuge tube and centrifuge for 5 minutes at $600g$.
5. Discard supernatant and resuspend pellet in 5 mL SC/-trp
6. Thaw an aliquot (1 mL) of PJ69-4A library cells in a water bath at RT.
7. Mix 5 mL of bait resuspension from step 5 with 1 mL of PJ69-4A cells in a 2 L sterile baffled flask.
8. Add 45 mL of 2x YPDA medium to the flask and swirl gently. Clean out remaining cells from the aliquot tube (step 6) using 1 mL of 2x YPDA twice.
9. Incubate flask overnight (20-24 hours) at 30°C with gentle shaking (30-50 rpm). At this point it is usual for the cells to clump together, making the culture look granulated.

Day 4

10. After overnight mating; transfer mating culture to 2 sterile 50 mL centrifuge tubes
11. Centrifuge for 10 minutes at $1000g$. During centrifugation; wash the 2 L baffled flask used for mating twice with 50 mL 0.5x YPDA medium. Use this medium for resuspending the cells after centrifugation and discarding of supernatant
12. Centrifuge for 10 minutes at $1000g$, and discard supernatant.
13. Resuspend the pellets in a total volume of 10 mL 0.5x YPDA medium
14. In order to determine effectiveness of mating; spread 100 µL of 1:10, 1:100, 1:1000 and 1:10 000 dilutions onto three types of plates: SC/-trp, SC/-leu and SC/-trp/-leu. Spread the cells using autoclaved glass beads
15. Spread the rest of the culture onto plates with SC/-his/-leu/-trp/+3AT. Use 200 µL pr plate
16. Incubate plates at 30°C while monitoring for 3-7 days

Day 7/8

17. After 3-4 days, start picking colonies using sterile toothpicks. Mark the plates where a colony is picked, so that the same colonies are not picked several times. Draw a 12 square grid on the back of 14 cm plates with SC/-ade/-his/-leu/-trp/+3AT and transfer one colony to each square of the grid. Colonies can be picked at different time points, but make a note of the date each colony was picked, and keep track of where in the grid they are placed
18. Calculate the number of viable colony-forming units (cfu) pr mL on SC/-trp plates, SC-/leu plates and SC/-leu/-trp plates:

$$\frac{cfu \cdot 1000\ {}^{\mu L}/_{mL}}{volume\ plated\ (\mu L) \cdot dilution\ factor} = no.\ viable\ cfu/mL \tag{2.3}$$

no. viable cfu/mL on SC/-leu = Viability of library strain

no. viable cfu/mL on SC/-trp = Viability of bait strain

no. viable cfu/mL on SC/-leu-trp = Viability of diploids

Use information to calculate mating efficiency

$$\frac{no.\ cfu/mL\ of\ diploids}{no.\ cfu/mL\ of\ limiting\ partner} \cdot 100 = \%\ diploid \tag{2.4}$$

Where limiting partner is the haploid strain with the lowest viability [59].

19. Colonies that grow on the SC/-ade/-his/-leu/-trp/+3AT plates are transferred to a flat-bottomed 96-well plate containing 150 µL YPDA medium per well. Incubate overnight on a shaker at 30°C, 200-250 rpm
20. Stamp out the cells on SC/-ade/-his/-leu/-trp/+3AT and SC/-ade/-his/-leu/-trp/+3AT/+x-α-gal plates as described below. Be sure to pay close attention to the positions of the colonies, and keep a table with continuous updates on the different colonies. Stamping: Sterilize the stamp using ethanol and a Bunsen burner, and allow to cool. Dip the stamp into the wells containing cells. Lift the stamp carefully so as not to lose any droplets, and turn it so that the stamp faces up. Turn the plates upside-down and allow them to rest on top of the stamp. Press the plate very lightly against the stamp, and remove it carefully without smearing the plate. Sterilize the stamp between each

plate

21. Incubate the plates at 30°C and check regularly. Write notes about controls continuously, including presence/absence, size and degree of colour of colonies.

2.3.5 Isolating plasmids from yeast cells

Isolating plasmids from yeast cells is more difficult than isolation from bacteria for several reasons. Most notably, yeast cells have a cell wall surrounding their plasma membrane, and their DNA is located within nucleus, so it is surrounded by more than one lipid bilayer. In this thesis, the USB® PrepEase® Yeast Plasmid Isolation Kit from Affymetrix was used to. The kit uses an enzyme solution to break down cell walls, turning the yeast cells into spheroplasts. Lysis solutions can then be used to break down lipid bilayers. Isolation of plasmids from yeast cells yields less DNA and more contaminations than bacteria.

Procedure

Experiment was performed according to the protocol for USB® PrepEase® Yeast Plasmid Isolation Kit from Affymetrix [71].

2.3.6 Verification of interactants with remating

In Y2H assays, yeast transformants are cultivated over a long period of time, increasing chances of random mutations or other errors occurring within the cells. After initial interactants have been identified, it is therefore prudent to make fresh transformants of both bait and prey-plasmids, and mate cells again to confirm interaction.

Procedure

1. Transform yeast cells strain PJ69-4A with pDBT-DEXI, pDBT (negative control) and pCBT-c-Myb (positive control)
2. Transform yeast cells strain PJ69-4α with plasmid pACT2 and pACT2 containing any interaction partners identified though the Y2H screening

3. Prepare mating between interactants and DEXI as well as empty pDBT. Include a control mating between c-Myb and empty pACT2.

Table 2.7: Remating bait with possible interaction partners
Several mating cultures should be prepared when checking interaction between bait protein DEXI and identified prey proteins (A,B,...). Cells containing plasmids pACT2 and pACT2 with identified interaction partners should be mated with empty pDBT vector as well as pDBT-DEXI. In addition, a control mating should be performed between pACT2 and pDBT-c-Myb.

	pACT2	pACT2-A	pACT2-B	pACT2-...
pDBT	X	X	X	X
pDBT-DEXI	X	X	X	X
pDBT-c-Myb	X			

4. Mate the strains as described in section 2.3.4. Note that no mating efficiency calculations is needed. After mating, only a small amount of mating culture ($\sim$ 2 µL) needs to be plated out

2.4 Mammalian cell techniques

Many mammalian cell lines have been immortalized and are commonly used as model systems in laboratories. In this thesis, human Jurkat T-cells was used to express DEXI for investiating sublocalization and to create a DEXI-containing lysate for GST pull-down. Jurkat cells are derived from peripheral blood of a male with acute T cell leukaemia. The cell line grows fast and is suitable for cell transfection.

2.4.1 Growth and maintenance of Jurkat cells

Cultures of mammalian cell lines are prone to infections, and care should be taken to avoid contaminants. All work with mammalian cell lines is carried out under laminar flow (LAF) hoods, using sterilized equipment. Jurkat cells are cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium, designed to support lymphoblastoid cell lines. Additional supplements of sodium pyruvate, non-essential amino acids (NOAA), and foetal bovine serum (FBS) are added to optimize growth. In addition, penicillin-streptomycin (pen-

strep) is added to the growth medium to protect cells against bacterial infections, and β-mercaptoethanol to protect against accumulation of reactive oxygen species.

Cells are cultivated in culture flasks at 37°C in a 5% CO_2 atmosphere. The cells were counted using BioRad TC20™ automated cell counter, and their concentration was maintained between $0.3 \cdot 10^6$ and $2.0 \cdot 10^6$ cells/mL

2.4.2 Transfection of Jurkat cells

Jurkat cells can be transiently transfected to express exogenous DNA. In this thesis, elecroporation was used to temporarily weaken cell membrane in order for plasmids to pass. Electroporation was carried out using ECM 830 Electro Square Porator™ from BTX® Harvard Apparatus. For every transfection performed, a control transfection with plasmid EGFP-N3, expressing GFP, was performed in parallel to the experiment. 24 h after transfection, cells with GFP were compared to cells without GFP using a flow cytometer (Attune® acoustic focusing cytometer from Applied Biosystems), in order to determine the transfection efficiency. Generally, a transfection efficiency of $\geq 40\%$ was considered successful.

Procedure

Day 1 - Seed cell culture

For each transformation of Jurkat T cells, $15 \cdot 10^6$ cells should be used, ideally from a culture with concentration between $0.6 \cdot 10^6$ and $1 \cdot 10^6$ cells/mL.

1. Measure cell concentration using an automated cell counter
2. Dilute to 100 mL of $0.4 \cdot 10^6$ cells/mL in a T175 flask, for a good growth culture with sufficient cells
3. Incubate overnight at 37°C, 5% CO_2.

Day 2 - Transfection

4. Count the cells using an automated cell counter. Harvest $15 \cdot 10^6$ cells per transfection by centrifuging at $300g$ for 8 minutes at RT. Include a transfection with EGFP-N3
5. Wash the cells twice by resuspending in RPMI media. Discard washing media by

spinning as in step 1.

6. While washing, prepare the following for each of your transfections:
   - Microcentrifuge tube containing desired amount of plasmid DNA
   - 4 mm electroporation cuvette
   - T75 flasks with 10 mL transfection media each. Pre-heat to 37°C
7. Resuspend the cells in 400 µL transfection media per transfection
8. Add 400 µL cells to each tube with DNA.
9. Transfer the cell-DNA mixes to 4 mm cuvettes and incubate at RT for 15 minutes
10. Mix lightly by flicking the cuvettes and tapping them gently on the tabletop to avoid air bubbles. Pulse the cuvettes once with 240 V for 25 ms
11. After the pulse, incubate the cells at RT for 15 minutes
12. Transfer the cells to the prepared flasks by first transferring 1 mL transfection media from the flasks to the cuvettes and then transfer the mix back into the flasks with pre-heated transfection medium. Try to avoid debris floating in the cuvette
13. Incubate at 37°C in 5% CO_2. Incubation time varies between plasmids transfected, and may need optimization
14. After 24 h, count cells and remove $1 \cdot 10^6$ cells transfected with EGFP-N3, and cells transfected with another plasmid.
15. Check the cells without GFP using a flow cytometer. Set gate on axis measuring side-scatter above the cell population. Check cells with GFP,and calculate how many of the cells in the population are above the gate. Confirm that the percentage is $\geq 40\%$

2.5 Imaging

The secondary aim of this thesis is to investigate the subcellular localization of DEXI in Jurkat T cells. To achieve this goal, cells transfected with FLAG-tagged DEXI is subjected to immunostaining with antibodies, and confocal microscopy. In confocal microscopy, it is possible to generate focal planes, and remove light from areas that are not in focus, leaving high-resolution images.

2.5.1 Immunostaining

In immunostaining, specific proteins can be coupled to fluorescent tags in order to visualize them with fluorescent microscopes, using antibodies. A primary antibody binds to the target protein, and a secondary antibody, conjugated with a fluorescent tag, binds to the primary antibody. When immunostaining intact cells, plasma membranes must be permeabilized to facilitate entry of antibodies to the cell interior. In this thesis, permeabilization is achieved by adding amphipatic molecules called saponins. Cells were fixed using paraformaldehyde (PFA). In addition the the immunostaining, cells were stained with Hoechst, a fluorescent dye that binds DNA, in order to visualize cell nuclei.

In this thesis, primary antibodies against DDK was used together with secondary antibodies conjugated to the dye Alexa Fluor® 555. Stained cells were studied with an Olympus FV1000/BX61 confocal microscope.

Procedure

1. Measure concentration of cell cultures
2. Transfer 1-1.5 million cells of interest to a 12 well plate
3. Add 1 mg/mL Hoechst in a 1:1000 ratio
4. Incubate for 15-20 min in 37°C, 5% CO_2
5. Transfer the cells to tubes and harvest the cells by centrifugation at 400 g
6. Wash once with 1 mL RPMI and spin as before.
7. Remove the supernatant and resuspend the pellet in 100 µL RPMI per million cells
8. Apply the resuspended cells to poly-L-lysine coated coverslips (one coverslip pr sample) and adhere for 10 min at RT in the dark. Check the adherence using a light microscope.
9. Carefully remove the media by pipetting (without touching the cells!) and fix the cells by applying 200 µL 3% PFA. Fix for 10 minutes at RT in the dark
10. Carefully remove PFA and wash with 200 µL PBS/2% FCS, incubate with the washing-media for 5 minutes before carefully removing the media. Do this twice
11. Permeabilize the cells with 200 µL PBS/0.05% Saponin/0.25% BSA for 5 min at RT in the dark.

12. Block the samples with 200 µL PBS/0.05% Saponin/0.25% BSA for 20 min RT in dark. Remove the media.
13. Dilute primary antibody in PBS/0.05% Saponin/0.25% BSA. Add 100 µL solution pr sample and incubate for 1 h RT in dark. Cover the samples to prevent evaporation. Remove the solution.
14. Wash three times with 200 µL PBS/0.05% Saponin/0.25% BSA. Incubate with the washing media for 5 min each time
15. Dilute secondary antibody in PBS/0.05% Saponin/0.25% BSA. Add 100 µL solution pr sample and incubate for 1 h RT in the dark. Cover the samples to prevent evaporation. Remove the solution
16. Wash three times with PBS/0.05% Saponin/0.25% BSA. Incubate with the washing media for 5 min each time
17. Let the sample nearly dry, add one droplet mounting media (∼45 µL), and place a cover glass on the top. Seal the edges with nail polish
18. Store in dark 4°C

3 - Results

3.1 Yeast two-hybrid screening

The main purpose of this thesis was to use the yeast two-hybrid (Y2H) system to find interaction partners for the protein encoded by *DEXI*, a gene suggested to be an MS risk factor. In the Y2H screening, bait-DB protein was encoded by plasmid pDBT-DEXI (see appendix D), prepared by someone in our lab. The plasmid was transformed into strain PJ69-4α of *S. cerevisiae*. The cDNA-AD protein was encoded by plasmid pACT2-library, already transformed into yeast strain PJ69-4A. Library plasmid and yeast strains were provided by Odd Stokke Gabrielsens group (UiO).

The diploid cells generated during mating will contain both plasmids, and interaction between DEXI and a library protein in a cell will bring the two domains of the Gal4 transcription factor together at the Gal4 binding site of DNA, facilitating expression of three reporter genes (see figure 1.7). A successive screening of these reporter genes was performed. Plasmids were extracted from clones found to express the genetic reporters, and the plasmid DNA was sequenced. Interesting candidates were then selected for verification by remating and a GST pull-down assay.

3.1.1 Autoactivation and toxicity test

Before the screening is initiated, a test is performed to reveal whether the protein DEXI might be toxic to the yeast strain PJ69-4α, and to establish whether DEXI autoactivates the reporter genes used in the screening (see section 2.3.3). Cells transformed with empty vector pDBT, known to be non-toxic, were used as positive control for the toxicity test, whereas cells transformed with a plasmid expressing c-Myb, known to activate expression of reporter genes in the absence of Gal4 activation domain, so-called autoactivation, were used as a positive control for the autoactivation test.

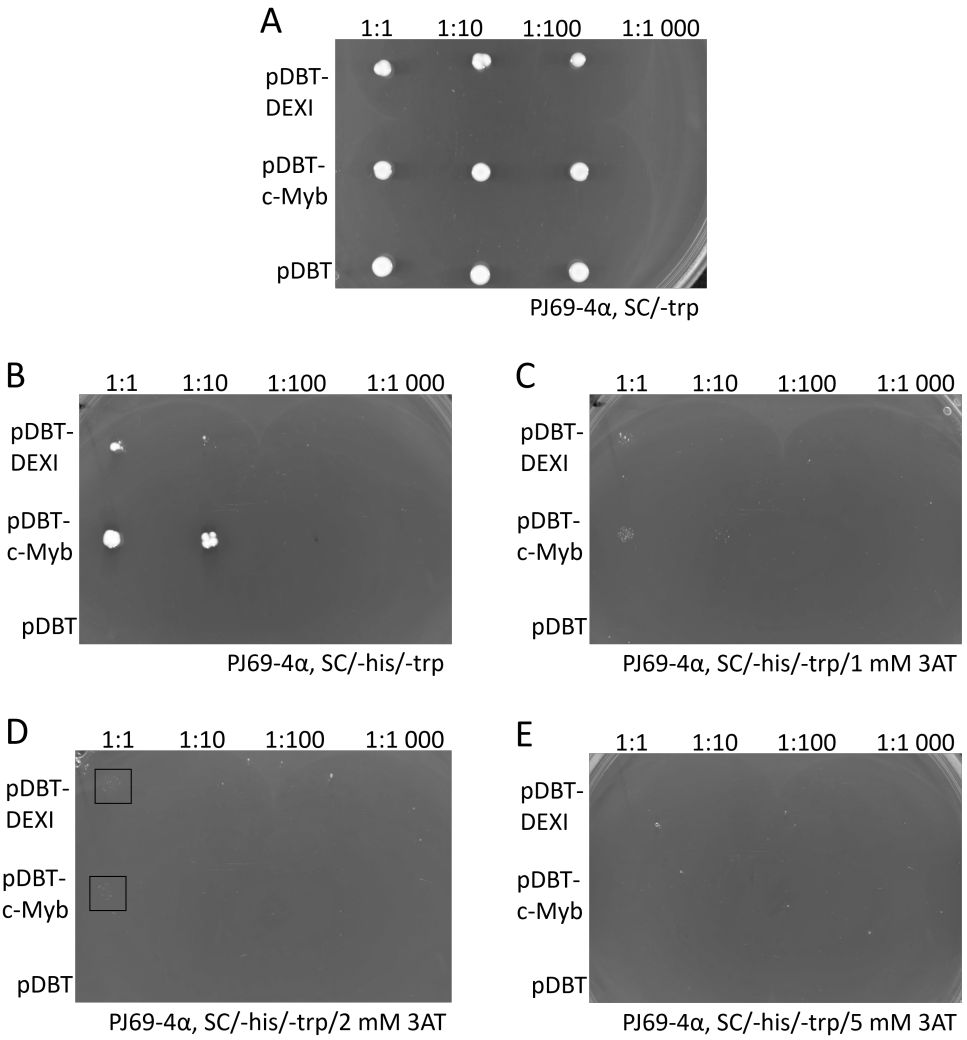


Figure 3.1: Autoactivation and toxicity test of PJ69-4α
Yeast colonies transformed with empty vector pDBT, pDBT-DEXI or pDBT-c-Myb were cultivated in 1:1, 1:10, 1:100 and 1:1 000 dilutions onto plates of different media. A) SC/-trp was used to check toxicity, B) SC/-his/-trp was used to check autoactivation. C-E) SC/-his/-trp plates containing increasing concentrations of 3AT (1 mM, 2 mM and 5 mM, respectively), to check degree of inhibition of autoactivation by 3AT.

It was found that yeast colonies containing pDBT-DEXI grew equally well as the positive control containing the empty pDBT vector, indicating that DEXI is not toxic to the yeast cells (figure 3.1A). It was also found that the yeast colonies transformed with pDBT-DEXI grew equally well as the positive control for autoactivation, yeast cells transformed with pDBT-c-Myb, revealing that DEXI is capable of autoactivating the reporter gene *HIS3* (figure 3.1B). Autoactivation of reporter genes will lead to false positive results and should be avoided. As described in section 2.3.3, 3AT, a competitive inhibitor for the gene product

of *HIS3* was added in various concentrations to establish the level of 3AT sufficient to inhibit baseline expression of *HIS3*. It was found that 1 mM and 2 mM 3AT were insufficient to inhibit growth. The growth was, however, sufficiently inhibited on plates containing 5 mM 3AT (figure 3.1C-E). Consequently, 5 mM 3AT was included in restrictive medium of successive steps of the Y2H screening.

3.1.2 Mating efficiency

The two yeast strains PJ69-4α, transformed with pDBT-DEXI, and PJ69-4A, transformed with pACT2 with cDNA library, were mated as described in section 2.3.4. To establish the mating efficiency, a sample was removed from the mating culture and four dilutions were made. These dilutions were spread onto plates selective for cells containing pDBT (SC/-trp), pACT2 (SC/-leu), or both (SC/-leu/-trp). The colony forming units (cfu) on each plate was estimated, and the numbers were used to calculate the mating efficiency using equations 2.3 and 2.4 from section 2.3.4.

All plates displayed abundant growth. More than 500 colonies were found on the lowest dilution of the SC/-leu plates, and more than 1000 colonies were found on the lowest dilution of the SC/-trp plates. On the SC/-leu/-trp plate plated with the lowest dilution, 69 colonies were observed. For the higher concentrations, colonies covered the entire plate, and their numbers became difficult to estimate precisely, referred to as a lawn. The results are shown in table 3.1.

Table 3.1: Number of colony-forming units (cfu)
Colonies were counted on plates streaked with 100 µL culture of different dilutions

Dilution	SC/-leu	SC/-trp	SC/-leu/-trp
1:10	lawn	lawn	lawn
1:100	lawn	lawn	>1000
1:1 000	>1000	≫1000	446
1:10 000	565	1004	69

For accuracy, cfu/mL was calculated based on the number of colonies on the plates with lowest concentration, as shown in table 3.2.

Table 3.2: Number of viable cfu/mL
Number of cfu pr mL was calculated using equation 2.3, 100 µL culture was plated, and dilution factor was 1:10 000

Plate	Number of colonies	No. viable cfu/mL
SC-Leu	565	$5.65 \cdot 10^7$
SC-Trp	1004	$1.00 \cdot 10^8$
SC-Leu-Trp	69	$6.90 \cdot 10^6$

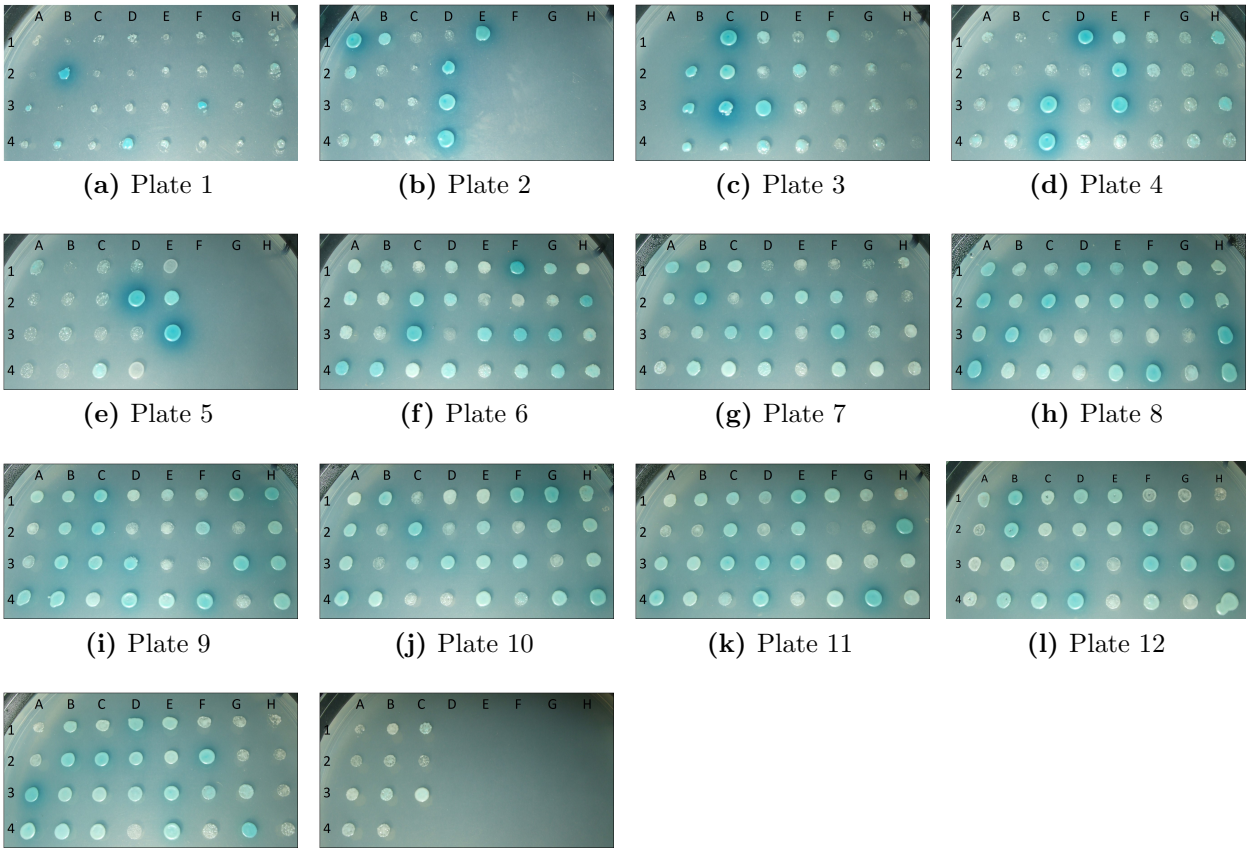
PJ69-4A was found to be the limiting partner, as it was the mating strain with the lowest viability. The mating efficiency was calculated to be approximately 12%, which is acceptable for a two-hybrid screening [59].

$$\frac{6.90 \cdot 10^6 \ cfu/mL}{5.65 \cdot 10^7 \ cfu/mL} \cdot 100 = 12.21\% \tag{3.1}$$

3.1.3 Two-hybrid Screening

The rest of the mating culture was then plated out onto 14 cm petri dishes containing SC/-his/-leu/-trp/+5 mM 3AT to screen for expression of the first reporter gene, *HIS3*. The plates were incubated at 30°C and monitored over several days. Due to the large amount of colonies on the plates, only colonies larger than 1 mm in diameter were selected. A total of 702 colonies were transferred to SC/-ade/-his/-leu/-trp/+3AT plates, selecting for both reporter genes *HIS3* and *ADE2*. Of these, 412 appeared to survive on media lacking adenine when incubated for 2-4 days at 30°C, and these surviving colonies were selected for stamping out on SC/-ade/-his/-leu/-trp/+3AT/+X-α-gal, to check for expression of the third reporter gene *MEL1*. Pictures of the colonies from these plates are shown in figure 3.2

For each of the three screening steps, the growth of clones was monitored daily. For the first two steps, notes were made on the appearance and size of each colony. For the last step, notes were made on the colour of the colonies. After screening for all three reporter genes, each clone was evaluated based on their growth. The number of positive candidates from each step was determined subjectively (see table 3.3). After screening, the clones were evaluated based on growth or colour from each step. 92 candidates were found to have



(a) Plate 1 **(b)** Plate 2 **(c)** Plate 3 **(d)** Plate 4

(e) Plate 5 **(f)** Plate 6 **(g)** Plate 7 **(h)** Plate 8

(i) Plate 9 **(j)** Plate 10 **(k)** Plate 11 **(l)** Plate 12

(m) Plate 13 **(n)** Plate 14

Figure 3.2: Screening plates with SC/-ade/-his/-leu/-trp/+ 5 mM 3AT/+ 0.04 mg/mL x-α-gal
Each colony is labelled with plate number and plate coordinate, for example 1-A1 for the top leftmost colony on plate 1. The intensity of the blue colour of each colony was evaluated. Clones with a middle (such as clone 6-E3) to strong (5-E3) colour were considered to be positive

sufficiently strong growth and colour to be selected for further investigation. The relative interaction strength of these 92 candidates were given a subjective grade from 1-4, based on their appearance for each step of the screening.

Table 3.3: Overview of successive screening for expression of reporter genes *HIS3*, *ADE2*, and *MEL1*
On plates selecting only for *HIS3*$^+$ cells, many more colonies appeared, but only those $\geq$ 1 mm were selected and moved to the next plate. On plates selecting for all three reporter genes, colonies with any amount were considered to be positive, but only the colonies with consistent growth and strong blue colour were considered to be interesting candidates.

Selected for reporter genes	Medium	Number of positive colonies
HIS3	SC/-his/-leu/-trp/+3AT	702
HIS3 and *ADE2*	SC/-ade/-his/-leu/-trp/+3AT	412
HIS3, *ADE2*, and *MEL1*	SC/-ade/-his/-leu/-trp/+3AT/+x-α-gal	130
Number of clones selected as particularly strong candidates		92

3.2 Identification of interaction candidates

In order to identify the library sequences within the 92 interaction candidates, DNA had to be retrieved from the cells and then sent to sequencing. Two strategies were used for this purpose: (1) colony PCR directly on yeast cell lysates followed by sequencing, and (2) plasmid isolation from yeast cells, transformation into bacterial cells, isolation of plasmids from bacteria, restriction enzyme digestion, and sequencing.

3.2.1 Colony PCR as strategy to identify interaction candidates

Fresh colonies of each of the 92 candidates were picked from plates and used as template in a colony PCR as described in section 2.1.9, using primers A097-pACT2-forward and A097-pACT2-reverse (see appendix B for primer sequences). The PCR reactions were checked using agarose gel electrophoresis (see section 2.1.8), and the PCR products were extracted from the gel as described in section 2.1.10. After confirming presence of DNA in the samples with a new agarose gel electrophoresis, samples were sequenced by GATC Biotech with the forward primer. Of the 92 candidates, 41 yielded a PCR product detectable by agarose gel electrophoresis. For most samples, however, the amount of PCR product was very low, and after extracting PCR product from gel, DNA could only be confirmed in 27 of the candidates.

The success rate of sequencing from these 27 colony PCR products was quite low, and only 15 colonies were successfully sequenced from colony PCR products.

3.2.2 Transformation into bacteria as strategy to identify interaction candidates

For the candidates where the colony PCR strategy proved unsuccessful for retrieving sequences, an effort was made to further amplify and purify the plasmid DNA. This alternative scheme for plasmid retrieval involved isolation of whole plasmid from yeast cells using the PrepEase kit (section 2.3.5). Because plasmids isolated from yeast cells are of a lower concentration and purity than plasmids isolated from bacteria, the plasmids isolated form yeast cells were used to transform *E. coli* XL1-Blue cells, and the plasmids were re-isolated with miniprep.

The diploid yeast cells contain both the pACT2-library and pDBT-DEXI plasmids. Since both plasmid have the same antibiotic marker, when isolating plasmids from yeast and using them to transform bacteria, one cannot know which of the two plasmids are actually present in the bacterial cells. Because we only wanted to sequence the pACT2 plasmids, a restriction enzyme digestion was performed to establish the identity of the purified plasmids. To achieve this, BglII was selected as a suitable enzyme, as it cuts in pACT2, but not pDBT (figure 3.3).

The vector pACT2 contains two binding sites for BglII, flanking the cDNA library sequences (figure 3.3). Any secondary bands that appear along with the main body of the plasmid should be the size of the inserts, which are unknown. An empty plasmid would contain a very small secondary fragment.

When running agarose gel, there is a possibility that small fragments (< 500 bp) are overlooked on the gel, both because small fragments may move completely across the gel and because light intensity under UV light is relative to DNA size. In order to not exclude potential interaction partners consisting of one or several small bands, candidates that were found to contain pACT2 with no visible extra bands were also sequenced. If these candidate

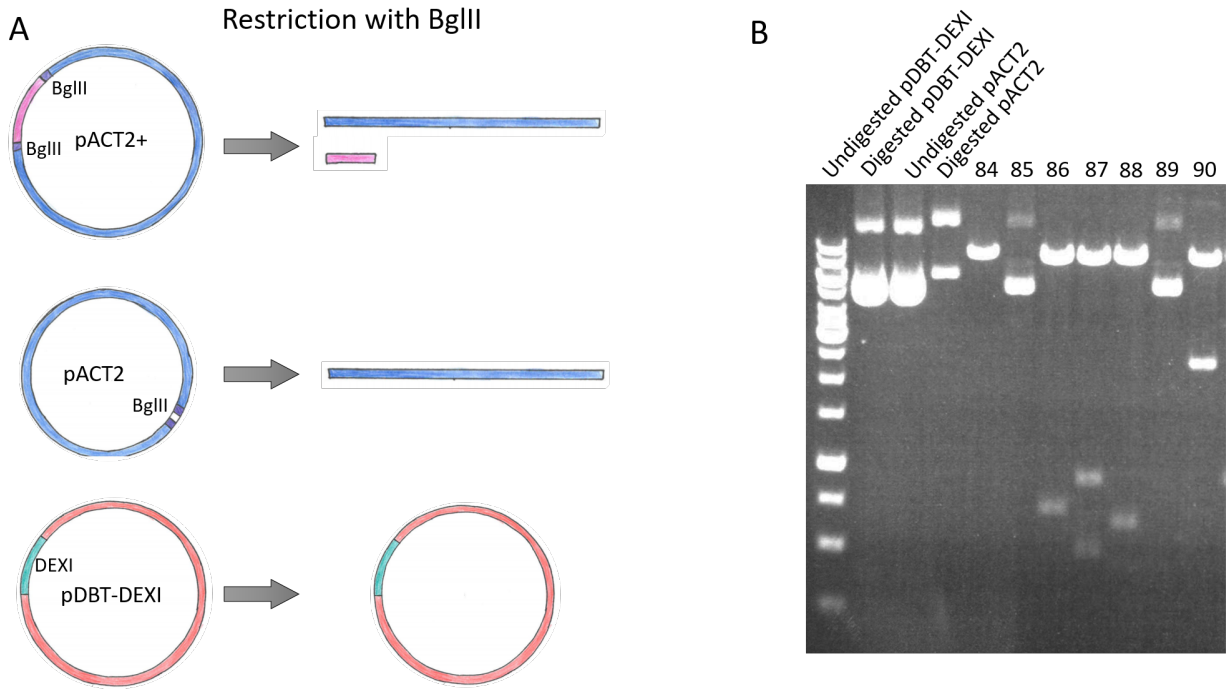


Figure 3.3: Identification of plasmids by restricion enzyme digestion with BglII
A) Schematic illustration of the restriction enzyme digestion of plasmids pACT2 with insert, ie cDNA library (pAC2T+), empty pACT2 and pDBT-DEXI. pACT2+ contains an insert flanked by BglII, generating two fragments. pACT2 without insert generates one detectable band and one fragment too small to detect. pDBT-DEXI generates a single, linear fragment. B) An selected result of restriction reaction on candidates 84-90 using BglII. Gel contains Digested and undigested pDBT-DEXI and pACT2 for comparison. In this example, candidates 85 and 89 seem to contain pDBT-DEXI, and the rest of the candidates contain pACT2 with various inserts.

contain only empty pACT2 vector, this will be revealed upon investigation of the sequences.

After miniprep and restriction analysis, all samples found to contain pACT2 plasmid were sent to sequencing. 54 novel sequences were retrieved in addition to two candidates already identified using colony PCR.

Of the 92 interaction candidates selected during Y2H screening, sequences from 13 were retrieved using the colony PCR strategy, 54 were retrieved using the isolation of plasmids strategy and two were retrieved using both. In total 69 sequences were retrieved.

3.2.3 *In silico* analysis of sequences obtained

Initially, sequences were trimmed of their flanking pACT2 sequences and identified with nucleotide BLAST (nBLAST)(https://blast.ncbi.nlm.nih.gov). Because the Y2H screening was performed based on protein products, sequences were translated to amino acids and used as a query in protein BLAST (pBLAST) to confirm their identity, and to check length of alignments. Each sequence was translated in the reading frame of the plasmid pACT2. Summary of Y2H screening and *in silico* analysis, can be found in table 3.4.

Table 3.4: Overview of the candidates from Y2H screening that gave rise to identifiable sequences
Clone number is an arbitrary name between 1-94 given to each candidate. Interaction strength is a subjective assesment of the expression of all reporter genes, based on notes made during screening. The size of insert describes the size of PCR product using A097-pACT2 primers, or the size of bands after digestion with BglII. Unknown size of bands from plasmids means that the restriction analysis indicated the presence of pACT2, but no additional insert could be seen on the gel. nBLAST and pBLAST are the results from BLAST-searches done on nucleic sequences and amino acid sequences, respectively. BLAST results with e-value higher than 0.01 were considered not significant. Alignment length is the length of alignment between the amino acid sequence and the pBLAST result from a candidate.

Clone	Interaction strength	Size of insert (bp)	Sequenced on	nBLAST	pBLAST	Alignment length (aa)
2	3	1000 + 500	plasmid	CAMLG	CAMLG	226
3	3	1000	PCR	CSNK2A1	not significant	
4	4	2500	plasmid	IVNS1ABP	not significant	
5	3	780	plasmid	CAMLG	CAMLG	266
6	3	750	plasmid	SNAPIN	SNAPIN	90
7	2	1000	PCR	TCRB variables	not significant	
8	2	700	plasmid	MARCH2	MARCH2	62
10	3	1000 + 500	plasmid	CAMLG	CAMLG	285
11	3	1500	plasmid	BCORL1	not significant	
12	3	1300	plasmid	BCORL1	not significant	
13	4	1000 + 500	plasmid	CAMLG	CAMLG	198
14	4	600	plasmid + PCR	MARCH2	MARCH2	62
15	4	800	PCR	CSNK2A1	not significant	
16	2	750	plasmid + PCR	MARCH2	MARCH2	62
17	4	500	plasmid			
18	4	unknown	plasmid	CAMLG	CAMLG	38
19	2	unknown	plasmid	CAMLG	CAMLG	38

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Table 3.4 Continued from previous page

Clone	Interaction	Size of insert (bp)	Sequenced on	nBLAST	pBLAST	Alignment length (aa)
23	3	750	plasmid	NDFIP1	not significant	
24	4	unknown	plasmid	CAMLG	CAMLG	38
25	2	unknown	plasmid	TCRB	not significant	
27	2	750	plasmid	CSNK2A1	not significant	
28	2	2900	plasmid	MTCH1	not significant	
29	2	2000	plasmid	AKIRIN2	not significant	
34	4	750	plasmid	GHITM	not significant	
36	4	1000	PCR	CAMLG	CAMLG	38
37	4	800	PCR	CSNK2A1	not significant	
38	4	600	PCR	PIG-M	not significant	
40	4	600	plasmid	CSNK2A1	not significant	
41	4	800	PCR	CSNK2A1	not significant	
42	4	1000	plasmid	CAMLG	CAMLG	201
43	3	1000	PCR	CAMLG	CAMLG	38
44	3	1000	plasmid	RP11-125—23	not significant	
45	3	900 + 500	plasmid	CAMLG	CAMLG	258
46	4	1000	PCR	CAMLG	CAMLG	38
47	3	900 + 500	plasmid	CAMLG	CAMLG	223
48	3	1000 + 500	plasmid	CAMLG	CAMLG	193
49	4	600	plasmid	CSNK2A1	not significant	
51	4	1000 + 500	plasmid	CAMLG	CAMLG	193
52	4	750	plasmid	MDK	MDK	142
53	3	1000 + 500	plasmid	CAMLG	CAMLG	264
54	4	400	PCR	RBMX	not significant	
55	4	900	plasmid	RP11-356—2	not significant	
56	4	900 + 500	plasmid	CAMLG	CAMLG	270
58	3	2500	plasmid	IVNS1ABP	not significant	
59	3	1200	plasmid	CAMLG	CAMLG	58
60	4	750	plasmid	CAMLG	CAMLG	284
61	3	1100	plasmid	MDM2 / ZFP36L2	not significant	
63	4	unknown	plasmid	CAMLG	CAMLG	38
64	3	1100	PCR	CAMLG	CAMLG	57
65	4	900	plasmid	CAMLG	CAMLG	276
66	3	900	plasmid	CAMLG	CAMLG	270
67	3	500	plasmid	CAMLG	CAMLG	57
69	3	600	plasmid	CSNK2A1	not significant	269
71	4	500 + 1000	plasmid	CAMLG	CAMLG	267
72	3	500 + 1000	plasmid	CAMLG	CAMLG	267
73	3	300 + 1700	plasmid	SLTM	SLTM	99
75	4	1000	plasmid	RP11-356—2	not significant	

Continued on next page

Table 3.4 Continued from previous page

Clone	Interaction	Size of insert (bp)	Sequenced on	nBLAST	pBLAST	Alignment length (aa)
77	4	500 + 1000	plasmid	CAMLG	CAMLG	269
79	4	unknown	plasmid	CSNK2A1	not significant	
80	3	unknown	plasmid	CAMLG	CAMLG	202
82	4	unknown	plasmid	CAMLG	not significant	
85	4	750	plasmid	RP13-36G14	not significant	
86	4	800 + 450	plasmid	CAMLG	CAMLG	270
87	4	600	plasmid	CSNK2A1	not significant	
89	3	2400	plasmid	IVNS1ABP	not significant	
90	3	800	plasmid	CAMLG	CAMLG	274
91	3	500	PCR	RP11-34F20	not significant	
93	4	1000	PCR	CAMLG	CAMLG	37
94	4	800 + 450	plasmid	CAMLG	CAMLG	272
						Concluded

3.2.4 Investigating interaction candidates

Of all the initial genes identified using nucleotide BLAST, five of them gave significant results for alignment on protein sequences as well. These five were CAMLG, MARCH2, MDK, SLTM and SNAPIN (see table 3.5).

Table 3.5: Possible interactions partners for DEXI
The table shows names and brief function of five proteins identified as potential interaction partners for DEXI.

CAMLG	Calcium signal-modulating cyclophilin ligand	Protein involved in calcium-dependent signalling from TCR, affecting proliferation downstream of T cell activation [72, 73]
MARCH2	Membrane-associated ring finger (C3HC4) 2	E3 ubiquitin-protein ligase. Negatively regulates autophagy [74]
MDK	Midikine	MDK Signalling activates mitogen-activated protein kinase (MAPK) and phosphoinositide 3 (PI3)-kinase [75]. Promotes lymphocyte survival and drives chemotaxis of immune cells [76]
SLTM	Scaffold attachment factor B (SAFB)-like transcription modulator	Transcription inhibitor involved in apoptosis [77]
SNAPIN	SNARE-associated protein	Involved in intracellular vesicle trafficking and recycling of synaptic vesicles. Plays a role in autophagy [78]

No interactions between any of these proteins were found using STRING database (https://string-db.org/).

Of the 69 sequences analysed, 33 was recognized as *calcium modulating ligand* (*CAMLG*) based on nucleotide BLAST. Only one of these candidates were not in reading frame. The candidates had alignments of varying lengths, that could be roughly put into six categories (see figure 3.4). Three of the candidates that contained short sequences (1-57 or 21-58 aa) of CAMLG were found to have been completely sequenced, indicating that interaction with DEXI took place with this small segment of CAMLG.

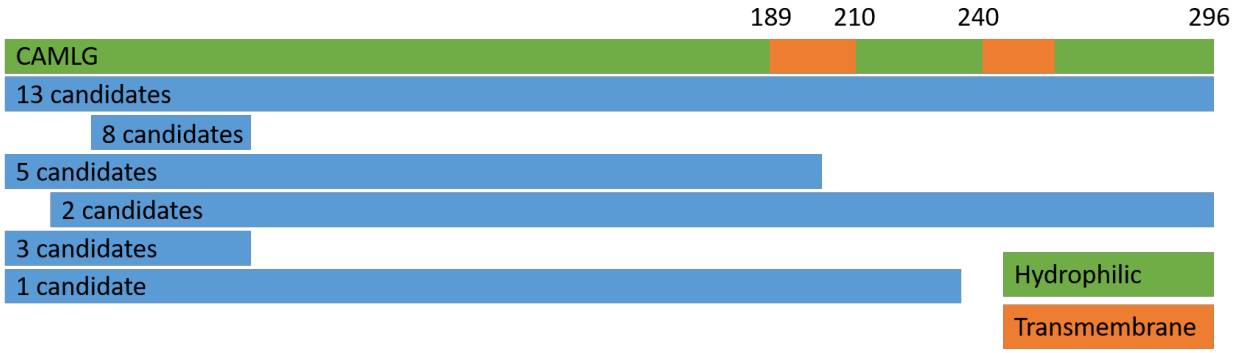

Figure 3.4: Schematic illustration of topological domains of CAMLG and alignment with interaction candidates
The illustration shows six categories of alignments found between different candidates and CAMLG. Many candidates contained full-length CAMLG, but some sequences only contain parts. A hydrophilic segment between amino acids 21-57 is found in all candidates. Topological domains of CAMLG found on UniProt (http://www.uniprot.org/uniprot/P49069, verified 21.10.17)

The three sequences that were recognized as MARCH2 all aligns over the last 62 amino acids of the protein, indicating that only parts of the protein is needed for interaction with DEXI. According to the Human Protein Atlas, MARCH2 may be found in cytosol of cells (http://www.proteinatlas.org, verified 22.10.17) . One of the candidates is recognized as full-length heparin-binding growth factor midikine, localized in cell nucleus [79]. The candidate recognized as SLTM covered 99 amino acids of the 1034 aa long protein, located in the cell nucleus [77]. SNAPIN, recognized by a candidate covering the last 90 aa of the 136 aa long protein, is located in cytosol [78].

Due to time pressure, only one of the five potential interaction partners was selected for further analysis. During the screening, CAMLG was early identified as the most interesting candidate, due to the many replications of the hit. Since the interaction verification tests were started before sequence analysis was fully completed, the best candidate that was available at time of experiment initiation, candidate 64, was selected. Candidate 64 was one of three candidates that aligned with CAMLG over the first 57 amino acids (see figure 3.4).

3.2.5 Re-mating

To verify that interaction takes place between candidate 64 and DEXI in the yeast system, and is not the result of a random mutation occurring during the screening process, the mating process was repeated as described in section 2.3.6. Empty pACT2 vector and pACT2-64 was used to transform yeast cells PJ69-4α, while empty pDBT plasmid and plasmids pDBT-DEXI and pDBT-Myb were used to transform PJ69-4A yeast cells. The cells were cultivated and mated as described in 2.3.6. Each mating culture was plated onto SC/-leu/-trp plates to verify that mating was successful, and to SC/-ade/-his/-leu/-trp/+x-α-gal/+3AT plates to check for interaction by screening for activation of reporter genes. Both candidate and empty pACT2 plasmid, was mated with cells containing empty pDBT plasmid as control, and a final control mating was performed with cells containing empty pACT2 plasmid and pDBT-Myb (figure 3.5).

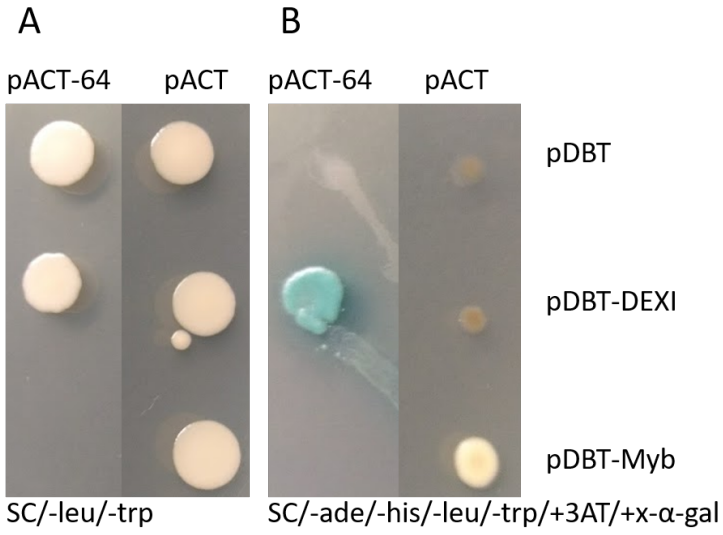

Figure 3.5: Remating PJ69-4α/A
Remating was performed with DEXI and candidate 64, as well as empty plasmids and plasmid containing autoactivating Myb as controls. A) Mated cells were cultivated on media selective for cells containing both plasmid derivatives to confirm the success of mating. B) Mated cells were simultaneously cultivated on selective media where only cells that express reporter genes *HIS3* and *ADE2* will survive. Cells expressing reorter gene *MEL1* will turn blue.

As can be seen in figure 3.5A, all five mating cultures contained cells able to survive on media lacking leucine and tryptophan, indicating that they are diploid cells containing both pDBT

and pACT2 derived plasmids. Reporter genes seem to be expressed in the mating culture containing both pACT-64 and pDBT-DEXI, confirming a possible interaction between the two proteins DEXI and CAMLG. Some expression of reporter genes is also seen in the culture containing autoactivating c-Myb.

3.3 Verification of interaction partner CAMLG using GST pull-down

We wanted to study interaction between DEXI and protein expressed by candidate 64, and decided to perform a glutathione S-transferase (GST) pull-down assay using DEXI and protein from candidate 64.

3.3.1 Cloning GST-64

In order to perform the assay, the sequence retrieved from candidate 64 was cloned into GST expression vector pGEX-6P-1. The vector contains an MCS just downstream of the gene encoding GST, and cloning a sequence into the MCS and transforming the plasmid into a suitable bacterial strain results in expression of a fusion protein (see appendix D for plasmid maps). The plasmid was named pGEX-64, and the fusion protein GST-64. PCR with Pfu polymerase (see section 2.1.9) was performed on pACT2-64 plasmid to amplify the CAMLG-sequence (figure 3.6). The PCR product was confirmed with agarose gel electrophoresis (section 2.1.8). Both PCR product and empty pGEX-6P-1 plasmid were then digested with BamHI and NotI successively (section 2.1.6). For each enzyme, incubation time was maximized to increase chance of binding in spite of the short distance between enzyme binding site and ends of PCR sequence. Alkaline phosphatase was added to pGEX restriction reaction to prevent religation of empty pGEX.

After running the restriction reaction on gel, the desired bands were excised from the gel and purified using the Wizard kit (see section 2.1.10). Samples of the cleaned DNA products

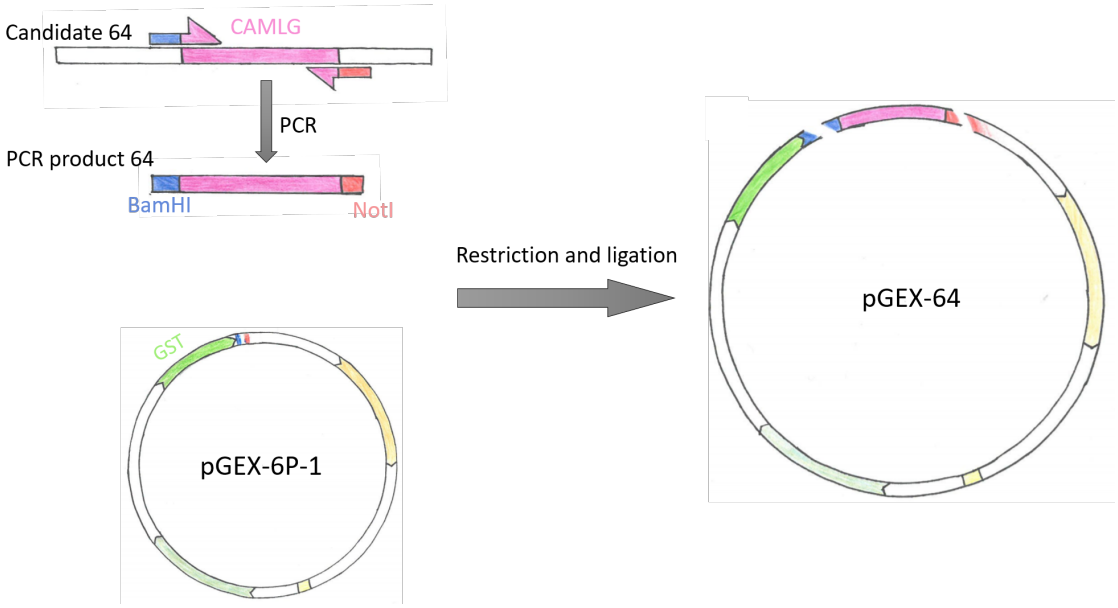

Figure 3.6: Schematic illustration of the cloning of pGEX-64
Candidate 64 contains partial CAMLG sequence. Primers were designed to flank this segment and contain binding sites for BamHI (blue) and NotI (red). Restriction digestion with these enzymes creates sticky ends of the PCR product 64 and plasmid pGEX-6P-1. PCR product 64 can then be ligated into the plasmid pGEX-6P-1, just upstream of the gene encoding GST (green), creating plasmid pGEX-64, encoding the fusion protein termed GST-64.

were run on another agarose gel to estimate the amount of DNA in each sample. This was done by comparing intensity of bands between the samples and DNA ladder (see section 2.1.7).

The amount of vector relative to insert used in the ligation reaction was determined by equation 3.2, as described in section 2.1.7

$$\frac{ng\ insert}{bp\ insert} / \frac{ng\ vector}{bp\ vector} = R \tag{3.2}$$

Using equation 3.2, the estimated concentrations of vector pGEX and insert 64, and the known sizes of 64 and pGEX, the volumes required for each reaction were calculated. Using these volumes, ligation reactions were performed as described in section 2.1.7. The resulting reaction mixtures were used to transform *E. coli* XL1-BLue using the electroporation protocol (see section 2.1.3), one transformation per ligation mixture. After incubation overnight, colonies were found on all plates with bacteria transformed with ligation reaction mixtures.

Plasmids were isolated from bacteria using miniprep (section 2.1.4), and then subjected to a restriction enzyme digestion to confirm the orientation and size of insert within the plasmid pGEX. Once it was established that the plasmid contained the correct insert, it was used to transform *E. coli*.

3.3.2 Protein expression of GST-64 in *E. coli* BL21, and protein extraction

For the purpose of expressing proteins and making lysate, the BL21(DE3) strain is especially well suited (see section 2.1.1). The bacteria were transformed with pGEX-64 or empty plasmid pGEX, using the heat-shock protocol as described in section 2.1.3. Transformed bacteria were plated out on LB/amp/chloramphenicol plates and incubated overnight at 37°C.

Only two colonies grew from the transformation with pGEX-64. Since protein expression and preparation of protein lysates requires a larger input, one of the colonies was picked and streaked onto a new LB/amp/chloramphenicol plate for amplification. The plate was incubated overnight at 37°C. Protein expression was induced as described in section 2.2.1. A sample of the culture was taken out before adding IPTG, which induces expression of proteins encoded by plasmid pGEX-6p-1. Another sample was removed after IPTG induction. Cell lysing was performed with Triton™ X-100 without sonication (see section 2.2.2). Samples removed before and after induction with IPTG and lysate from cells transformed with both pGEX and pGEX-64 were run on an SDS-PAGE and stained with Coomassie, as described in section 2.2.6. A picture of the stained gel is shown in figure 3.7.

As can be seen in the figure, a protein sized 25 kDa is induced in lysates from cells transformed with the pGEX plasmid. This is the expected size of GST. In the sample removed from pGEX-64 culture after induction with IPTG, a protein sized between 25 kDA and 37 kDA is seen. The estimated size of the protein from candidate 64 is ∼8.5 kDa, and the total size of GST-64 (∼33.5 kDa) matches the size of the induced band seen in the gel. There does seem to be some overflow in the gel, with bands appearing in lanes that were not loaded.

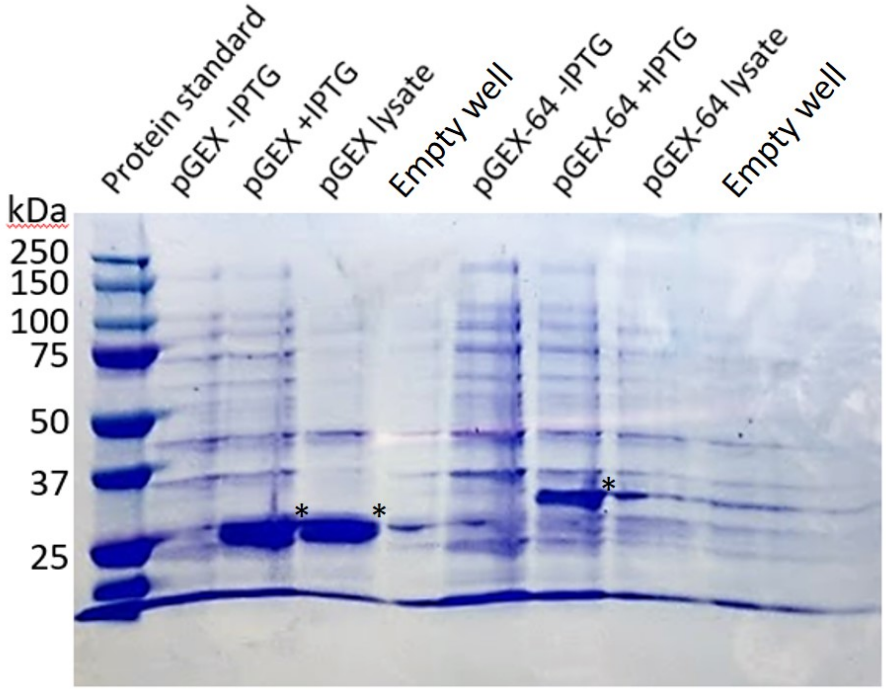

Figure 3.7: c
The figure shows a 10% criterion gel containing cell lysates from BL21 cells transformed with pGEX and pGEX-64. Samples labeled "-IPTG" were removed from the cell cultures prior to adding IPTG. Samples labeled "+IPTG" were taken out after protein expression was induced with 0.4 mM IPTG. SDS loading dye with β-mercaptoethanol was added to the samples before they were heated to 95°C for 2 minutes. Samples labeled "lysate" contains the final lysates prepared by adding Triton™ X-100 and ultracentrifugation. The expected sizes of induced proteins are 25 kDa for GST and ∼33.5 kDa for GST-64. Asterisks indicate proteins induced by IPTG

In order to extract GST and GST-64 from the respective lysates, a cleaning protocol using glutathione sepharose (GS) beads was performed (see section 2.2.3). The cleaning protocol was performed on GST and GST-64 lysate. The samples were run on an SDS-PAGE and stained with Coomassie (sections 2.2.5 and 2.2.6) (see figure 3.8A).

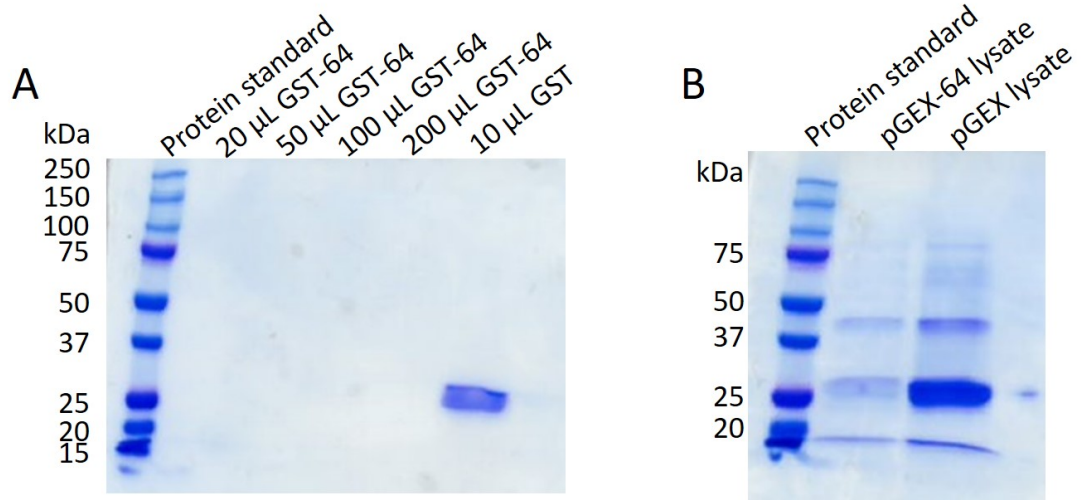

Figure 3.8: SDS-PAGE on cleaned GST-64 and GST proteins, and lysates from cells transformed with pGEX or pGEX-64
The figure shows 10% criterion gels stained with Coomassie Blue. A) The gel contains 20-200 µL of cleaned GST-64 protein solution and 10 µL cleaned GST protein solution. B) The gel contains 3 µL lysate from cells transformed with pGEX-64 and pGEX.

As seen in figure 3.8A, the protein is not visible in any volumes of the cleaned GST-64 solution, but clearly present in the GST solution. To check the presence of protein GST-64 in cell lysates, a new SDS-PAGE was conducted on the cell lysates loaded in figure 3.7. In the new gel (figure 3.8B), only weak bands could be seen in the lane loaded with pGEX-64 lysate. The GST band is present, but the size of the band indicates that there is no fusion protein GST-64 present in the lysate. Consequently, the experiment was repeated and new lysates were prepared from cells transformed with pGEX-64, and SDS-PAGE with Coomassie staining was performed (figure 3.9).

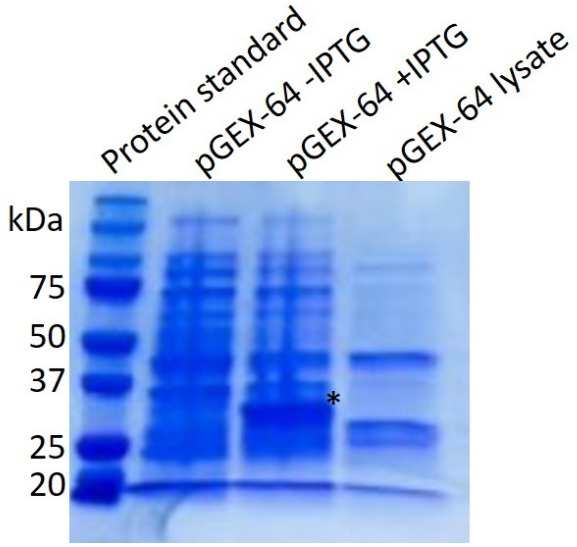

Figure 3.9: SDS-PAGE on samples from cells transformed with pGEX-64
The figure shows a Coomassie stained 10% criterion gel containing samples from the second attempt at making lysate from cells transformed wih pGEX-64. Asterisk indicates a protein whose expression is induced after addition of 0.4 mM IPTG

As before, it is apparent that expression of a protein of the expected size of GST-64, is induced upon incubation with IPTG. This protein, however, cannot be seen in the lysate, although a band that could represent GST without the added peptide can be seen.

3.3.3 Testing alternative lysis protocols

Since protein GST-64 is clearly present in the cell, but not detectable in the cell lysates prepared using Triton™ X-100, several alterations were made to the protocol in an attempt to detect protein in lysate, or determine whether it precipitates during ultracentrifugation. The alterations includes two alternative lysis protocols, and an additional investigation of the pellets generated by ultracentrifugation.

The expression of protein was induced as described in section 2.2.1. The 2 mL resuspension was divided into three fractions and lysed as described in 2.2.2, using BugBuster®, and Triton™ with or without sonication. After centrifugation, pellets were resuspended in dH_2O sonicated. Samples were applied to a criterion gel for SDS-PAGE and subsequent staining with Coomassie Blue (fig 3.10), as described in sections 2.2.5 and 2.2.6.

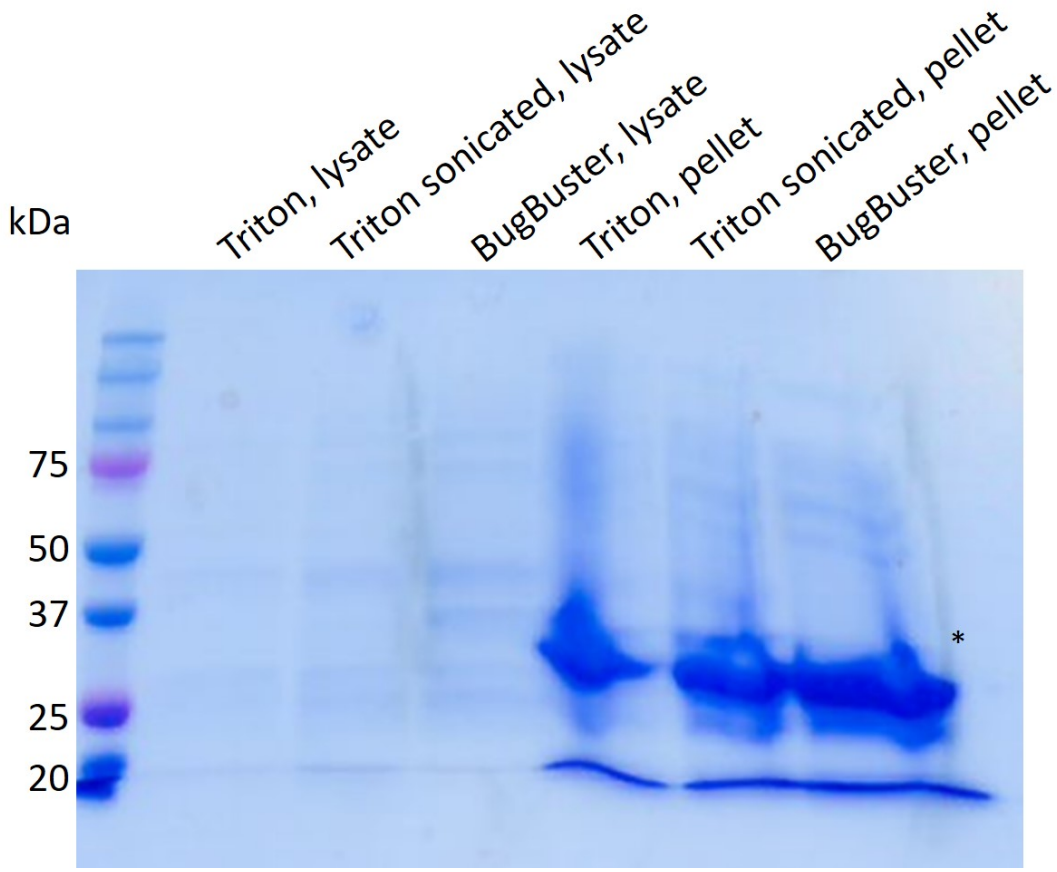

Figure 3.10: SDS-PAGE with samples from cells transformed with pGEX-64
10% criterion gel containing samples of cells transformed with pGEX-64, lysed using different protocols. One sample was treated with Triton™ X-100 and ultracentrifugation, one with Triton™ X-100, sonication and then ultracentrifugation, and one with BugBuster lysis buffer and ultracentrifugaion. Supernatants were transferred to new tubes, pellets were resuspended in dH_2O and sonicated. Gel was loaded with 3 µL sample + 30 µL SDS loading dye (heated to 95°C for 2 minutes) and stained with Coomassie blue.

It seems as though the protein GST-64 appears together with cell debris during the ultracentrifugation step of lysis, regardless of cell lysis protocol, and is therefore not detected in the cell lysate.

3.4 Sub-localization of DEXI in Jurkat cells

We wanted to investigate the subcellular localization of DEXI in Jurkat T cells in order to evaluate the physiological relevance of interaction partners identified using the Y2H assay.

In our lab there had been previous success with tagging DEXI with a FLAG/DDK tag and use antibodies agains this tag, so this method was applied in this thesis. A plasmid encoding DEXI with a C-terminal DDK-Myc tag, pCMV6-DEXI-DDK-Myc (see appendix D), was already available in our lab, and had already been used to investigate DEXI sublocalization. In this thesis we set out to verify the results from previous experiments and at the same time investigate if localization changes when expressing N-terminal tagged DEXI.

3.4.1 Cloning N-terminal tagged DEXI

A Gateway® entry vector containing DEXI prepared using conventional subcloning was available in our lab. A Gateway® destination vector containing 3xFLAG tag was provided by Anne Simonsen (UiO). The Gateway® strategy (see section 2.1.12) was performed using these vectors to generate plasmid pDEST-3xFLAG-DEXI, encoding DEXI with three N terminal FLAG tags.

3.4.2 Optimization of DEXI overexpression in Jurkat cells

The pDEST vector had not been used to overexpress DEXI in Jurkat cells in our lab before, so an optimization step was included to determine the optimal conditions for transfection. Jurkat cells were prepared and transfected as described in section 2.4.2, using 1, 3 and 5 µg plasmid. Cells were harvested 24 and 48 hours after transfection. In addition, a transfection was prepared with the plasmid EGFP-N3, to be used for checking transfection efficiency by flow cytometry (see section 2.4.2). RIPA cell lysates were prepared from the cells of each transfection, and their protein concentrations estimated by a Bradford assay (see section 2.2.4). SDS-PAGE (section 2.2.5) followed by Western blot using α-DDK (section 2.2.7) was performed using 70 µg protein from each sample (figure 3.11).

From the Western blot it would appear that 1 µg plasmid is not sufficient for proper expression of *DEXI* in Jurkat cells, whereas decent expression is evident in cells transfected with both 3 and 5 µg, and after 24 as well as 48 hours of incubation. It seems that transfection with 3 µg and incubation at 48 h yields marginally more 3xFLAG-DEXI, so these conditions

was applied in following experiments.

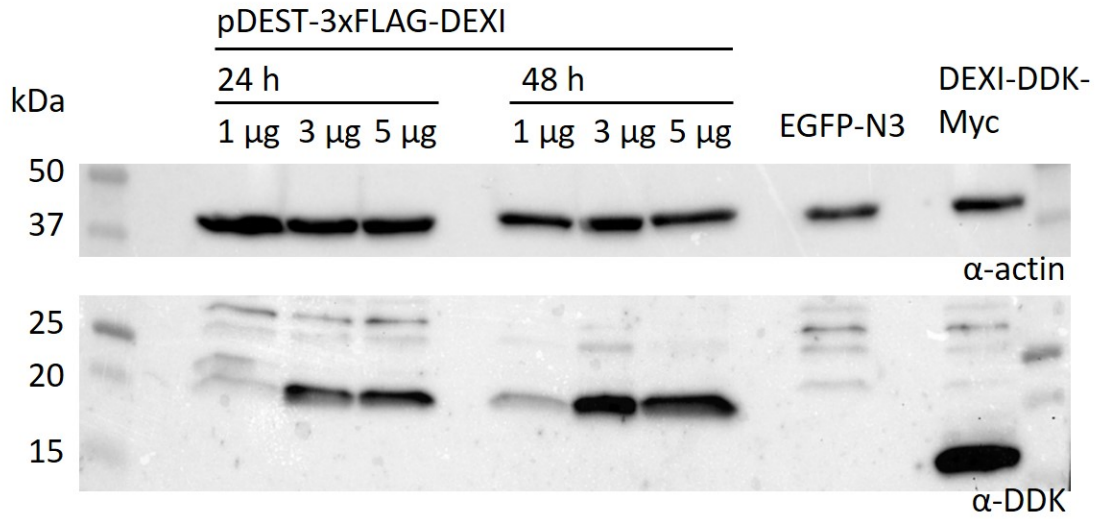

Figure 3.11: Optimization of DEXI expression in Jurkat
The figure shows a Western blot probed with α-DDK, stripped and then reprobed with α-actin. Wells contain RIPA lysates from Jurkat cells transfected with various amounts of pDEST-3xFLAG-DEXI, harvested 24 or 48 h after transfection. Each of these wells contain approximately 70 µg protein. A negative control for DEXI is provided by cells transfected with 1 µg plasmid EGFP-N3, and a positive control for DEXI is provided loading 45 µg DEXI-DDK-Myc.

3.4.3 Immunostaining and confocal microscopy

To locate the intracellular localization of DEXI in Jurkat cells, DEXI was overexpressed in these cells using two different plasmids, pCMV6-DEXI-DDK-Myc and pDEST-3xFLAG-DEXI. These plasmids encode the protein DEXI in fusion with the same tag (flag/DDK), C-terminal for DEXI-DDK-Myc and N-terminal for 3xFLAG-DEXI. The transfected cells were stained as described in section 2.5.1, using primary antibody against DDK. When studied with confocal microscopy, stained cells transfected with pDEST-3xFLAG-DEXI were too few to draw conclusions of DEXI localization. From stained cells transfected with pCMV6-DEXI-DDK-Myc (figure 3.12), it appears that DEXI is located in cytosol or plasma membrane. This concurs with the results from previous experiments performed in our lab (Eriksson, Keshari, Berge, unpublished).

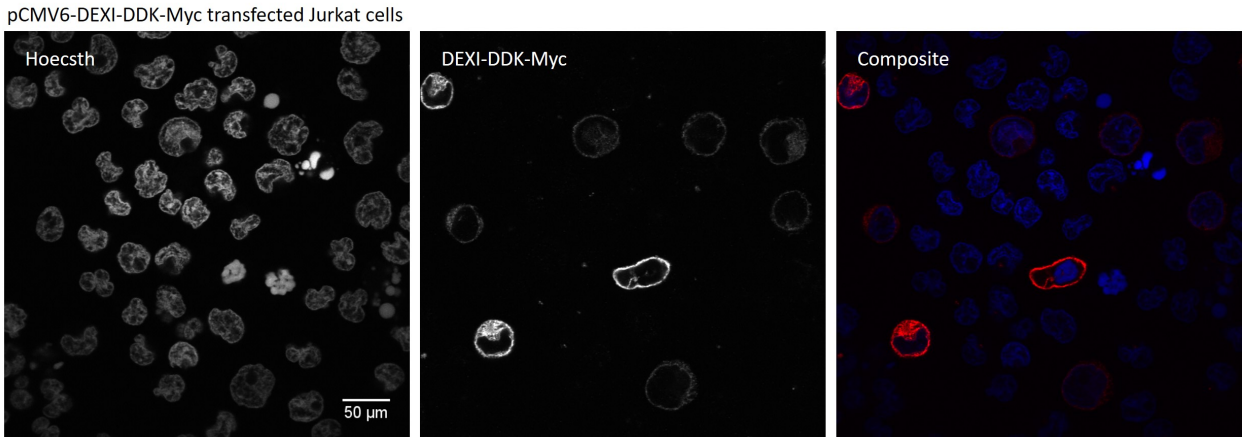

Figure 3.12: Sublocalization of DEXI in Jurkat cells
Jurkat cells transfected with pCMV6-DEXI-DDK-Myc and stained Hoechst and primary antibody α-DDK + secondary antibody conjugated with Alexa Fluor®555

4 - Discussion

In this thesis, a yeast two-hybrid assay was performed to investigate potential interaction partners for DEXI from a cDNA library from human thymic tissue. After mating yeast cells and screening for expression of three reporter genes (see section 1.4.1), five proteins were identified as potential interacting partners. One of the candidates was selected for further investigation. The candidate was confirmed to lead to expression of reporter genes in the Y2H system, but further attempts to confirm interaction by using an independent method, a GST pull-down, was not completed.

The secondary aim of the thesis was to investigate the subcellular localization of DEXI in Jurkat cells using both N-terminally and C-terminally tagged DEXI. Immunostaining and confocal microscopy indicated that C-tagged DEXI is localized in cytoplasma or plasma membrane, but localization of N-tagged DEXI could not be determined.

4.1 Yeast two-hybrid screening

When performing initial tests to investigate the effects of expressing DEXI in yeast cells, it was found that the protein did not infer any detrimental effects on the viability of the yeast cells. It was, however, discovered that DEXI was able to autoactivate the reporter system used. As the function of DEXI is not known, there is a possibility that the protein have some latent transcription activation activity. Such an activation activity is found in $\sim$ 5% of all proteins [58], and could explain the observed autoactivation. The autoactivation was successfully inhibited with 3AT. The first two screenings against reporter genes yielded many colonies, but a major fraction of the colonies were lost in the successive screening steps (see table 3.3).

After having selected 92 interesting interaction candidates, DNA retrieval and sequencing was performed. The initial attempt of performing colony PCR directly on cell lysate proved to be very inefficient. Colony PCR is known to be less effective than PCR performed on

purified DNA, due to the amount of cell debris and proteins in the sample. Similarly low-quality yield is expected when isolating plasmids from yeast cells, so sequencing directly from these plasmids rarely yields results. Sequences were, however, successfully obtained by plasmid isolation and amplification in bacteria.

When sequencing, only forward primer was used. Because we cannot be sure where the sequencing terminates, we cannot know whether the entire insert is sequenced (figure 4.1), unless the vector can be found in both ends of the sequence. This uncertainty should be kept in mind when assessing potential interaction candidates based on their sequence.

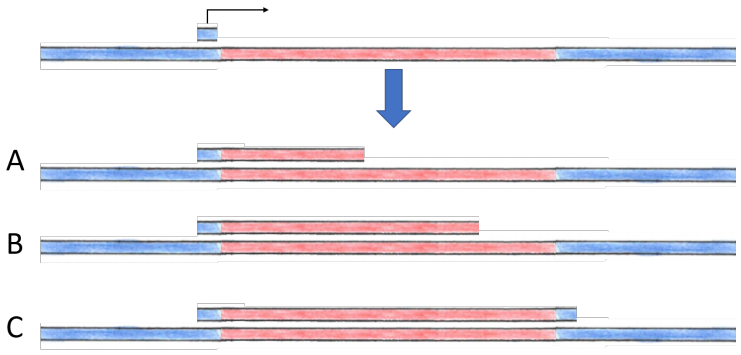

Figure 4.1: Schematic illustration of sequencing using one primer
The primer binds to the pACT2 vector, just upstream of the insert. Sequencing is initiated here, but is not known where sequencing terminates. If sequencing terminates before insert is fully sequenced, we cannot know how large the insert actually is (A and B) compared to the retrieved sequence. The exception is if sequencing continues long enough to include the pACT2 primer in the other end of the insert (C)

After sequences were obtained they were investigated *in silico*. Initially they were stripped of flanking pACT2 vector sequences, and used as queries in BLAST. 21 unique genes were returned. Of these, only 5 were confirmed when BLAST was performed on amino acid sequences in the reading frame of the plasmid.

4.1.1 Addressing false positives

The yeast two-hybrid assay is prone to yield false positive results, with clones containing sequences that cannot be identified constituting up to 67% of the results [80]. In this thesis, the Gal4 transcription factor was used for the Y2H. Gal4-AD is a strong activator of

transcription, which allows the detection of weak interactants, which can potentially reveal interesting results. It does, however, allow for more background than activation domains from other transcription factors may yield [58]. During the Y2H screening performed in this thesis, 5 mM 3AT was used. There is a possibility that a stricter 3AT inhibition could have prevented the appearance of some false positives.

There are many potential reasons why the Y2H give rise to false positive results. In hybrid proteins generated with random cDNA libraries, out-of-frame sequences will appear in five out of six fusion proteins [81]. Some of these might bind to the bait simply by containing several hydrophobic residues, leaving them intrinsically sticky. Many reported false positives of Y2H are clones containing short (typically less than 20 aa), out-of-frame sequences. It has been suggested that these peptides are selected because of common short linear motifs (SLiMs) [80]. These out-of-frame sequences may have high affinity with the bait, if the bait contains SLiMs, or other conserved protein domains [80]. One should also keep in mind that prey proteins that are in reading frame are generated from a cDNA library, and are not necessarily full-length proteins. Such protein-domains may behave differently *in vivo* than their full-length origins [58].

Even if a result is not affected by any of the methodological reasons for false positives, the system enables interaction between proteins that is biologically improbable. Using hybrid proteins can potentially change conformation of your protein, which may affect its interaction abilities. There is also a possibility that the structure of your bait is dependent on post-translational modification that is not facilitated in yeast cells. Interaction may occur as an artefact between proteins that would otherwise never come into proximity, due to them not being expressed at the same time in cell cycle, or in the same organelles [58]. False positives could also occur because the biological function of the protein involves binding to many different proteins, or because of non-specific binding properties the protein might have [82].

Another possible confounding factor when it comes to analysis, is the fact that yeast cells can support transfection with two plasmids simultaneously. Thus there is a possibility that the sequence retrieved from the yeast cells is not, in fact, responsible for the interaction leading to activation of reporter genes [58, 81].

Since the Y2H assay is so prone to false positives it is necessary to validate any results using independent methods to confirm interaction.

4.1.2 Assessment of interaction candidates

The five candidates that yielded a protein alignment were CAMLG, MARCH2, MDK, SLTM and SNAPIN.

CAMLG appears to be the most interesting candidate, as it was replicated many times, and several of the replicants contained long sequences ($\sim$ 300 aa). Interestingly, the function of CAMLG is involved in T cell activation, which is highly relevant for MS. It has been found that CAMLG deficient T cells proliferate less after activation and eventually dies, indicating that the protein is essential downstream of T cell activation [72]. CAMLG contains several hydrophobic domains long enough to span a membrane, and a hydrophilic N-terminal domain, suggested to regulate the function of the membrane-spanning domain. Immunostaining and confocal microscopy indicate that the protein is localized in cytoplasmic vesicles, with the hydrophilic N-terminal located in cytoplasm [73].

Another interesting candidate is the E3 ubiquitin ligase MARCH2, recently found to regulate autophagy [74]. All three candidates that were identified as MARCH2 were aligned over the last 62 amino acids of the protein, a segment containing a PDZ domain binding motif [74].

Midikine (MDK) is a heparin-binding growth factor that has been shown to be significantly upregulated in experimental autoimmune encephalitis (EAE), the rodent model for MS [83]. It has also been shown that mice that are deficient for midikine have an attenuated reponse to MOG-induced EAE [75].

SLTM is a general inhibitor of gene expression. The protein shares a 34% identity heat shock protein SAFB1 [77]. Heat-shock proteins are listed as common false positives of Y2H screenings [58].

Finally, SNAPIN is a toll-like receptor 2 (TLR2) interacting protein that has been shown to be highly expressed in macrophages of patients with rheumatoid arthritis. Evidence points

to SNAPIN playing a role in lysosomal trafficking and maturation as well as autophagy [78].

The immune-related functions of CAMLG, midikine, and SNAPIN makes them highly interesting candidates for further studies in light of the association between DEXI and MS, as well as other immune-related diseases. Two of the candidates, MARCH2 and midikine, are involved in autophagy. This makes autophagy an interesting focal point for further functional studies of DEXI. SLTM may constitute a less interesting interaction candidate, due to its shared identity with known false positives of Y2H, but it could still constitute a legitimate interaction that could teach us something about the function of DEXI. The yeast two-hybrid system is a useful method that could contribute to generating hypotheses about protein-protein interactions, but all proposed interaction partners must be validated using independent methods before conclusions can be drawn. One possible method that could be used is the GST pull-down assay.

4.2 GST

Following the Y2H screening we wanted to confirm interaction of one candidate using a separate method, and decided to perform a GST pull-down with candidate 64, containing partial CAMLG. The candidate sequence was cloned into vector pGEX-6p-1 and used to transform *E. coli* BL21(DE3). The encoded fusion protein GST-64 could not be detected in the lysate. Upon investigating protein was found to precipitate during centrifugation step of lysing for three different lysis protocols. This is a common problem in protein expression, and might result from misfolding of the protein as a result of the high rate of protein production. To overcome the problem, optimization of the protocol can be performed, or alternative methods could be considered, discussed in section 4.4.

4.3 Subcellular localization

DEXI has, due to its transmembrane regions and its leuzine sipper motif, been predicted to span the plasma membrane [56]. Preliminary sublocalization experiments performed in our lab indicates that C-terminally tagged DEXI is localized in plasma membrane or cytosol of Jurkat T cells. Since terminal tags potentially can affect the subcellular localization of proteins, we wanted to investigate whether the N-tagged DEXI would localize to the same areas as C-tagged. Due to time retrains, only one immunostaining experiment was performed, and the quality of the image procured of N-tagged was not sufficient to draw conclusions. An image of C-terminally tagged DEXI, however, seemed to be in concurrence with images generated previously.

According to The Human Protein Atlas (http://www.proteinatlas.org), there is evidence for DEXI expression in nuclear speckles and cytosol. When using saponin to immunostain cells, it is important to keep in mind that the detergent is quite mild, and does not facilitate entry of antibodies across the nuclear envelope. So when using only saponin protocols, nuclear localization cannot be detected. To achieve this, an immunostaining protocol using stronger detergents, such as methanol, should be applied.

4.4 Concluding remarks and future work

In this study, a yeast two-hybrid system was used to identify possible interaction partners for DEXI. Five potential interaction partners were identified, and one of them was subjected to a remating, replicating the result. To verify the occurrence of interaction in yeast cells, remating could be performed on the remaining four candidates.

A GST pull-down experiment using GST fused to one of the candidates, GAMLG, was initiated, but due to time restraints the experiment was not completed. It was discovered that the GST-fusion protein precipitates during the lysing of cells, implying that an optimization of the protein expression and cell lysation protocols is required, initially by decreasing

incubation time and temperature when expressing recombinant protein in BL21(DE3) cells (section 2.2.1). Once this problem is overcome, the GST pull-down should be completed. If problems with GST-fusion expression persist, one can consider switching roles for the proteins, and rather generate a GST-DEXI clone and use this to pull down CAMLG from lysate. A GST pull-down could also be performed on the rest of the candidates.

Overexpressing C-terminal tagged DEXI in Jurkat cells and immunostaining with a saponing protocol indicated that DEXI is located in plasma membrane or cytosol of cells. To validate this result, immunostaining could be performed using alternate protocols allowing for staining of nucleus. In addition, it could be interesting to see if localization is changed when using a N-terminal tag.

There are some aspects to keep in mind when deciding further studies. When studying hybrid proteins, there is a chance that the structure or behaviour of the protein will change as a result of the modification, including protein localization and binding affinities. It experiments are repeated with different types of tags, it could indicate whether binding affinity or localization is affected by the tag. In addition, structure of mammalian proteins may be reliant on post-translational modifications that will not occur when using bacterial or yeast model systems. Because of these things, it would be interesting to study the interactions in its native form, for example by performing immunoprecipitation, where crude lysate and bait protein extracted from mammalian cells can be used. Finally, in Y2H, interaction is revealed between proteins independently of their expression pattern in cells. The interaction could be an artefact, as the proteins might never be in actual proximity under physiological circumstances.

In light of the connection to the T cell driven immunopathology of MS, it could be interesting to study the effect of overexpression or knock-down studies of DEXI in Jurkat T cells. In relation to the proposed interaction with CAMLG and midikine, two proteins with function related to immunoregulation, it could be interesting to study the effect DEXI might have on cell activation or cytokine production. In addition, it could be interesting to study the effect on lysosomal trafficking and autophagy, as two of the proposed candidates, SNAPIN and MARCH2, have functions related to these mechanisms.

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Appendix A: Abbreviations

3AT	3-amino-1,2,4-triazole
3C	Chromosome confirmation capture
AA	Alopecia areata
AD	Activation domain
Ade	Adenine
AID	autoimmune disease
APC	antigen presenting cell
BBB	blood-brain barrier
BD	DNA binding domain
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary DNA
CIITA	*Class II transactivator*
CIS	Clinically isolated syndrome
CLEC16A	*C-type lectin-like domain family 16A*
c-Myb	Human cMyb
CNS	Central nervous system
DEXI	*Dexamethasone-induced gene*
dH_2O	Destilled water
DIS	Dissemination in space
DIT	Dissemination in time
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DTT	Dithiotreitol
E. coli	*Escherichia coli*
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
EDTA	Ethylenedinitrilotetraacetic acid (EDTA)
eQTL	Expression quantitative trait loci
EtBr	Ethidium bromide
EtOH	Ethanol
FBS	Foetal bovine serum
FCS	Foetal calf serum
GFP	Green fluorescent protein
HCl	Hydrogen chloride
His	Histidine
HLA	Human leukocyte antigen
HRP	Horseraddish peroxidase
Ig	Immunoglobulin
IHF	*E. coli* integration host factor

Int	Bacteriophage λ integrase
IPTG	Isopropyl-β-D-tiogalactopyranosid
JIA	Juvenile idiopathic arthritis
kb	Kilobase
KCl	Potassium chloride
LB	Lysogeny broth
Leu	Leucine
LiAc	Lithium acetate
LPS	lipopolysaccharide
M	Molar (mol/L)
MBP	Myelin basic protein
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
Mw	Molecular weight (g/moL)
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEAA	Non-essential amino acids
OD	Optical density
ON	Overnight
PAGE	Polyacrylamide gel electrophoresis
PAI	Primary adrenal insuffieciency
PAMP	pathogen-associated molecular patterns
PBC	Primary biliary cirrhosis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIC	Protease inhibitor cocktail
PLP	Myelin proteolipid protein
PMSF	Phenylmethanesulfonyl fluoride
PPMS	primary progressive MS
PRR	pathogen-recognition receptors
PVDF	Polyvinylidene fluoride
RA	Rheumatoid arthritis
RIPA	Radioimmunoprecipitation assay buffer
RIS	Radiologically isolate syndrome
RNA	Ribonucleic acid
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute medium
RRMS	relapsing-remitting MS
RT	Reverse transcriptase
RT	Room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SC	Synthetic complete

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
SOB	Super optimal broth
SOCS1	*Suppressor of cytokine signalling 1*
SPMS	secondary progressive MS
ssDNA	DNA sodium salt
T1D	Type 1 diabetes
TAE	Tris-acetate ethylene diamine tetraacetate
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween
TCR	T cell receptor
TE	Tris-ethylene diamine tetraacetate
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
Tris-EDTA	Tris-ethylene diamine tetraacetate
Trp	Tryptophan
UV	Ultraviolet
VLA4	\textalpha 4-integrin
x-α-gal	5-bromo-4-chloro-3-indolyl-α-D-galactopyranosid
YPDA	Yeast peptone dextrose adenine

Appendix B: Materials

Chemicals	**Supplier**	**Cat. no**
3-amino-1,2,4-triazole (3AT)	Sigma-Aldrich	A8056
L-Adenine hemisulphate	Sigma-Aldrich	A9126
Agarose, SeaKem® LE	Lonza	50004
Ammonium sulphate	Sigma-Aldrich	A4418
Ampicillin sodium salt	Sigma-Aldrich	A9518
Bacto agar	OXOID	LP0011
Bacto peptone	Formedium	PEP03
Bromophenol blue	Sigma-Aldrich	B0126
Calcium chloride ($CaCl_2$)	Sigma-Aldrich	C7902
Dithiotreitol (DTT)	QIAGEN	
Drop-Out supplement amino acid mix (-ade/-his/-leu/-trp)	Clontech	630428
Ethanol	Sigma-Aldrich	
Ethidium bromide	MP Biomedicals	190202
Ethylenedinitrilotetraacetic acid (EDTA)	Merck Millipore	108452
Foetal bovine serum (FBS)	Gibco	10500064
Chloramphenicol	Sigma-Aldrich	C0378
Glucose $\geq$ 99.5 %	Sigma-Aldrich	G7021
Glycerol $\geq$ 99.5%	Sigma-Aldrich	G5516
Glycine	Sigma-Aldrich	G7126
L-Histidine HCL monohydrate	Sigma-Aldrich	H5659
Hoechst 33342	Thermo Scientific	H1399
Hydrochloric acid (HCl)	Sigma-Aldrich	H1758
Isopropanol	Sigma-Aldrich	59304
Kanamycin sulfate	Amresco	A9518
L-Leucine	Sigma-Aldrich	L8912
Lithium acetate dihydrate (LiAc)	Sigma-Aldrich	L6883
Magnesium chloride ($MgCl_2$)	Merck Millipore	105833
Magnesium sulfate ($MgSO_4$)	Sigma-Aldrich	M5921
Methanol	Sigma-Aldrich	32213
N,N-Dimethylformamide (DMF)	Sigma-Aldrich	D4551
Non-essential amino acids	Gibco	11140035
Paraformaldehyde	Sigma-Aldrich	P6148
Penicillin-Streptomycin	Thermo Scientific	15140122
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich	P7626
Polyethylene glycol (PEG) 3350	Sigma-Aldrich	202444
Potassium chloride (KCl)	Sigma-Aldrich	P9541
Saponin from quillaja bark	Sigma-Aldrich S7900	
Skim milk powder	Sigma-Aldrich	70166
Sodium chloride (NaCl)	Merck Millipore	106404
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich	L3771

Chemicals	Supplier	Cat. no
Sodium hydroxide (NaOH)	Applichem	A3910
Sodium hypochlorite (NaOCl)	Sigma-Aldrich	425044
Sodium pyruvate	Gibco	11360-039
Tris-base	Fisher Scientific	BP152-1
Triton™ X-100	Sigma-Aldrich	T9284
Trizma® base	Sigma-Aldrich	T1503
Trizma® hydrochloride (Tris-HCl)	Sigma-Aldrich	T3253
Tryptone	OXOID	LP0042
L-Tryptophane	Sigma-Aldrich	T0254
Tween® 20	Sigma-Aldrich	P1379
X-α-gal	Clontech	630407
Yeast extract	OXOID	LP0021
Yeast nitrogen base	BD	291940
β-mercaptoethanol	Applichem	A1108

Enzymes	Supplier	Cat. no
FastAP Thermosensitive Alkaline Phosphatase	Thermo Scientific	EF0654
FastDigest™ BamHI	Thermo Scientific	FD0054
FastDigest™ BglI	Thermo Scientific	FD0083
FastDigest™ EcoRI	Thermo Scientific	FD0274
FastDigest™ EcoRV	Thermo Scientific	FD0303
FastDigest™ NotI	Thermo Scientific	FD0593
FastDigest™ SmaI	Thermo Scientific	FD0663
Gateway® LR clonase® II Enzyme Mix	Thermo Scientific	11791020
GoTaq® DNA polymerase	Promega	M3005
Pfu Turbo DNA polymerase	Agilent	600250-52
T4 DNA ligase	Thermo Scientific	EL0011

Commercial kits	Supplier	Cat. no
EndoFree® Plasmid MAXI Kit	QIAGEN	12362
PrepEase® Yeast Plasmid Isolation Kit	Affymetrix	79220
QIAprep® Spin Miniprep Kit	QIAGEN	27104
Wizard® SV Gel and PCR Clean-Up System	Promega	A9281

Antibodies	Supplier	Cat. no
Anti-DDK monoclonal antibody	OriGene	TA50011-100
Anti-actin antibody produced in rabbit	Sigma-Aldrich	A2066
F(ab')2-Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor®555	Thermo Scientific	A21425

Standards and buffers	Supplier	Cat. no
BugBuster®	Novagen	70921
Clarity™Western ECL Substrate	Bio-Rad	1705060
4-15% Criterion™TGX™	Bio-Rad	5671083
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	11836170001
Coomassie Brilliant Blue R-250 Staining Solution	Bio-Rad	1610436
6x DNA loading dye	Thermo Scientific	R0611
dNTP	Applied Biosystems	362271
Drop-Out supplement amino acid mix (-Ade, -His, -Leu, -Trp)	Clontech	630428
Dulbecco's phosphate buffered saline (PBS)	Sigma-Aldrich	D8537
Extra thick blot filter paper	Bio-Rad	1703967
10x FastDigest Buffer	Thermo Scientific	B64
GeneRuler 1 kb DNA ladder	Thermo Scientific	SM0311
GeneRuler 100 bp DNA ladder	Thermo Scientific	SM0241
Glutathione-sepharose™4B beads	GE healthcare	17075601
GoTaq buffer	Promega	M792A
Immun-blot® PVDF membrane	Bio-Rad	162-017
10% Mini-PROTEAN® TGX™	Bio-Rad	456-1033
Pfu reaction buffer	Agilent	600153-82
Polysine® adhesion slides	VWR	631-0107
Precision Plus Protein™ All Blue Standard	Bio-Rad	161-0373
Precision Plus Protein™ Dual Colour Standard	Bio-Rad	161-0374
Quick Start™ Bovine Serum Albumin Standard	Bio-Rad	500-0206
Quick Start™ Bradford 1x Dye Reagent	Bio-Rad	500-0205
Restore™ PLUS Western Blot Stripping Buffer	Thermo Scientific	46430
RIPA Lysis and Extraction Buffer	Thermo Scientific	89900
RPMI	Gibco	21875-034
T4 ligase buffer	Thermo Scientific	123962
10x TGS Buffer	Bio-Rad	161-0732
Tris buffered saline with Tween® 20 (TBS-T)	Sigma-Aldrich	91414

Machines and equipment	Supplier
2720 Thermal Cycler	Applied Biosystems
Attune® Acoustic Focusing Cytometer	Applied Biosystems
ChemiDoc™ Touch Imaging System	Bio-Rad
NanoDrop 2000c	Thermo Scientific
pHenomenal™	VWR
Ultrospec 2100 pro	Amersham Biosciences

Software	Supplier
Attune® Cytometric Software	Applied Biosystems
ImageJ 1.50g	http://imagej.nih.gov/ij
Image Lab 5.2.1	Bio-Rad Laboratories
NanoDrop 2000c	Thermo Scientific

Primers	Sequence
A097-pACT2-forward	5'-GCGTATAACGCGTTTGGAAT-3'
A097-pACT2-reverse	5'-GCACGATGCACAGTTGAAGT-3'
CAMLG-forward	5'-AATTAGGATCCATGGCC1TCGCTACCGA-3'
CAMLG-reverse	5'-AATTAGCGGCCGCGCAGCTGTTAGTACTGTTG-3'

Yeast strains	Genotype (described in [84])
PJ69-4α	MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4(deleted) gal80(deleted) LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ
PJ69-4A	MATA trp1-901 leu2-3,112 ura3-52 his3-200 gal4(deleted) gal80(deleted) LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ

Appendix C: Recipes

Adenine solution, 50x

L-adenine hemisulfate salt	1 g
dH_2O	1 L

Autoclave 121°C, 20 minutes
Store at 4°C

Ampicillin stock solution, 100 mg/mL

Ampicillin sodium salt	500 mg
Autoclaved dH_2O	5 mL

Work in fume hood
Make 1 mL aliquots and store at -20°C.

Binding buffer

1 M Tris-HCl, pH 8.0	5 mL
5 M NaCl	3 mL
0.5 M EDTA	20 mL
20% Triton X-100	5 mL
dH_2O	to 100 mL
Just before use, add	
Protease inhibitor cocktail	1 tablet pr 50 mL buffer
1 M DTT	50 µL pr 5 mL buffer (final concentration 10 mM)

Chloramphenicol, 100 mg/mL

Chloramphenicol	100 mg
Ethanol	1 mL

Sterile filter, store at -20°C

Colony PCR buffer, 10x

1M Tris-HCl, pH 8.5	6.25 mL
1M KCl	11 mL
dH_2O	32.75 mL

Sterile filtrate

De-staining solution for Coomassie gel staining

Ethanol	400 mL
Acetic acid	100 mL
dH_2O	500 mL

DTT, 1 mM

DTT	1.54 g
dH_2O	10 mL

Sterile filter, store at -20°

Glycerol-yeast extract-tryptone (GYT) medium

Glycerol	2 mL
Yeast extract	25 mg
Tryptone	50 mg
dH_2O	To a total of 20 mL

Autoclave, 121°C for 20 minutes

Histidine solution, 50x

L-histidine-HCl monohydrate	1 g
dH_2O	1 L

Autoclave 121°C, 20 minutes
Store at 4°C

IPTG, 1 M

IPTG $\geq$ 99%	2.383 g
dH_2O	to 10 mL

Sterile filter, and store at -20°C

Jurkat growth medium

RPMI media	500 mL
Foetal bovine serum	50 mL
Penicillin tryptomycine	5 mL
Non-essential amino acids	5 mL
Sodium pyruvate	5 mL
β-mercaptoethanol	1.75 µL

Jurkat transfection medium

RPMI media	500 mL
Foetal bovine serum	25 mL
Non-essential amino acids	5 mL
Sodium pyruvate	5 mL
β-mercaptoethanol	1.75 µL

Leucine solution, 50x

L-leucine	5 g
dH_2O	1 L

Autoclave 121°C, 20 minutes
Store at 4°C

Lysogeny broth (LB) medium

Trypton	20 g
Yeast extract	10 g
NaCl	20 g
dH_2O	To a total volume of 2 L
1M NaOH	To adjust pH to 7.2
Optional:	
Bacto agar	6 g pr 400 mL

Autoclave 121°C, 20 minutes

Magnesium chloride ($MgCl_2$), 25 mM

$MgCl_2$	0.255 g
dH_2O	to 50 mL

Sterile filtrate

One-step buffer

1M LiAc	10 mL
PEG3350	20 g
dH_2O	To a total volume of 45 mL
Dissolve completely	
DNA sodium salt from salmon testes, incubated on heating block at 95°C for 5 minutes, and then cooled on ice	2.5 mL
dH_2O	To a total volume of 50 mL

Sterilize tube by incubating 3 x 30 min on waterbath at 65°C.
Store at 4°C

PMSF, 50 mM

PMSF	8.7 mg
Isopropanol	to 1 mL

Sterile filter, store at -20°C

Potassium chloride (KCl), 1M

KCl	3.73 g
dH_2O	To a total of 50 mL
Sterile filtrate	

SDS-PAGE loading buffer, 6x

1.5 M Tris-HCL, pH 6.8	5.8 mL
SDS (Mw=288.4 $^{g}/_{mol}$)	2.5 g
Glycerol	7.5 mL
Bromophenolblue (Mw=691.9 $^{g}/_{mol}$)	$\sim$ 3 mg
dH_2O	To a total of 25 mL

Freeze at -20°C in aliquots of 1 mL.
Add 50 µL of β-mercaptoethanol per mL before use.

Super optimal broth (SOB) medium

Bacto tryptone, (2% $^{v}/_{w}$)	2 g
Bacto yeast extract, (0.5% $^{v}/_{w}$)	0.5 g
NaCl, (10mM)	0.058 g
$MgSO_4 \cdot 7H_2O$, (10mM)	0.247 g
$MgCl_2$, (20mM)	0.20 g
dH_2O	to 100 mL

Synthetic complete (SC) medium

DO supplement amino acid mix (-Ade, -His, -Trp and -Leu)	1.2 g
Glucose	40 g
Ammonium sulphate	10 g
Yeast nitrogen base	3.4 g
dH_2O	To a total volume of 2 L
Optional:	
Bacto agar	8 g pr 400 mL

Autoclave at 121°C for 20 minutes.
After autoclaving, add 8 mL of 50x amino acid solutions pr 400 mL SC medium according to necessity.

Tris/Glycerol/EDTA $(TGE)_{500}$ buffer

1.0 M Tris-HCl, pH 8.0	10 mL
0.5 M EDTA	1 mL
50% Glycerol	100 mL
5.0 M NaCl	50 mL
dH_2O	to 500 mL

Autoclave, 121°C, 20 min
Optional:
1 table protease inhibitor cocktail pr 50 mL buffer

Tris/EDTA/NaCl (TEN) buffer

1.0 M Tris-HCl, pH 8.0	5 mL
0.5 M EDTA	1 mL
5.0 M NaCl	10 mL
dH_2O	to 500 mL

Autoclave, 121°C, 20 min

Tris-HCl, 1M

Tris-base (121.1 g/mol)	121.1 g
dH_2O	$\sim$ 800 mL
HCl	To adjust pH to 8.5 or 8.0
dH_2O	To a total volume of 1000 mL

Autoclave at 121°C for 20 minutes.

Triton X-100, 20%

Triton X-100	10 mL
dH_2O	40 mL

Sterile filtrate

Tryptophane solution, 50x

L-tryptophane	1 g
dH_2O	1 L

Autoclave at 121°C for 20 minutes.
Store at 4°C.

Transfer buffer for Western blot

25 mM Tris, 192 mM glycin, 10% methanol

Tris base (Mw=121.14 $^{g}/_{mol}$)	15 g
Glycin (Mw=75.067 $^{g}/_{mol}$)	72 g
Methanol	500 mL
dH_2O	To a total of 5.0 L

X-α-gal, 20 mg/mL

X-α-gal	25 mg
Dimethylformamide (DMF)	1.25 mL

Make aliquots of 100 µL and store at -20°C.

Yeast peptone dextrose adenine (YPDA) medium

	0.5x	**1x**	**2x**
Yeast extract	20 g	40 g	80 g
Bacto peptone	40 g	80 g	160 g
Glucose	40 g	80 g	160 g
dH_2O to a total of	4 L	4 L	4 L

Adjust pH to 5.0-5.5 using HCl
If making plates, add 8 g bacto agar per bottle.
Autoclave (121°C, 20 min)
Add 50x adenine solution:
6 mL to 400 mL 0.5x
12 mL to 400 mL 1x
24 mL to 400 mL 2x

Appendix D: Plasmids

pCMV6-DEXI-DDK-Myc

DEXI (Myc-DDK-tagged)-Human Dexi homolog (mouse) (DEXI) bought from OriGene (catalogue number RC207463). Plasmid encodes DEXI with a C-terminal DDK-Myc tag.

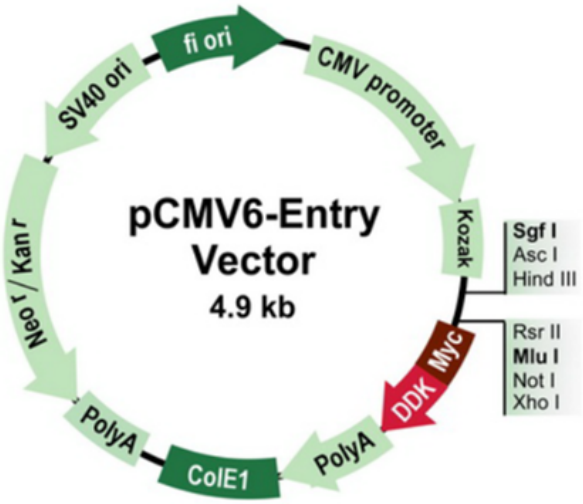

Figure D.1: pCMV6 plasmid
pCMV6-DEXI-DDK-Myc was bought from OriGene. DEXI is cloned into the MCS of the plasmid, not shown i the map. Image taken from OriGene (http://www.origene.com, validated 22.10.17)

pENTR-3C-DEXI

DEXI was cloned from pCMV6-DEXI-DDK-Myc to Gateway® entry vector pENTR 3C to form pENTR-3C-DEXI. Cloning was performed by Pankaj Keshari in our group.

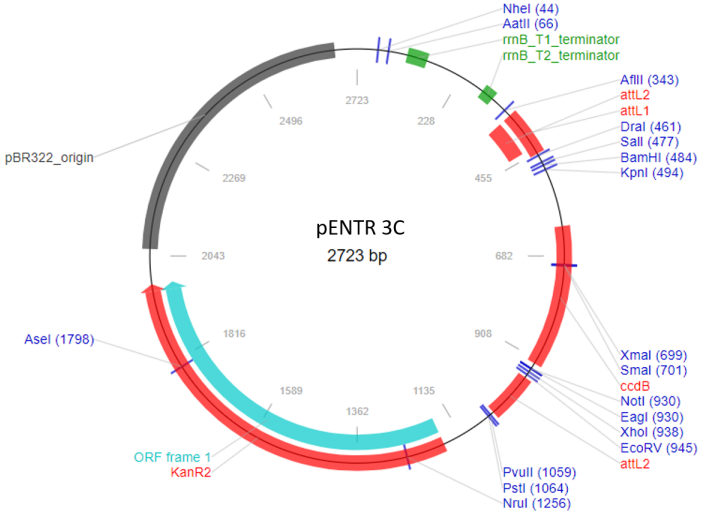

Figure D.2: pENTR 3C plasmid
Image taken from AddGene (http://www.addgene.org, validated 22.0.17)

pDEST-3xFLAG

Gateway® destination vector, containing N-terminal triple FLAG tag. Kindly provided by Anne Simonsen (UiO).

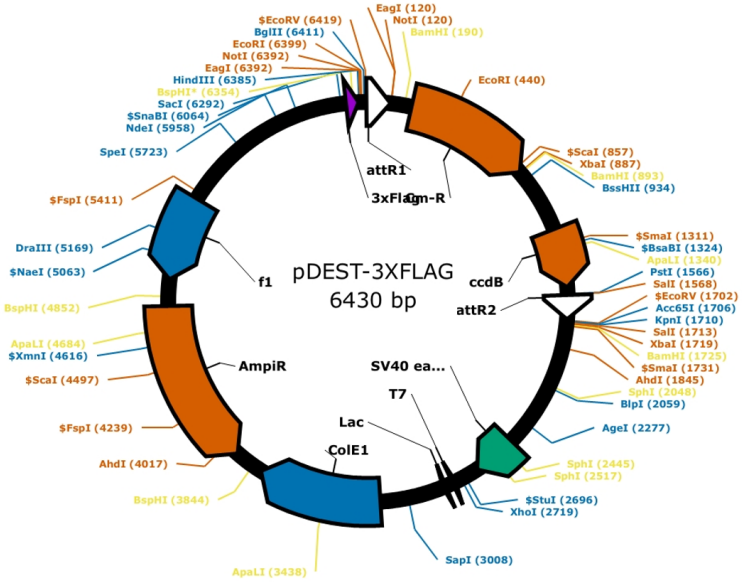

Figure D.3: pDEST-3X plasmid

pEGFP-N3

Plasmid encodes green fluorescent protein. Kindly provided by Anne Spurkland (UiO).

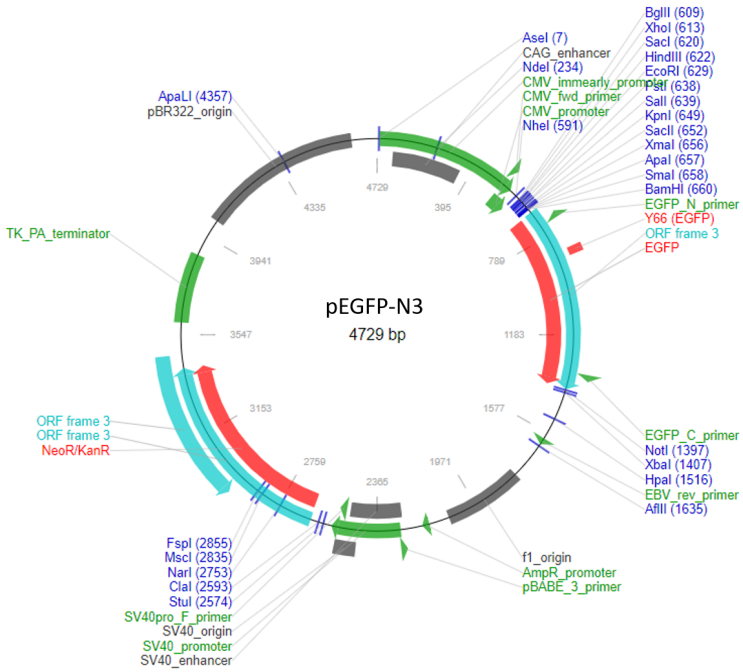

Figure D.4: pEGFP-N3 plasmid
Image taken from AddGene (http://www.addgene.org, validated 22.0.17)

pDBT

Plasmids pDBT and pDBT-c-Myb were kindly provided by Odd Stokke Gabrielsen (UiO) [85]. Cloning of pDBT-DEXI was performed by someone in our group. DEXI was cloned from pCMV6-DEXI-DDK-Myc into vector pDBT between recognition sites for EcoRI and NotI within the multiple cloning site (MCS) of the plasmid. The resulting pDBT-DEXI plasmid had a total size of 7464 bp

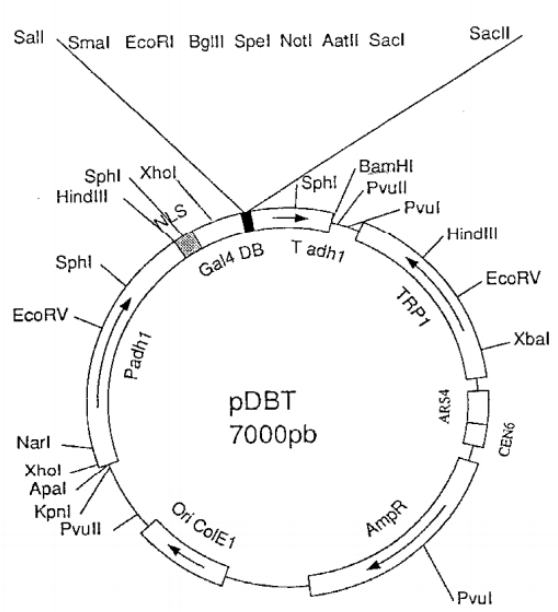

Figure D.5: pDBT plasmid
pDBT-DEXI and pDBT-c-Myb were generated by cloning DEXI and c-Myb into MCS of pDBT, resulting in a plasmid encoding DEXI-Gal4DB or c-Myb-Gal4DB fusion proteins used in Y2H

pACT2

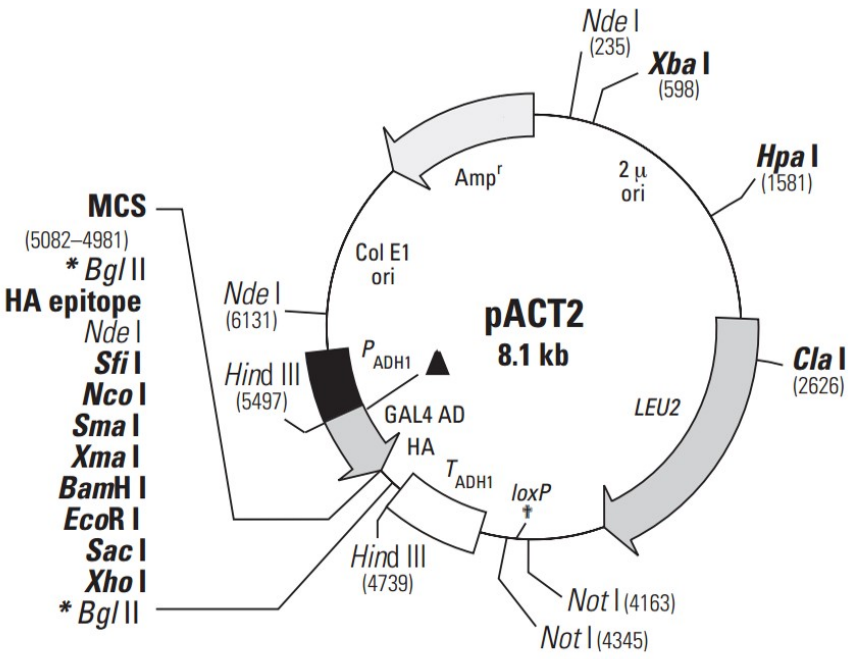

Figure D.6: pACT2 plasmid
cDNA library were cloned into pACT2 between recognition sites for XhoI and EcoRI within the MCS of the plasmid resulting in a plasmid encoding cDNA library protein-Gal4AD hybrids used in Y2H

pACT2 plasmid and pACT2 cloned with Human Thymus Matchmaker™ cDNA Library from Clontech (catalogue number 638827) were gifts from Odd Stokke Gabrielsen (UiO) [85].

pGEX-6P-1

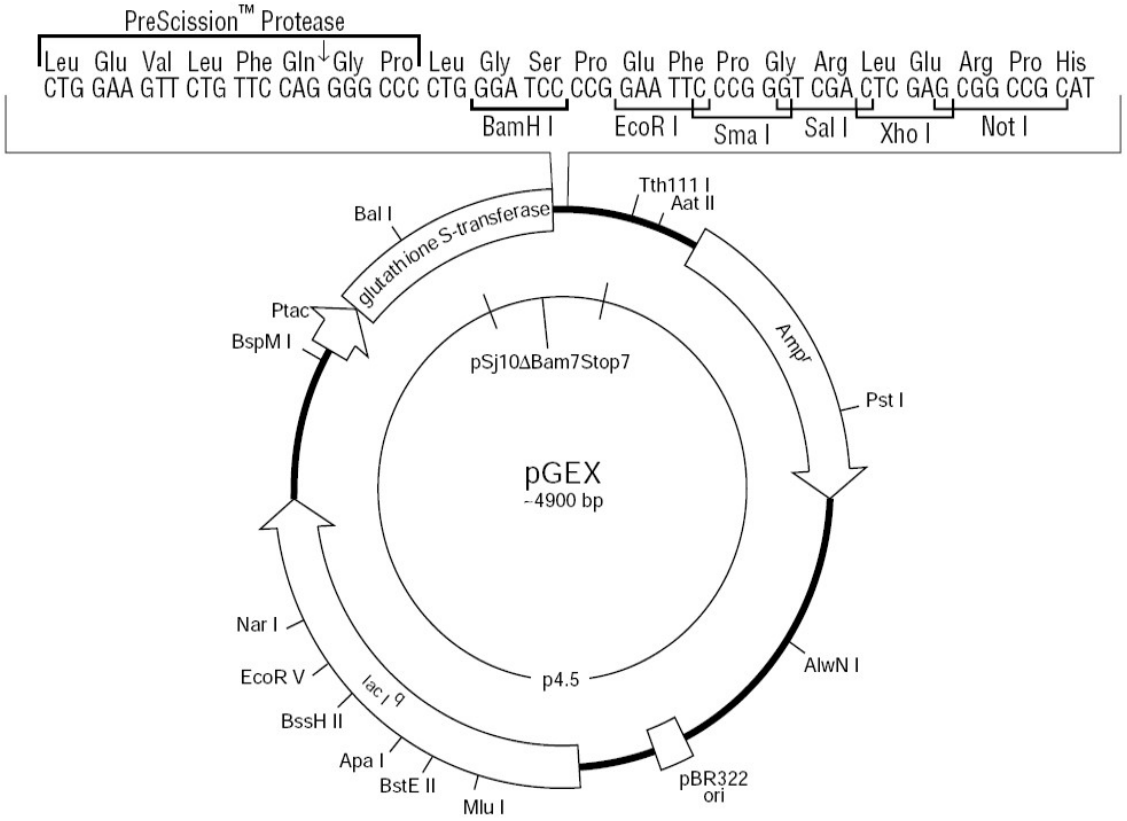

Figure D.7: pGEX-6p-1 plasmid
Image taken from GE healthcare website (http://www.gelifesciences.com, validated 22.10.17)

GST expression vector pGEX-6p-1 from GE healthcare (catalogue number 28-9546-48) was kindly provided by Anne Spurkland (UiO).