

CHAPTER 4

INVESTIGATION INTO PROTEIN-PROTEIN INTERACTIONS BETWEEN RAT BRAIN SECRETORY PROTEINS AND AN *O. savignyi* cDNA LIBRARY BY MEANS OF THE GAL4 TWO-HYBRID SYSTEM

4.1. INTRODUCTION

Protein-protein interactions are essential to cellular mechanisms at all levels in biologically responsive systems. These interactions occur extracellularly, and include ligand-receptor interactions, cell adhesion, antigen recognition, immune response and virus-host recognition (Young 1998). Intracellular protein-protein interactions occur in the formation of multi-protein complexes, during the assembly of cytoskeletal elements, between receptor-effector as well as effector-effector molecules of signal transduction pathways and even assembly of transcriptional machinery. These interactions can be defined as the interaction between specific amino acid regions or interacting pockets of the two proteins, which can be studied by various methods such as FRET (fluorescence resonance energy transfer), chemical cross-linking, surface plasmon resonance biosensors, affinity chromatography, immunoprecipitation and fluorescence gel retardation, to name just a few. All of the latter techniques have their advantages as well as disadvantages. One great disadvantage is that most of them require the availability of one protein binding-partner (bait) and is performed on protein level. Therefore, after identification of the interacting partners, one needs to determine their amino acid sequences in order to clone them for further studies. Also, these techniques do not always reflect the *in vivo* reactions, which are usually more complex than *in vitro* reactions. One technique, the two-hybrid assay, is performed *in vivo* and results in the immediate gene isolation of the interacting proteins or protein domains (Frederickson 1998). Since no data is available on the SNAREs and secretory proteins of *O. savignyi*, we exploited the two-hybrid system.

4.1.1. The yeast two hybrid system

In July 1989, Stanley Fields and Ok-kyu Song published the first paper describing a novel genetic system to study protein-protein interaction by taking advantage of the properties of the GAL4 protein of the yeast, *Saccharomyces cerevisiae* (Fields and Song 1989). They investigated the yeast proteins SNF1, SNF4 and the GAL4 protein, which is a transcriptional

activator required for the expression of genes encoding enzymes of galactose utilization. It consists of two separable and functionally essential domains: a N-terminal DNA-binding domain, which binds to specific DNA sequences (UAS_G) and a C-terminal activation domain containing acidic regions, which is necessary to activate transcription by directing the RNA polymerase II complex to transcribe the gene downstream of the UAS (Figure 4.1). By generating two hybrid proteins GAL4(1-147)-SNF1 and SNF4-GAL4(768-881), transcription of reporter genes regulated by UAS_G occurred (Fields and Song 1989). Their results further indicated that the DNA-binding domain alone did not activate transcription, nor did the GAL4(1-147)-SNF1 or SNF4-GAL4(768-881) fusion proteins.

Today, we know that many eukaryotic trans-acting transcription factors are composed of physically separable, functionally independent domains. These include a DNA-binding domain (DNA-BD) that binds to a specific enhancer-like sequence, which in yeast is referred to as an upstream activation sequence (UAS). In the case of the native yeast GAL4 protein, the two domains are part of the same protein, but in principle any activation domain (AD) can be paired with any DNA-BD to activate transcription.

In the MATCHMAKER™ GAL4 two-hybrid system that was used in this study, the DNA-BD (amino acids 1–147) and the AD (amino acids 768–881) are both derived from the yeast GAL4 protein. Two different cloning vectors are used to generate fusions of these domains to genes encoding proteins that potentially interact with each other. The recombinant hybrid proteins are co-expressed in yeast and are targeted to the yeast nucleus. An interaction between a bait protein (fused to the DNA-BD) and a library-encoded protein (fused to the AD) creates a novel transcriptional activator with binding affinity for a GAL4-responsive UAS. This factor then activates reporter genes having upstream GAL4-responsive elements in their promoter, and this makes the protein-protein interaction phenotypically detectable. If the two hybrid proteins do not interact with each other, the reporter genes will not be transcribed (Figure 4.1.).

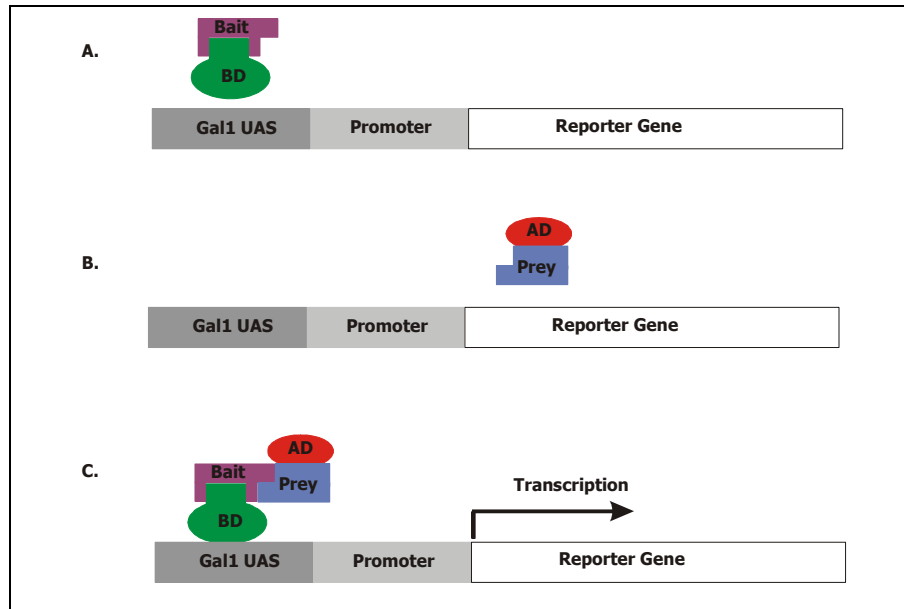


Figure 4.1. Schematic diagram of the GAL4-based two-hybrid system. (A) The DNA-BD/bait hybrid binds to the Gal1 UAS but cannot activate transcription without the activation domain. (B) In the absence of bait protein, the AD/library fusion protein cannot bind to the Gal1 UAS and thus does not activate transcription. (C) Interaction between the bait and library protein *in vivo* activates transcription of the reporter gene.

Constructing a DNA Binding Domain-Bait chimeric protein

Performing a two-hybrid experiment is a complex process involving many different steps, and there are a number of potential problems. When constructing a BD/Bait construct, the technical problems of cloning the gene of interest into a suitable plasmid and the variety of *in vivo* criteria that should be met, are the greatest source of problems (MacDonald 2001). These are discussed in greater detail below.

i. Cloning the gene of interest

In this study the bait proteins were cloned into the pAS2_1 vector (Figure 4.2). This vector includes both bacterial (Col E1) and yeast (2μ) origins of replication and a phenotypic selection marker (*TRP1*) suitable for selection in the yeast strain of choice, AH109. The pAS2_1 vector contains a cycloheximide resistant marker (*CYH2*) that is useful for eliminating false positives that have lost bait plasmid and also ampicillin resistance gene (*Amp^r*) for selection in bacteria. The multiple cloning site (MCS) in all reading frames was used for directional cloning of the bait (see methods section). Great care was taken to ensure that the bait was cloned in the correct reading frame, i.e. in the reading frame of the GAL4 BD, which forms the N-terminal part of the fusion protein and ensuring that cloning of the gene does not create an in-frame stop codon.

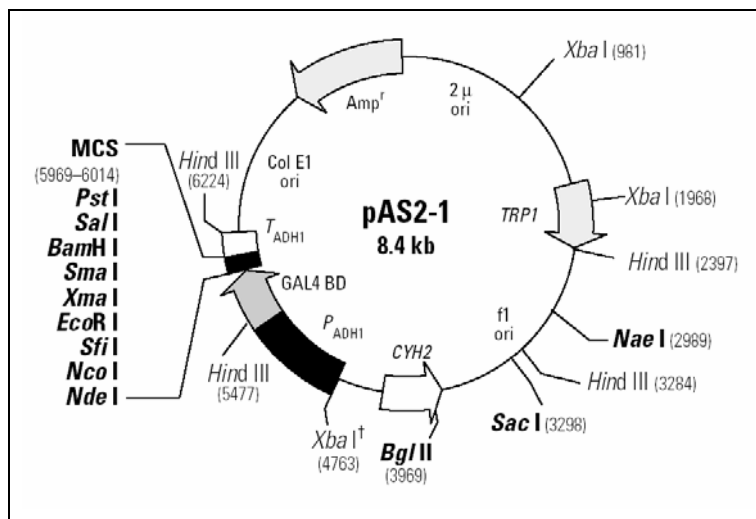


Figure 4.2. pAS2-1 map and MCS (Clontech Laboratories 1997a). Unique sites are in bold. *pAS2-1* is a cloning vector used to generate fusions of a bait protein with the GAL4 DNA-BD (amino acids 1–147). *pAS2-1* carries the *CYH2* gene for cycloheximide sensitivity. The hybrid protein is expressed at high levels in yeast host cells from the full-length *ADH1* promoter (P_{ADH1}). The *Xba I* site at bp 4763 (†) is methylation sensitive. *pAS2-1* contains the *TRP1* gene for selection in *Trp*⁻ auxotrophic yeast strains.

Since the interacting complex must be able to localize to the nucleus, care must be taken to choose the correct domain(s) of the protein of interest as bait. In the case of cytosolic proteins such as α -SNAP and Rab3a, full-length constructs were used. For syntaxin, which is an integral membrane protein, both a full-length construct and a truncated construct (lacking the C-terminal transmembrane region) was used to ensure localization to the nucleus.

ii. In vivo criteria

a. Levels of expression: Vector Promoters, Terminators and Copy number

Currently, most two-hybrid vectors utilize the yeast *ADH1* promoter to drive expression of the fusion protein and transcription is terminated at the *ADH1* transcription termination signal. The *ADH1* promoter is available in full-length and truncated (*ADH1**) forms, which results in lower expression levels. The *pAS2_1* plasmid used in this study contains a full-length *ADH1* promoter that results in high levels of constitutively expressed BD/bait fusion protein (Clontech Laboratories 1997a). This enables the detection of weak interactions, but can also increase toxicity and background activation of reporter genes. There is a common belief that higher expression will increase the number of interactions identified. However, in most cases the same interactions are detected with low expression and could even result in better detection of interactions. Therefore, great care should be given to choosing a promoter or even testing a high- and low strength promoter (MacDonald 2001).

The majority of two-hybrid vectors utilize the yeast 2μ origin of replication to maintain plasmids at high copy number (15-30 copies per cell). This elevated copy number has the advantage of increased bait protein expression, but the disadvantage that the copy number is variable and may not be the same for bait and target. This explains the prolonged time needed for cells to amplify plasmids before they are able to activate more stringent reporters such as *ADE2* (MacDonald 2001).

b. Fusion domains, localization to the nucleus and dimerisation

Although different fusion domains (AD and BD) work similarly, there are several potential problems that can sometimes be addressed by switching to an alternative fusion domain. These include poor stability or incorrect folding of the fusion protein or obstruction of a binding site. Unfortunately these are difficult to diagnose, and deciding which domain to use is a matter of trial and error. Some interactions work better with the LexA system, others with GAL4. It has even been found that some interactions are directional, i.e. they do not work if the BD and AD inserts are reversed (MacDonald 2001). The most commonly used fusion domains are derived from the GAL4 and LexA proteins, which both bind DNA as dimers. GAL4 contains a nuclear localization signal (NLS) while LexA fusions enter the nucleus without a NLS. The mechanism by which the LexA fusions enter the nucleus is unknown to date. However, by comparing large and small LexA-baits, it was found that small constructs enter the nucleus more successfully, probably due to the fact that they are below the size exclusion limit of the nuclear pore (MacDonald 2001). Both Gal4 and LexA fusion domains have been shown to exhibit increased efficiency when multiple binding sites are present in the reporter gene promoter, therefore much attention has been given to constructing suitable reporter genes in various yeast cells.

c. Protein Folding

Most two-hybrid vectors produce fusions in which the protein of interest (bait) is fused to the carboxyl terminus of the transcription factor domain (DNA-BD). This could be troublesome if the bait protein requires the N-terminal domain for interaction and therefore this orientation could block the interaction site. The possible solutions are to switch to an amino-terminal fusion approach, such as the LexA system, or use smaller fractions of the bait protein (MacDonald 2001). The latter is however, only viable if the domain structure of the protein is known, as there is a risk of eliminating the interacting site.

Constructing an Activation Domain-Fusion Library

The power of the two-hybrid system is to a very large extent based on its use to screen AD-fusion libraries for novel genes that interact with the protein of interest. AD-fusion libraries have several critical features (Bartel and Fields 1997):

- AD-fusion libraries are fusion libraries, i.e. each clone encodes a fusion of the AD to whatever polypeptide is encoded in-frame by the cloned insert.
- AD-fusion libraries are expression libraries, i.e. the fusion proteins must be expressed in yeast cells for the assay to work.
- AD-fusion libraries are cDNA libraries, since only protein-coding sequences are of interest.
- AD-fusion libraries must be constructed (or converted) to plasmid vectors to allow transformation, maintenance and selection.

Choice of AD vector

System II utilizes pACT2 as AD vector, which is one of the earliest AD vectors and is an improved version of the pACT1 vector. pACT2 contains an expanded polylinker region, a HA epitope tag and truncated ADH1 promoter, which results in constitutive regulated medium expression levels (Clontech Laboratories 1997a). It also contains yeast and bacterial *ori*, ampicillin resistance and a *LEU2* selection marker (Figure 4.3). The *LEU2* marker can be used for selection in Leu⁻ auxotrophic yeast strains, as well as *E. coli* strains containing the *leu6* mutation (for distinguishing bait and prey plasmids during plasmid rescue).

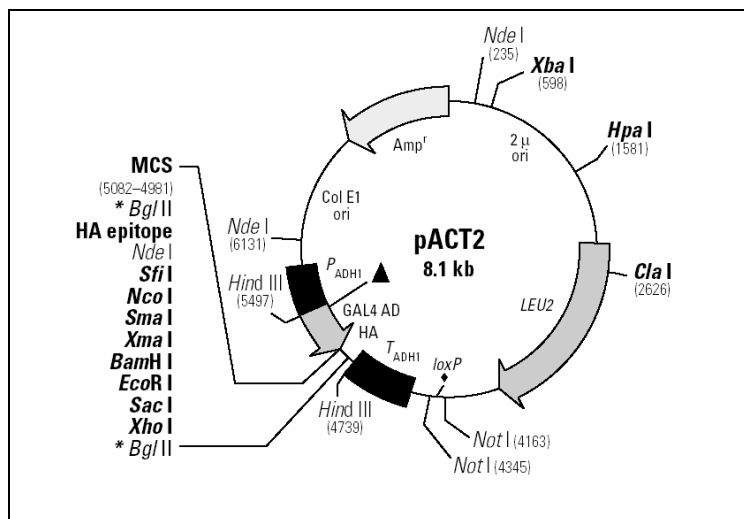


Figure 4.3. pACT2 map and MCS (Clontech Laboratories 1997a). Unique sites are in bold. The hybrid protein is expressed at medium levels in yeast host cells from an enhanced, truncated ADH1 promoter and is targeted to the yeast nucleus by the SV40 T-antigen nuclear localization sequence (\blacktriangle). pACT2 contains the LEU2 gene for selection in Leu⁻ auxotrophic yeast strains.

Source of RNA

Libraries should always be constructed from RNA derived from the appropriate tissue and at a specific differentiation stage. In ticks it has been described that the total protein and RNA increased sixfold in the salivary glands of fed male *A. americanum*. In fed male *D. andersoni* salivary glands, RNA increased approximately 3.5 times. Feeding *D. andersoni* in the presence of females increased total RNA by 25% over those fed in the absence of females (Bior *et al.* 2002). Therefore during this study, ticks were fed prior to RNA isolation. Since fusion libraries are cDNA libraries, it is recommended that library construction start with poly (A)⁺ RNA. Since obtaining sufficient high quality poly (A)⁺ RNA from fed tick salivary glands are a laborious process, the SMART technology (Clontech), which enriches and amplifies poly (A)⁺ from total RNA, was used during this study.

Insert length, priming method and cloning

Unlike normal cDNA libraries, useful AD fusion libraries do not require full-length cDNA. Based on the assumption that a minimum polypeptide length required for an interaction is in the range of 50 residues, some researchers create libraries with quite short inserts. Shorter inserts also favor more efficient ligation of vector to insert. However, if one wants to detect multiple interactions or interactions that require complex protein folding, longer inserts are required. To date, two priming methods are used for cDNA synthesis. The first method uses random priming. This randomly primed reaction ensures that the library will contain clones

encoding fusions to the amino-terminal and internal domains. This method also generates a wide size range, and subsequent size fractionation is used to control the insert length. The second priming method uses an oligo (dT)₂₅d(A/G/C)-primer, which will result in clones enriched in carboxyl-terminal domains (Bartel and Fields 1997; Clontech Laboratories 1997b). Directional cloning, which increases the library complexity two-fold, can be achieved by two methods. The first entails the ligation of adaptor to the ds cDNA followed by subsequent restriction digestion, phosphorylation and ligation into dephosphorylated vector (Clontech Laboratories 1997b). The second method is the one used in this study. In this case, primers containing directional cloning sites are used during SMART cDNA synthesis and directional cloning is achieved after restriction enzyme digestion and ligation (Clontech Laboratories 2001a).

Library complexity

Library complexity is defined as the number of independent clones present in the original, unamplified library. It is most likely the most critical determinant of whether or not screens of an AD-fusion library will be successful. The more independent clones, the higher the complexity of the library, and better the chances of finding even a very rare interacting protein. Generally, a library should contain at least 1×10^6 independent clones (Bartel and Fields 1997). In order to obtain sufficient material for yeast transformation (following amplification of the library), the titer must be $>10^8$ cfu/ml for plasmid libraries (Clontech Laboratories 1998). If sufficient information is available regarding the organism, the sequence representation can be determined (e.g. by using probes against a house-hold gene) to further address library quality. In mammalian libraries, a β -actin probe that cross-reacts with all mammalian β -actin cDNA is mostly used. Human cDNA libraries must show a minimum β -actin frequency of 0.10%, and all other mammalian libraries a minimum frequency of 0.05%. Non-mammalian cDNA libraries can be screened with a ubiquitously expressed species-specific probe (Clontech Laboratories 1998).

Two-Hybrid Yeast: Their Promoters, Phenotypes and Reporter Genes

i. Yeast promoters

A region containing a loosely conserved sequence (TATA box) precedes all yeast structural genes and determines the transcription start site. Many genes are also associated with *cis*-acting elements, i.e. DNA sequences to which transcription factors and other *trans*-acting regulatory proteins bind and affect transcription levels. The term "promoter" usually refers to

both the TATA box and the associated *cis*-regulatory elements. In this text, "minimal promoter" will refer specifically to the TATA region, exclusive of other *cis*-acting elements. The minimal promoter (or TATA box) in yeast is typically approximately 25 bp upstream of the transcription start site. One type of *cis*-acting transcription element in yeast is upstream activating sequences (UAS), which are recognized by specific transcriptional activators and enhance transcription from adjacent downstream TATA regions. The enhancing function of yeast UASs is generally independent of orientation; however, it is sensitive to distance effects if moved more than a few hundred base pairs from the TATA region. In most cases, the reporter genes (*lacZ*, *HIS3*, *ADE2* and *LEU2*) are under control of artificial promoter constructs comprised of a TATA and UAS (or operator) sequence derived from another gene. For GAL4-based systems, either a native GAL UAS or a synthetic UAS_{G-17-mer} consensus sequence provides the binding site for the GAL4 DNA-BD (see Figure 4.4).

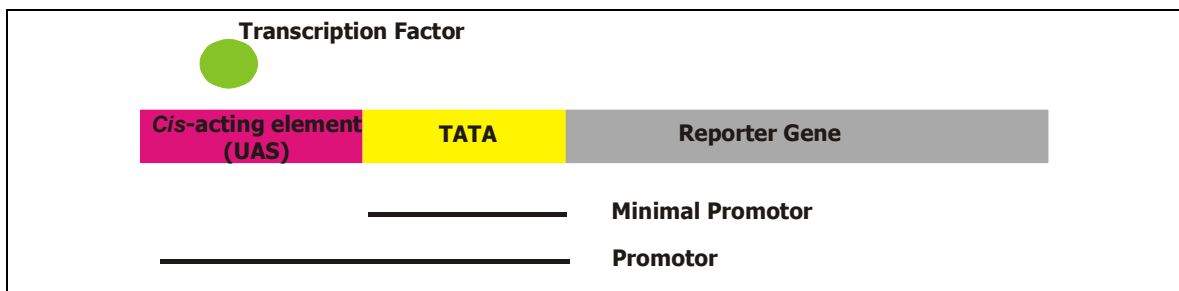


Figure 4.4. Schematic presentation of a yeast promoter.

ii. Yeast phenotypes

Various yeast strains are available for use in two-hybrid assays. All the strains used during this study use the GAL4 promoter system. Their genotype and most important applications are listed in Table 4.1. Strain AH109 was used most since it has an additional reporter gene for adenine. This allows for the selection on not only SD/-His/-Leu/-Trp (TDO, Triple drop out) but also SD/-His/-Leu/-Trp/-Ade (QDO, quadruple drop out).

Table 4.1. MATCHMAKER yeast strain genotypes and applications (Clontech Laboratories 1998; Clontech Laboratories 1999).

Strain	Genotype	Application (s)
AH109	MAT α , <i>trp1-901</i> , <i>LEU2-3</i> , <i>112</i> , <i>ura3-52</i> , <i>HIS3-200</i> , <i>GAL4Δ</i> , <i>gal80Δ</i> , <i>LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3</i> , <i>GAL2_{UAS}-GAL2_{TATA}-ADE2</i> , <i>URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ</i>	Two-hybrid library screening using <i>HIS3</i> , <i>ADE2</i> and <i>MEL1</i>
Y187	MAT α , <i>ura3-52</i> , <i>HIS3-200</i> , <i>ADE2-101</i> , <i>trp1-901</i> , <i>LEU2-3</i> , <i>112</i> , <i>GAL4Δ</i> , <i>meF</i> , <i>gal80Δ</i> , <i>URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ</i>	Two-hybrid assay of known proteins; Quantitative β -gal assays; Mating partner of CG-1945 and Y190
CG-1945	MAT α , <i>ura3-52</i> , <i>HIS3-200</i> , <i>ADE2-101</i> , <i>lys2-801</i> , <i>trp1-901</i> , <i>LEU2-3</i> , <i>112</i> , <i>GAL4-542</i> , <i>gal80-538</i> , <i>cyh2</i> , <i>LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3</i> , <i>URA3::GAL4</i>	Two-hybrid library screening with a highly sensitive <i>HIS3</i> reporter; cycloheximide counterselection

iii. Reporter genes in AH109

The yeast strain AH109 (MATCHMAKER system 3), virtually eliminates false positives by using three reporter genes: *ADE2*, *HIS3* and *MEL1* (or *lacZ*), all under the control of distinct GAL4 upstream activating sequences (UASs) and TATA boxes (Figure 4.5). These promoters yield strong and specific responses to GAL4. As a result, two major classes of false positives are eliminated: those that interact directly with the sequences flanking the GAL4 binding site and those that interact with transcription factors bound to specific TATA boxes. The *ADE2* reporter alone provides strong nutritional selection, while the option of using *HIS3* selection reduces the incidence of false positives and allows one to control the stringency of selection. Furthermore, one has the option of using either *MEL1* or *lacZ*, which encode α -galactosidase and β -galactosidase respectively. Because α -galactosidase is a secreted enzyme, it can be assayed directly on X- α -Gal indicator plates employing blue/white screening.

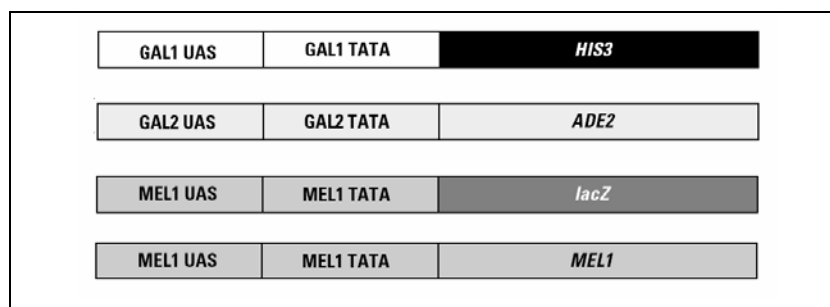


Figure 4.5. Reporter gene constructs in the yeast strains AH109. The *HIS3*, *ADE2*, and *MEL1/lacZ* reporter genes are under the control of three completely heterologous GAL4-responsive UAS and promoter elements, i.e. *GAL1*, *GAL2*, and *MEL1*, respectively.

4.1.2. Using the two-hybrid system for the identification of binding partners of SNAREs and secretory proteins.

In literature, there are many examples of using SNARE- and secretory proteins as bait for the identification of binding partners. These are listed in Table 4.2. The examples listed include cross-species screening, various BD/bait fusion constructs and libraries used.

Table 4.2. The use of various SNAREs and secretory proteins in two-hybrid assays.

Bait	Prey	Proteins identified by two-hybrid assay	Reference
Syntaxin Baits			
Syntaxin 1A & 1B (WT and cytosolic domain)	VAMP 1 & 2 (Cytosolic domains) Munc 18-a WT	Three-hybrid proof that Munc inhibit SNARE assembly	(Perez-Branguli <i>et al.</i> 2002)
Rat Syntaxin 1A (181-288)	Fragmented human brain cDNA library (Obtained from Clontech)	α -SNAP (10 clones) β -SNAP (13 clones) SNAP-25 (3 clones) Syntaphilin (2 clones) Syntaxin 1A (5 clones) SNAP-29	(Su <i>et al.</i> 2001)
Syntaxin 1A (1-265) Syntaxin 2 (1-266) Syntaxin 3 (1-263) Syntaxin 4 (1-273) Syntaxin 5 (1-284)	Rat SNAP-25 (WT) Human SNAP-23 (WT) Mouse SNAP-23	Specificity of interactions	(Araki <i>et al.</i> 1997)
Syntaxin 4 (1-273)	Fragmented human B lymphocyte cDNA library (Obtained from Clontech)	SNAP23	(Ravichandran <i>et al.</i> 1996)
Rab Baits			
Rab6a (WT) Rab6a (Val22) Rab6a (Ile126) Rab6a (Leu72) Rab6a (Asn27) Rab5 (WT)	Full length poly (A) ⁺ mouse brain (Stratagene Protocol)	Rab GDI SNIF4 Rabkinesin6	(Janoueix-Lerosey <i>et al.</i> 1995) (Stephens and Banting 2000)
Rab3a (WT) Rab3a (Q81L)	Full length poly (A) ⁺ rat brain (Stratagene Protocol)	Rabin 3 (3 clones) PRA1 (Rab6/5 partner C)	(Martincic <i>et al.</i> 1997)
SNAP Baits			
dSNAP (<i>Drosophila</i> SNAP)	<i>Drosophila</i> ovary cDNA library	<i>Drosophila</i> syntaxins 1, 5 & 16	(Xu <i>et al.</i> 2002)
Bovine α -SNAP (WT)	Fragmented human leukocyte cDNA library (Obtained from Clontech)	Various syntaxin isoforms Novel syntaxin 18 isoform	(Hatsuzawa <i>et al.</i> 2000)

Mouse SNAP-25	Fragmented rat brain library	HRS (Hepatocyte growth factor regulated tyrosine kinase substrate) & Syntaxin 1B	(Kwong <i>et al.</i> 2000)
SNAP-25B	Rat brain library	EHS1 (EH/SH3 domain protein) / Intersectin	(Okamoto <i>et al.</i> 1999)
Human SNAP-23 (WT)	Fragmented human B lymphocyte cDNA library	Syntaxin 11	(Valdez <i>et al.</i> 1999)
γ -SNAP	IIB-Mel-J cell cDNA library	Cytosolic thiolase (22 clones) Gaf-1 (32 clones)	(Chen <i>et al.</i> 2001)
Other Proteins used as Baits			
Rat NSF (N-terminal)	Rat lung cDNA library (Obtained from Clontech)	Rab6	(Han <i>et al.</i> 2000)
Munc 18-1 Munc 18-2	Various syntaxin constructs	Specificity of Munc-syntaxin interactions	(Hata and Sudhof 1995)
Cytoplasmic synaptotagmin I	Rat brain library (Life Technologies Protocol)	Synaptotagmin I	(Sugita <i>et al.</i> 1996)
β -Arrestin 1 (WT)	Fragmented rat brain cDNA library (Obtained from Clontech)	NSF	(McDonald <i>et al.</i> 1999)
Kinesin heavy chain (814-963)	Human brain library	SNAP25 & SNAP23	(Diefenbach <i>et al.</i> 2002)
Oxysterol-binding protein (WT)	B-cell library	VAP-A (syntaxin-like protein in ER/Golgi)	(Wyles <i>et al.</i> 2002)

In our study, syntaxin, Rab3a and α -SNAP constructs were investigated as baits. Therefore emphasis will be placed on studies utilizing these proteins as bait. Studies by Su *et al.* and Ravichandran *et al.*, in which syntaxin was used as bait for screening AD-fusion libraries, mostly identified various SNAPs (Ravichandran *et al.* 1996; Su *et al.* 2001). In both cases truncated syntaxin baits were used. In the case of Su *et al.*, the inhibitory N-terminal was removed, but the transmembrane region was kept intact (amino acids 181-288). In contrast, the group of Ravichandran *et al.* removed only the transmembrane region of syntaxin. In both cases, cross-species two-hybrid assays were performed using a fragmented cDNA library. Interestingly, 6 different interacting proteins were identified from brain tissue, while only one partner was identified from B lymphocyte tissue. In the case of Perez-Branguli *et al.* wild type syntaxin was used in a three-hybrid assay (Perez-Branguli *et al.* 2002). During their study they were able to show that wild type syntaxin does bind to its partners Munc and VAMP when they are expressed in high enough levels. Similarly, the study of Araki *et al.*

indicates that syntaxin lacking the transmembrane region is successful in binding SNAP25 (Araki *et al.* 1997). From these studies it is clear that (i) various constructs of syntaxin are useful as bait, (ii) depending on the tissue type, different binding partners were identified and (iii) fragmented libraries were most successful.

In the case of Rab3a, it is evident that wild type and GTPase deficient constructs must be used for the identification of Rab_{GTP} and Rab_{GDP} binding partners, respectively. In both studies listed, full-length poly (A)⁺ rat brain libraries were used. The use of Rab3a for screening cross-species and various non-neuronal tissues has not been described to date.

Regarding the use of α -SNAP as bait, the study of Hatsuzawa *et al.* is valuable since it describes the cross-species use of α -SNAP as bait. They were able to use bovine α -SNAP as bait for screening a human leukocyte fragmented cDNA library and successfully identified various leukocyte syntaxin isoforms (Hatsuzawa *et al.* 2000).

4.2. HYPOTHESIS

- Cross-species two-hybrid assays are possible between rat brain SNAREs or secretory proteins (bait) and a salivary gland cDNA library of *Ornithodoros savignyi* (prey).

4.3. AIMS

- Construction of both a full-length poly(A)⁺ and a truncated *Ornithodoros savignyi* salivary gland plasmid AD-fusion library.
- Construction of wild type syntaxin, Rab3a and α -SNAP BD/bait fusion constructs.
- Construction of a transmembrane depleted syntaxin BD/bait fusion construct.
- Construction of a GTPase deficient Rab3a BD/bait fusion construct.
- Two-hybrid assay using rat brain BD/bait constructs and tick salivary gland plasmid AD-fusion libraries.

4.4. MATERIALS

The MATCHMAKER™ GAL4 two hybrid system 2 was a kind gift from Dr. A. Dhugra, University of Pennsylvania, USA while system 3 was purchased from Clontech Inc. The Super SMART™ cDNA synthesis kit was obtained from Clontech (Southern Cross Biotechnology). KC8 *E. coli* cells were a kind gift from Dr. Hannelie Moolman-Smook, University of Stellenbosh, South Africa. Recombinant NSF, ATPase deficient NSF (D127) and α -SNAP were kinds gifts from Proff. Whiteheart and Rothman of the Memorial Sloan-Kettering Cancer Institute, New York, USA. Recombinant syntaxin, SNAP-25, VAMP, Rab3a and synaptotagmin were a gift from Prof. R.H. Scheller, Stanford University Medical Center, USA. NucleoSpin® Plasmid Quick Pure, NucleoBond® PC2000 and NucleoSpin® Extract kits were from Macherey-Nagel, Germany (Separations). PCR nucleotide mix (10 mM deoxynucleotide solution), *Sfi*I restriction enzyme and Shrimp alkaline phosphatase were from Roche Diagnostics. Peptone, agar and yeast nitrogen base without amino acids were from Difco (Labretoria). Yeast extract and tryptone were purchased from Oxoid Ltd. (England). Deoxyribonucleic acid sodium salt type III from salmon testes, 3-amino-1,2,4-triazole (3-AT), cycloheximide, , RNase Inhibitor, PEG4000, 425-600 micron glass beads, Triton X-100 and all the various amino acids used were from Sigma. Dextrose, Isopropyl β -D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), L-adenine hemisulphate, lithium acetate, ammonium acetate and ampicillin were from ICN (Separations). DNA polymerase I large (Klenow) fragment, *Taq* Polymerase, *Pfu* DNA polymerase, Proteinase K and various restriction enzymes were from Promega (Wisconsin, USA). TaKaRa Ex Taq (5 U/ μ l) and T4 DNA Ligase (350 U/ μ l) were from Takara Bio Inc., Japan (Separations). Primers were synthesized by Inqaba Biotech (Pretoria, South Africa). NaCl, ethylene diamine tetra-acetic acid (EDTA), Tris(hydroxymethyl)aminomethane, methanol, isopropanol and chloroform were obtained from Merck, Germany. Sodium dodecyl sulphate (SDS) was from BDH Laboratory Supplies LTD., England.

4.5. METHODS

4.5.1. Full-length GAL4 AD/ library construction

cDNA synthesis and amplification using the Super SMART System™

Tick feeding, salivary gland dissection, RNA isolation, double strand cDNA synthesis and amplification by LD-PCR were performed as described in Chapter 3. In order to allow restriction enzyme digestion and directional cloning, different primers were used. The primers used for plasmid library construction contained a *Sfi* I restriction site, which allows

(i) directional cloning of transcripts into the pACT2 plasmid using a single restriction enzyme step and (ii) a very low abundance restriction enzyme cutting site to limit extensive digestion of the tick transcripts. The sequences of the primers that were used are given in Table 4.3.

Table 4.3. Primers used for synthesis and amplification of cDNA during cDNA library construction.

Primer	Sequence
SMART IV	5' AAGCAGTGGTATCAACGCAGAGTGGCCATGGAGGCCGGG 3' <i>Sfi</i>
CDS III / 3'PCR	5' ATTCTAGAGGCTCCATGGCCGACATG(T) ₃₀ NN 3' <i>Sfi</i>
5' PCR	5' AAGCAGTGGTATCAACGCAGAGT 3'

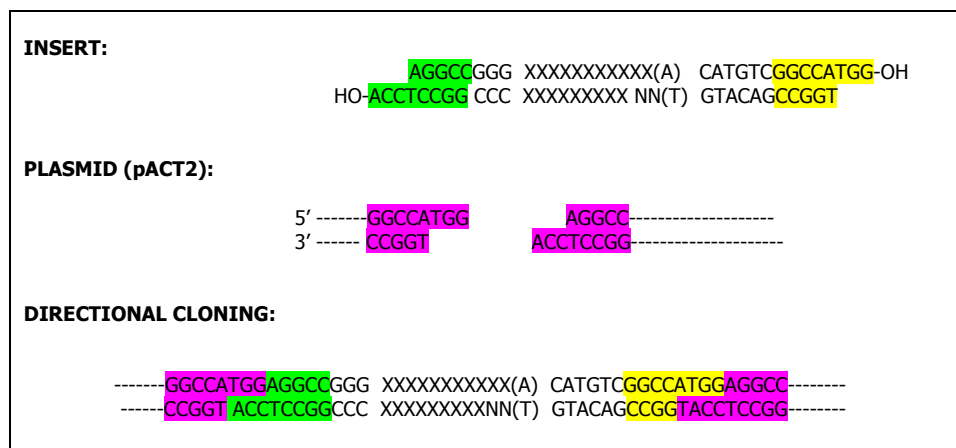


Figure 4.6. Schematic representation of directional cloning using *Sfi* digestion.

ds cDNA Polishing and size fractionation

Directly after LD-PCR amplification the ds cDNA was polished using the protocol described by Clontech (Protocol # PT3041-1, SMART PCR cDNA synthesis manual). Briefly, the 100 μ l LD-PCR reaction was divided into two 50 μ l samples. To each, 4 μ l Proteinase K (10 μ g/ μ l) were added and incubated at 45 $^{\circ}$ C for 1 hour, before inactivation at 90 $^{\circ}$ C for 10 minutes. The tubes were immediately chilled (2 min) in ice water to allow for specific re-annealing of strands before the addition of 3 μ l T4 DNA Polymerase (5 U/ μ l) and incubation at 16 $^{\circ}$ C for 30 minutes to allow synthesis of full-length ds cDNA. The reaction was terminated by incubation at 72 $^{\circ}$ C for 10 minutes. The dsDNA was purified using the NucleoSpin[®] extract kit (as described previously) and eluted in 50 μ l buffer NE (5 mM Tris-HCl, pH 8.5). The yield and purity was determined on the Gene Quant Pro system. Typically 1,7-2,5 μ g of SMART polished ds cDNA was obtained with a 260nm/280nm ratio greater than 1,8.

Construction of a GAL4 AD/ plasmid library

Polished SMART DNA as well as purified pACT2 plasmid was digested with *Sfi*I at 50°C for 2 hours, before inactivation of the reaction with the addition of 2 µl Proteinase K (20 µg/ml) and incubation at 45°C for 1 hour. The latter reaction was terminated at 75°C for 30 minutes, and the digested products purified using the NucleoSpin extract kit. To avoid ligation of the complementary *Sfi*I generated overhangs of the pACT2 plasmid, the digested plasmid was dephosphorylated with the addition of 10µl Shrimp Alkaline Phosphatase (1 U/µl) and incubated for 30 minutes at 37°C. The reaction was terminated with the addition of Proteinase K and subsequent heat inactivation (as described previously). To ensure that pACT2 was successfully dephosphorylated prior to ligation of the library, some plasmid was incubated with T4 DNA ligase and analyzed with agarose gel electrophoresis.

In order to optimize library ligation into pACT2, various conditions were tested. These are listed in Table 4.4. Briefly, the various ratios of plasmid and library (insert) were combined with 1µl T4 Ligase (100 U/µl) and incubated overnight at 16°C, followed by heat inactivation. The ligated products were precipitated in the presence of tRNA and electroporated into BL21 *E. coli* cells (as described in Chapter 3).

Table 4.4. Ligation of the GAL4 AD / plasmid library using the pACT2 vector (8100 bp).

Average Insert size	250 bp			500 bp			1000 bp			1500 bp		
Ligation Ratio	1/1	2/1	5/1	1/1	2/1	5/1	1/1	2/1	5/1	1/1	2/1	5/1
Insert (ng)	4.6	9.3	23	9.3	18.5	46.3	18.5	37	92.6	27.8	55.6	138.9
Vector (ng)	150	150	150	150	150	150	150	150	150	150	150	150
Sample #	1	2	3	2	4	5	4	6	7	8	9	10

After electroporation, the cells were diluted in 1 ml LB-broth and incubated at 30°C with moderate shaking for 60 minutes. Following incubation, 10µl of cells from each sample were diluted with 40 µl LB-Broth and plated onto a 1.5% agar plate containing ampicillin (50 µg/ml). The plates were inverted and incubated at 37°C overnight while the remaining transformation mixtures were stored at 4°C. The next day, the confluent and semi-confluent plates were selected as successful transformation ratios and the remaining transformation mixtures of these ratios were plated among 50 large plates (150 mm diameter) and again incubated overnight. Before pooling the library, the percentage of clones containing inserts

was determined (see the following section). Finally, the cells were scraped from all of these plates, pooled into 500 ml of LB-Broth containing 25% glycerol (on ice) and stored in 1 ml aliquots at -70°C .

Determining the number of recombinant/independent clones

In order to determine the percentage of recombinant clones in the library, at least 15 isolated colonies were randomly picked from nearly confluent plates, inoculated into 1 ml LB-Broth containing ampicillin and grown overnight. The following day the samples were subjected to miniprep plasmid isolation (as described in Chapter 3), *EcoRI* and *HindIII* digestion and analysis on agarose electrophoresis. A transformation mixture was only added to the pooled library if at least 10 out of 15 clones contained inserts.

Titering plasmid libraries

Prior to freezing the library the titer has to be determined to ensure a representative library. In general the titer should be at least 10-fold higher than the number of independent clones and at least 10^8 cfu/ml for long-term storage. To determine the titer, 1 μl of the pooled library is added to 1 ml LB broth in a 1.5 ml microcentrifuge tube (this is dilution A, $1:10^3$) and mixed by vortexing. 1 μl of Dilution A is added to 1 ml LB broth to create dilution B ($1:10^6$). For plating the cells, 1 μl of dilution A was diluted in 50 μl LB broth, mixed and the entire mixture plated. Two aliquots (50 μl and 100 μl) from dilution B were also plated. Following overnight incubation at 37°C , the number of colonies was counted and the titer (cfu/ml) calculated according to the following formulas:

- Colony# Dilution A $\times 10^3 \times 10^3 = \text{cfu/ml}$
- (Colony # Dilution B / plating volume) $\times 10^3 \times 10^3 \times 10^3 = \text{cfu/ml}$

Amplification and large-scale plasmid isolation of library

In order to amplify the cells from the stored library to obtain sufficient plasmid for a library scale transformation of yeast cells, 1 ml of frozen plasmid library was thawed on ice and diluted into 2 litres of LB broth containing ampicillin (250 μl of cells / 500 ml broth). Each 500 ml culture was placed in a 2-litre flask to ensure sufficient oxygenation during amplification at 30°C with vigorous shaking. Cultures were grown until $A_{600} \sim 0.5 - 0.6$ before collecting the cells with centrifugation (5000 $\times g$, 30 minutes). Large-scale plasmid isolation was subsequently performed using the NucleoBond™ PC2000 system. Briefly, the cells were resuspended in 90 ml of buffer S1 (50 mM Tris-HCl, 10 mM EDTA, 100 $\mu\text{g/ml}$ RNase A, pH

8.0) and lysed with the addition of 90 ml buffer S2 (1% SDS, 200 mM NaOH) and incubation at room temperature for 3 minutes. The released chromosomal DNA was precipitated with the addition of 90 ml pre-cooled buffer S3 (2.8 M potassium acetate, pH 5.1), mixed by inversion and incubated on ice for 5 minutes. The lysate was clarified by filtering the suspension through a NucleoBond® folded filter in a large funnel and loaded on a pre-equilibrated NucleoBond® AX2000 column (20 ml buffer Equilibration buffer N2, 100 mM Tris-HCl, 15% ethanol, 900 mM KCl, 0.15% TritonX100, adjusted to pH 6.3 with H₃PO₄). The column was subsequently washed twice with 50 ml of buffer N3 (100 mM Tris-HCl, 15% ethanol, 1.15 M KCl, adjusted to pH 6.3 with H₃PO₄) before eluting the plasmid with 25 ml buffer N5 (100 mM Tris-HCl, 15% ethanol, 1 M KCl, adjusted to pH 8.5 with H₃PO₄). During elution, 1 ml fractions were collected, which were immediately precipitated by the addition of 800 µl isopropanol per fraction and centrifuging the samples at 15 000 x g for 30 minutes at 4°C. The pellets were washed with 70% ethanol, vacuum-dried and dissolved in 50 µl water. Yields were determined spectrophotometrically as described previously.

4.5.2. Truncated GAL4 AD/ library construction

The methodology for creating a fragmented library whereby the coding strand is truncated from the 3' end is given in Figure 4.7. The approach utilizes random hexa-nucleotides and the DNA polymerase I large (Klenow) fragment. Klenow is useful in this case since each of the annealing temperatures of the random hexanucleotides differs, and therefore amplification must be done at low temperatures to allow optimal annealing. In this case extension was performed at 37°C, which is the optimal temperature of the Klenow enzyme.



Figure 4.7. Schematic representation of fragmenting the full-length *SfiI* library using random primers.

SMART *SfiI* dsDNA (1000 ng) was incubated with 650 pmol random hexanucleotide, 2 µl dNTPs (10 mM of each nucleotide) and 2.5 µl Klenow buffer in a 25 µl reaction. The dsDNA was denatured at 94°C for 1 minute and snap cooled on ice for 5 minutes to allow annealing of the random hexanucleotides. Klenow enzyme (4U) was added and the reactions incubated at 37°C for 30 minutes before heat inactivation at 70°C for 15 minutes. Generating dsDNA from only the 5' ends (which are the correct reading frames), was achieved by adding 650 pmol of the 5'PCR primer, 1 µl of dNTP's (10 mM), 2.5 µl ExTaq buffer and ExTaq polymerase (5U) to yield a final volume of 50 µl. The reactions were incubated at 94°C for 30 seconds, followed by annealing of the 5' PCR primer at 60°C for 30 seconds and extension at 72°C for 6 minutes. The fragmented dsDNA was purified using the NucleoSpin™ Extract kit as described previously. In general, there was a 2- 2.5x increase in the amount of dsDNA. In order to directionally clone these fragments, the fragmented DNA and the pACT2 plasmid were digested sequentially with *SfiI* and *XhoI*. Ligation, transformation, titering of the fragmented library and determination of the percentage of recombinant clones were done as described previously.

4.5.3. Verification of yeast host strains and control vectors

It is important to verify the phenotype of the yeast strains used prior to transformation. This is achieved by plating the yeast cells on standard (SD) or dropout (DO) medium (6,7 g/l

yeast nitrogen base without amino acids, 20 g/l agar, 100 ml of the appropriate 10x dropout solution) and incubating cells at 30°C for 3-5 days until colonies appear. As a positive control, cells were plated on adenine supplemented YPD media (YPDA, 20 g/l peptone, 10 g/l yeast extract, 20 g/l agar, 20g/l dextrose, 0,03g/l adenine hemisulphate). In the case of testing cycloheximide (CHX) resistance, 10 µg/ml cycloheximide was added to the plates. Additional cultures were propagated from isolated colonies identified on the selection plates. The phenotypes of the various MATCHMAKER yeast strains are listed in Table 4.5.

Table 4.5. MATCHMAKER yeast strain phenotypes. (+) Indicates growth, while (-) indicates no growth in the various growth media.

Strain	Selection media							YPDA	YPD/CHX
	SD/-Ade	SD/-Met	SD/-Trp	SD/-Leu	SD/-His	SD/-Ura			
AH109	-	+	-	-	-	+	+	-	
Y187	-	-	-	-	-	+	+	-	
CG-1945	-	+	-	-	-	+	+	+	

4.5.4. GAL4 DNA-BD/Bait construction

Native constructs

The full-length recombinant clones containing inserts encoding native rat brain syntaxin, Rab3a and α -SNAP were sequenced to confirm their nucleotide sequences. Primers were designed to PCR amplify and directionally clone these inserts in the correct reading frame into the pAS2_1 plasmid. The sequences of the primers are given in Table 4.6.

To amplify the open reading frame (ORF) of the native inserts, purified plasmid (50 ng), forward and reverse primers (5 pmol) were used. All other conditions were identical to that of 3' RACE described in Chapter 3. The amplified inserts were analyzed on agarose gel electrophoresis and purified using the NucleoSpin[®] Extract kit. Sequential digestions with the appropriate restriction endonucleases were performed on both the inserts and the pAS2_1 plasmid. The digested inserts and plasmids purified using the NucleoSpin[®] Extract kit were ligated overnight at 16°C, precipitated with tRNA and electroporated into DH5 α *E. coli* cells.

Table 4.6. Primers used for the amplification of native bait constructs.

Name	Sequence	Tm (°C)
Syntaxin bait constructs		
Syntaxin forward	GGA ATT <u>CCA TAT GAA</u> GGA CCG AAC CCA G <i>NdeI</i>	66.6
Syntaxin reverse	CCA ATG CAT TGG <u>TTC TGC AGC</u> TAT CCA AAG ATG CC <i>PstI</i>	70.6
Rab3a bait constructs		
Rab3a forward	GGA ATT <u>CCA TAT GGC</u> TTC CGC CAC AGA C <i>NdeI</i>	64.9
Rab3a reverse	CCG <u>GAA TTC</u> TCA GCA GGC ACA ATC CTG <i>EcoRI</i>	65.1
α-SNAP bait construct		
α-SNAP forward	GGA ATT <u>CCA TAT GGA</u> CAA CTC CGG GAA G <i>NdeI</i>	67.5
α-SNAP reverse	CCG <u>GAA TTC</u> TTA GCG CAG GTC TTC CTC <i>EcoRI</i>	69.0

Truncated syntaxin construct

Syntaxin is an integral membrane protein that is targeted to the plasma membrane. If this occurs a protein-protein interaction could still occur, but the complex would not be able to migrate into the nucleus and activate the two-hybrid marker genes. Therefore, a truncated syntaxin (1-265) construct lacking the transmembrane region was constructed and used as a bait molecule (Figure 4.8).

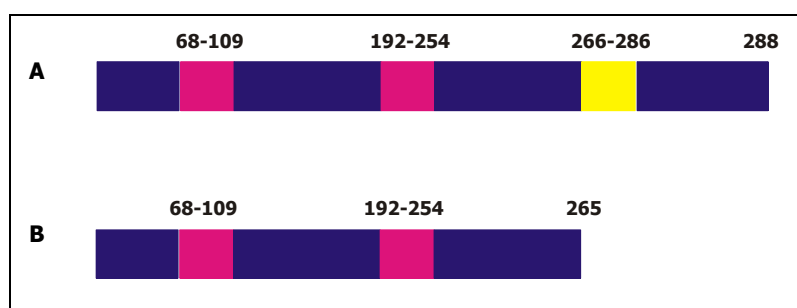


Figure 4.8. Schematic presentation of (A) native syntaxin 1 and (B) truncated syntaxin 1 bait constructs. The coiled-coiled regions are indicated in pink and the transmembrane region in yellow.

The reverse primer is given in Table 4.7 and the forward primer of the native construct was used. All procedures are identical to those described for constructing the full-length native constructs.

Table 4.7. Reverse primer used for the amplification of the syntaxin 1-265 construct.

Name	Sequence	Tm (°C)
Truncated syntaxin	CCA ATG CAT TGG TTC <u>TGC AGT</u> TCT TCC TGC GCG CC <i>Pst</i> I	72.2

GTPase deficient Rab3a T36N

When designing a bait molecule, one has to incorporate functional relevance into two-hybrid screens. One such an example is the identification of the Rab6-dependent trafficking machinery. Rab6 is a small GTPase that, like other Rabs, regulate membrane trafficking through the hydrolysis of GTP. This enables the use of mutant Rab6 proteins, constitutively 'locked' in either the GTP or GDP-bound state, to screen two-hybrid libraries for interacting proteins. When using the GDP-bound state the guanine nucleotide dissociation inhibitor protein (Rab GDI) was identified. In contrast, Rabkinesin6 was found to interact with the wild-type or GTP-locked forms, but not the GDP-locked form (Stephens and Banting 2000).

This approach was followed for Rab3a in this study (Figure 4.9). Both native Rab3a as well as a GTP-deficient Rab3a containing a T36N mutation (locked in the GDP form) was used. This mutation was selected based on the mutational analysis of Rab3a as described by Burstein *et al.* In their studies it was shown that, for the T36N mutant, the intrinsic GDP dissociation rate constant ($k_{off (GDP)}$) was 61-fold higher than that of wild-type Rab3a, indicating a GDP binding function (Burstein *et al.* 1992).

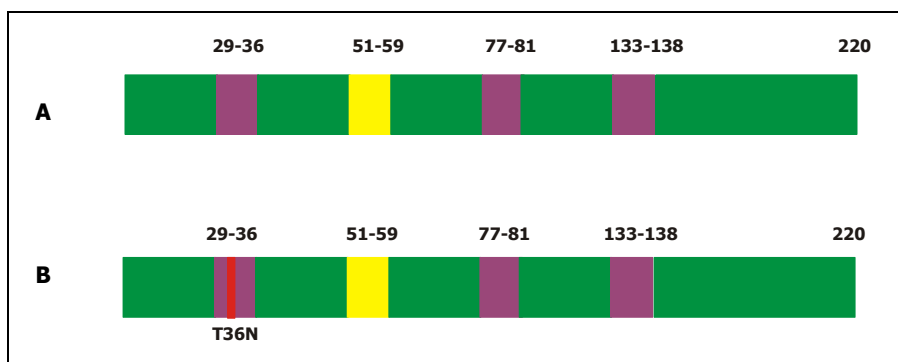


Figure 4.9. Schematic presentation of (A) native Rab3a and (B) mutated Rab3a bait constructs. The GTP-binding regions are indicated in purple, the effector-binding regions in yellow and the mutated site in red.

Site directed mutagenesis, based on the principle described in the QuickChange™ Site-directed mutagenesis Kit by Stratagene (La Jolla, California, USA), was used to generate the T36N mutant from the native Rab3a construct. In this protocol, PCR is used to introduce site-specific mutations to any double-stranded supercoiled plasmid containing the insert of

interest. Two complementary primers containing the desired mutation are used to create mutated plasmids with staggered nicks after linear amplification. *Pfu* DNA polymerase (isolated from *Pyrococcus furiosus*) is used to replicate both plasmid strands with high fidelity using its 3'-5' proofreading exonuclease activity. The product is then treated with *DpnI* (target sequence: 5' Gm⁶ATC-3') in order to remove the methylated parental template. The mutated plasmid is transformed into *E. coli* cells where the bacterial ligase system repairs the nicks to create double-stranded plasmids.

The mutagenesis reaction (50 μ l) contained 50 ng of wild-type Rab3a pAS2_1 plasmid, 125 ng of both the forward and reverse T36N primers (see Table 4.8), 1 x *Pfu* DNA polymerase reaction buffer, 2.5 mM of each dNTP and 3U *Pfu* DNA polymerase. The cycling parameters were 95°C for 30 sec, 55 °C for 1 minute and 68°C for 8 minutes repeated for 12 cycles in a Perkin Elmer GeneAmp PCR system 9700. After PCR amplification, 20 U of *DpnI* was added to the 50 μ l reaction and incubated for 3 hours at 37°C. The mutated plasmids were subsequently isolated using the High Pure Plasmid Isolation Kit (Roche), precipitated with tRNA and sodium acetate (pH 5), electroporated into DH5 α *E. coli* cells and plated on LB/Amp plates. The nucleotide sequences of positive clones were obtained in order to confirm mutagenesis.

Table 4.8. Primers used for the site-directed mutagenesis of Rab3a. *The mutated nucleotide is underlined.*

Name	Sequence	Tm (°C)
Rab3a T36N forward	GTG GGC AAA <u>A</u> AC TCG TTC CTC TTC	60
Rab3a T36N reverse	GAA GAG GAA CGA <u>G</u> TT TTT GCC CAC	60

4.5.5. Small-scale yeast transformation

Small scale yeast transformation was performed according to the method of Dr. Moolman-Smook, Department of Medical Biochemistry, University of Stellenbosch, South Africa (personal communication). Yeast cells were plated on the appropriate plates (YPDA for non-transformed cells or SD for transformed cells) and incubated for a maximum of 2 days at 30°C. A fresh yeast colony (25-50 μ l) was resuspended in 1 ml water (in a 2 ml tube), vortexed, and cells collected by centrifugation (13000 rpm, 30 sec). Yeast cell membranes were made porous (competent) with the addition of 1 ml lithium acetate (LiAc, 100 mM) and incubation at 30°C for 5 minutes without shaking. For transformation, the following was added to the pelleted LiAc treated cells in the listed order: 240 μ l 50% PEG 4000, 36 μ l LiAc

(1M), 25 μ l heat denatured salmon sperm DNA (2 mg/ml) and 500 ng plasmid in a final volume of 50 μ l water. The mixture was vortexed for at least a minute and heat-shocked at 42°C for 20 minutes in a water bath. The cells were collected with centrifugation, resuspended in 250 μ l water and plated on the appropriate agar for selection of the plasmid transformed into the AH109 yeast cells. Plates were incubated upside down at 30°C for 2-5 days.

4.5.6. GAL4 DNA-BD/Bait test for autonomous reporter gene activation

The DNA-BD/bait protein constructs were tested for transcriptional activation function before using it to screen a library by plating the transformed host strain (AH109) on SD/-Trp, SD/-His/-Trp (DDO, Double drop out) and SD/-His/-Trp/-Leu (TDO, Triple drop out). If cells survived on DDO or TDO, the construct was not suitable for screening the library. A colony-lift assay (see 4.5.8) for activation of the *lacZ* reporter gene expression was also done to confirm that the bait construct does not activate the *lacZ* gene.

4.5.7. Sequential library-scale transformation of AH109 yeast cells

For the library-scale transformation of yeast the 30 x TRAFCO protocol described by Agatep R, Kirkpatrick, R.D., Parchaliuk, D.L., Woods, R.A. and Gietz, R.D. was used (<http://tto.trends.com>). Since sequential transformation was done, AH109 yeast cells containing the DNA-BD/bait construct was inoculated in 50 ml SD/-Trp and grown overnight at 30°C with shaking. The cell titer was determined, the volume of cell suspension yielding a total of 7.5×10^8 cells calculated, the cells collected with centrifugation and diluted in 150 ml pre-warmed YPDA medium. The cells were incubated at 30°C with shaking for 3-4 hours until the cell titer reached 2×10^7 cells/ml before harvesting the cells once more by centrifugation (3000 x *g*, 5 min). The cells were washed with 75 ml water, collected by centrifugation, resuspended in 3 ml lithium acetate (100 mM) and incubated for 15 minutes at 30°C without shaking, again collected and the supernatant removed. The following were added to the cells in the listed order: 7,2 ml 50% PEG4000, 1 ml lithium acetate (1 M), 1,5 ml heat denatured salmon-sperm DNA (2 mg/ml), 250 μ g DNA-AD/ library pACT2 plasmid in a final volume of 1 ml water. The mixture was vigorously vortexed for 1 minute until the cell pellet was totally resuspended and then incubated at 30°C for 30 minutes. Subsequently, the cells were heat shocked at 42°C for 40 minutes and mixed by inversion for 15 seconds every 5 minutes. The cells were collected, gently resuspended in 40 ml water and 1 ml aliquots plated on 40 large SD/-Trp/-Leu plates. Plates were incubated at 30°C for 3-5 days until co-

transformed colonies appeared. Apart from sequential transformation of AH109 cells with the DNA-BD/bait and DNA-AD/ library constructs, cells were also sequentially transformed with the control vectors. These are listed in Table 4.9.

Table 4.9. Control vectors of the MATCHMAKER™ GAL4 two-hybrid system 2.

Control vector	Name	Selection
β-gal positive control	pCL1	<i>LEU2</i>
DNA-BD/p53	pVA3	<i>TRP1</i>
AD / T-antigen	pTD1	<i>LEU2</i>
DNA-BD / lamin C	pLAM5'	<i>TRP1</i>
Positive controls for a two-hybrid interaction		
Plasmids	pVA3 + pTD1	<i>TRP1, LEU2</i>

4.5.8. Two-hybrid screening of reporter genes (See appendix 1)

Co-transformed cells were scraped from the plates, collected with centrifugation, resuspended in 10 ml SD/-Trp/-Leu, 3 x 1 ml aliquots were plated on SD/-Trp/-Leu/-His (TDO, Triple drop-out) plates and incubated at 30°C for 2-8 days, or until colonies appeared. The remaining cells were stored in 1 ml aliquots at -70°C in SD/-Trp/-Leu containing 25% glycerol. TDO positive colonies were transferred to duplicate TDO master plates with sterile wood sticks and incubated at 30°C overnight. One master plate was stored at 4°C as back up while colonies from the second master plate were plated onto SD/-Trp/-Leu/-His/-Ade (QDO, Quadruple drop-out) for further selection. QDO positive colonies were transferred to two master plates. Again, one master plate was stored at 4°C while the other was used for the colony-lift β-galactosidase filter assay (see 4.5.8) to test activation of the *lacZ* gene.

4.5.9. Colony-lift β-galactosidase filter assay (Clontech Laboratories 2001b)

QDO positive colonies were grown on a master plate for 2-4 days at 30°C until 1-3 mm in diameter. Using forceps, a sterile dry filter paper was placed over the surface of the plate of colonies and gently rubbed to help colonies cling to the filter. Holes were poked through the filter into the agar in three asymmetric locations to orient the filter. When the filter was evenly wetted it was carefully lifted from the agar plate with forceps and transferred (colonies facing up) to a pool of liquid nitrogen. Using the forceps, the filter was completely submerged for 10 seconds, removed from the nitrogen and allowed to thaw at room

temperature. This freeze-thaw treatment to permeate the cells, was repeated three times. Finally, the filter was placed on a filter presoaked in 100 ml buffer Z (16,1 g/l $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; 5,50 g/l $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; 0,75 g/l KCl; 0,46 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; pH 7.0) containing 167 μl of X-gal solution (20 mg/ml) and incubated at room-temperature. The filter was checked periodically for the appearance of blue colonies. The time it takes colonies producing β -galactosidase to turn blue varies, typically from 30 minutes up to 3 hours. Prolonged incubation (>8 hr) may give false positives. Yeast transformed with the β -galactosidase positive control plasmid (pCL1) turns blue within 20-30 minutes, while cells co-transformed with plasmid for a two-hybrid interaction (pVA3 and pTD1) give a blue signal within 60 minutes. The colonies, which activated the *lacZ* gene, were identified on the master QDO plate and selected for further screening using nested PCR.

4.5.10. Nested-PCR screening of positive clones

Nested-PCR screening was performed according to the method of Dr. Moolman-Smook, Department of Medical Biochemistry, University of Stellenbosch, South Africa (personal communication). PCR directly from the yeast cells were performed by inoculating $\sim 1 \mu\text{l}$ of cells into the PCR mixture (15 pmol pACT2 forward and reverse primers, 1x *Taq* DNA polymerase buffer A, 1.5 mM MgCl_2 , 200 μM dNTP's and water to a final volume of 20 μl). *Taq* DNA in Buffer A (Promega) was used since buffer A (50 mM Tris-HCl, 100 mM NaCl, 0,1 mM EDTA, 1 mM DTT, 50% glycerol and 1% Triton X100) aids in disrupting the yeast cells. The cells were disrupted at 94°C for 7 minutes in Perkin Elmer GeneAmp PCR system 9700 and then cooled to 80°C after which 5 μl of enzyme mix (1.25 U *Taq* DNA polymerase diluted in 1x buffer A) was added. PCR was performed for 30 cycles of denaturation (94°C, 30 sec), primer annealing (60°C, 30 sec) and extension (72°C, 2 min). For the nested PCR, 1 μl of the above PCR product was used as template in a second PCR with the nested primers (Table 4.10) and identical composition otherwise. Nested PCR was performed for 30 cycles of denaturation (94°C, 30 sec), primer annealing (55°C, 30 sec) and extension (72°C, 2 min) before analyzing the products on ethidium bromide agarose gel electrophoresis.

Table 4.10. Nested PCR primers.

Name	Sequence	Tm (°C)
pACT2 Forward	CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CC	63.7
pACT2 Reverse	GTG AAC TTG CGG GGT TTT TCA GTA TCT ACG AT	63.9
pACT2 Forward Nested	TGT ATG GCT TAC CCA TAC GAT GTT CC	60.2
pACT2 Reverse Nested	GGG TTT TTC AGT ATC TAC GAT TCA TAG	55.2

4.5.11. Plasmid isolation from yeast

Plasmid isolation was performed according to the method of Dr. Moolman-Smook, Department of Medical Biochemistry, University of Stellenbosch, South Africa (personal communication). Yeast cells were grown in 1 ml TDO at 30°C overnight with shaking before adding 4 ml YPDA medium and growing cells for a further 4 hours. Cells were collected by centrifugation (3000 x *g*, 5 minutes), the supernatant removed and cells resuspended in 200 µl Smash-and-Grab buffer (1% SDS, 2% Triton X-100, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8). Glassbeads (~ 100 µl, 425-600 micron) and 200 µl phenol: chloroform: isoamylalcohol (25:24:1) were added and the mixture vortexed vigorously for 3 minutes to break open the yeast cells. The mixture was centrifuged (13 000 rpm, 5 minutes) and the aqueous layer removed. In order to precipitate the plasmid from the solution, 0.5 volumes of ammonium acetate (7.5 M, pH 5) and 2 volumes of 100% ethanol was added and the mixture centrifuged at 4°C (13000 rpm for 25 minutes). The pellet was washed with 70% ethanol, vacuum dried and dissolved in 20 µl water.

4.5.12. AD/library plasmid rescue via transformation in KC8 *E. coli*

KC8 *E. coli* cells have a defect in *leuB*, which can be complemented by *LEU2*. Thus, KC8 cells can be used to rescue AD/library plasmids (which carry *LEU2*) from yeast co-transformants that also contain a DNA-BD/bait plasmid. Plasmids isolated from QDO and *LacZ* positive colonies were electroporated into electro-competent KC8 *E. coli* cells. In order to select cells containing AD/library plasmids, cells were plated on M9 minimal medium containing ampicillin and lacking leucine.

4.5.13. Sequencing of AD/library inserts

KC8 cells containing AD/library plasmid were grown overnight in 1 ml LB/amp medium at 30°C with shaking. Plasmid was isolated from the cells using the High Pure plasmid isolation kit (Roche) as described previously. Automated nucleotide sequencing of the insert was

performed using the GAL4 AD sequencing primer (5' TACCACTACAATGGATG 3') with the Big Dye Sequencing kit on an ABI Prism 377 DNA sequencer (Perkin Elmer Applied Biosystems, USA) as described in Chapter 3. Sequences obtained were analyzed using the BioEdit Program. DNA and deduced protein sequences were analyzed using various databases and programs. These are described in the results section.

4.6. RESULTS AND DISCUSSION

4.6.1. Full-length cDNA GAL4 AD / Plasmid library construction

Figure 4.10 shows a typical gel profile of ds cDNA synthesized using the SMART cDNA synthesis and amplification system. First strand synthesis was done using 120 ng total RNA in a final volume of 11 μ l. After amplification of 2 μ l of first strand product, a smear from 0.3 -2 kb is visible after the optimal number of cycles (24 cycles) as determined in Chapter 3.

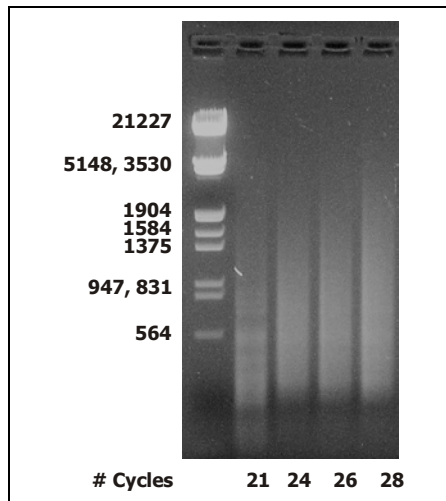


Figure 4.10. Analysis of ds cDNA amplification by LD-PCR using Super SMART™ technology.

Following amplification, the ds cDNA was polished, purified and size fractionated using the NucleoSpin® Extract system. The latter utilizes conventional binding of DNA to a silica membrane in the presence of chaotropic salts. DNA fragments between 200-2000 bp are subsequently eluted from the column with 50 μ l alkaline buffer. Using spectrophotometry, we were able to recover more than 95% of the loaded sample with an A260/280 exceeding 1.9.

The purified dsDNA was digested with *Sfi*I (in the presence of acetylated BSA) for 2 hours at 50°C and again purified using the NucleoSpin® Extract system. Figure 4.11 shows the gel profile of a polished purified sample and a sample following *Sfi*I digestion and the second purification step. In both cases, an intense smear ranging between 200 and 950 base pairs is visible centering around 550 bp. Therefore it can be concluded that *Sfi*I is also a rare cutter in *O. savignyi*, and does not completely digest the tick salivary gland dsDNA to small fragments. Therefore, we continued with the *Sfi*I digested dsDNA for constructing the GAL4 AD / plasmid library.

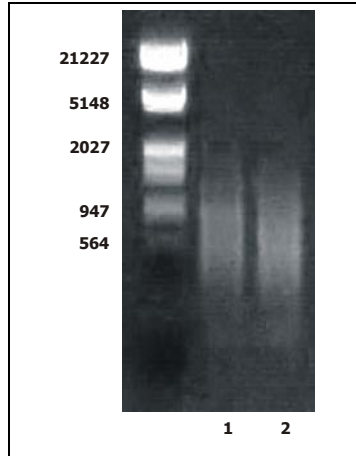


Figure 4.11. Agarose gel electrophoresis of (1) polished ds cDNA and (2) purified *Sfi*I digested ds SMART DNA.

Following *Sfi*I digestion of the pACT2 plasmid, the 5' phosphate groups were removed using Shrimp Alkaline Phosphatase (SAP). Since incomplete dephosphorylation would result in the ligation of intact pACT2 plasmids during ligation, we incubated dephosphorylated pACT2 with T4 DNA Ligase and performed agarose gel electrophoresis to test for complete dephosphorylation. The results in Figure 4.12 indicate that both *Sfi*I digestion and SAP dephosphorylation were successful.

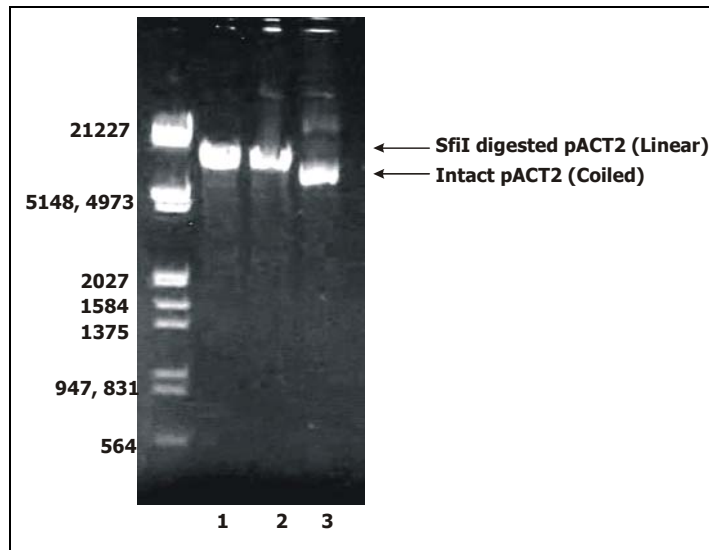


Figure 4.12. Agarose gel electrophoresis of (1) *Sfi*I digested pACT2, (2) *Sfi*I digested pACT2 treated with T4 Ligase and (3) untreated intact pACT2.

Ligation of the *Sfi*I digested dsDNA and dephosphorylated *Sfi*I digested pACT2 plasmid was optimized as described in Table 4.4. Indicated in Figure 4.13 are the results obtained from

the various vector: insert ratios obtained for an average insert of 500 bp and 150 ng vector which yielded the highest transformation efficiency. Both the 2:1 and 5:1 ratios exceeded 3×10^7 cfu/ μ g.

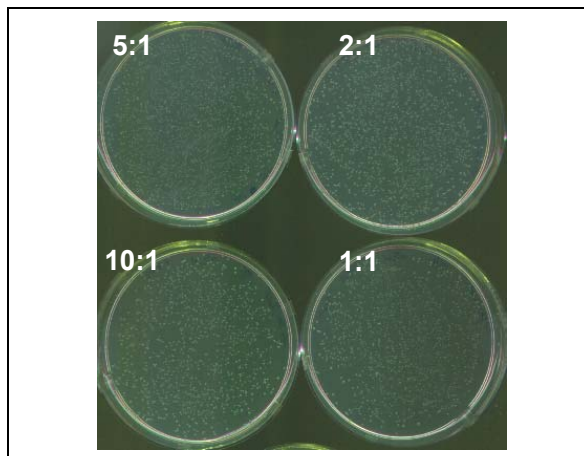


Figure 4.13. Transformation of various insert: vector ratios into electro competent BL21 *E. coli* cells.

The number of independent colonies was determined by restriction enzyme digestion of the fusion plasmids isolated from various clones. From Figure 4.14 it is obvious that numerous clones have similar molecular masses. To investigate whether these clones are identical, DNA sequencing was performed. The sequencing data indicated that clones with similar molecular masses do however contain different DNA sequences, but a high number of *Sfi*I-*Sfi*I linked inserts were detected among the clones that were sequenced (results not shown). Therefore, the library was not used for two-hybrid screens, and a fragmented library containing a *Sfi*I and *Xho*I site for directional cloning was created.

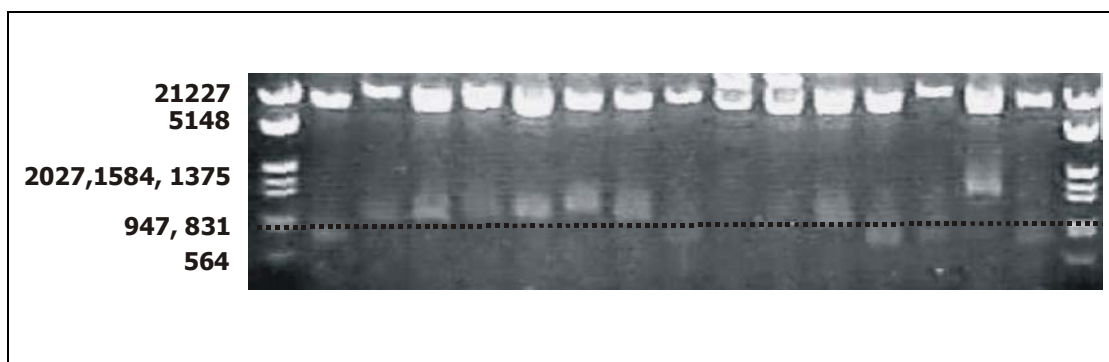


Figure 4.14. Agarose gel electrophoresis of *Sfi*I digested plasmids isolated from GAL4 AD/library transformed BL21 *E. coli* cells. The first and last lanes correspond to molecular mass markers while lanes 2-16 each corresponds to a single colony.

4.6.2. Truncated GAL4 AD / Plasmid library construction

Apart from the truncated cDNA library being directionally cloned via two different restriction sites (*Sfi*I and *Xho*I), it also allows for truncation of the coding strands from the 3' end, creating truncated C-terminal ends of the encoded proteins. This is useful since the SNARE proteins syntaxin and VAMP are anchored to the membrane via their C-terminal tails and could therefore now be identified in a two-hybrid assay that require translocation of the fusion protein complex to the nucleus.

As described in the methods section, the random hexa-nucleotides containing a *Xho*I site were annealed to the SMART dsDNA (1200 ng) and extended with Klenow enzyme at low temperatures. After converting the truncated strands to dsDNA the DNA was purified using NucleoSpin® columns. Using spectrophotometry we were able to calculate a two-fold increase in concentration during the truncation process. A typical gel electrophoresis profile of the fragmented *Xho*I digested library is shown in Figure 4.15. Once again a smear from 200- 950 bp was observed that center around ~550-600 bp, indicating *Xho*I to be a rare cutter.

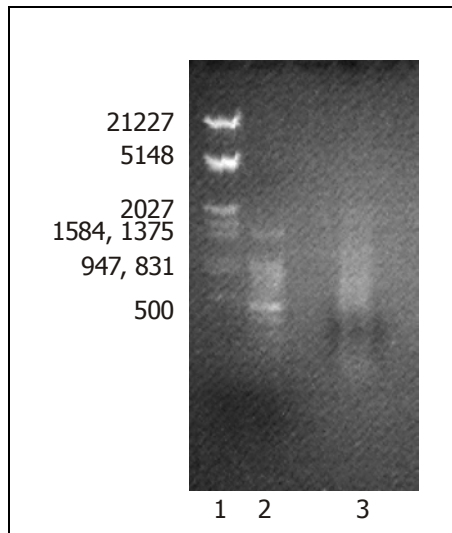


Figure 4.15. Agarose gel electrophoresis of the *Xho*I digested fragmented dsDNA. Lanes 1 and 2 corresponds to different molecular mass markers, lane 3 corresponds to the *Xho*I digested, truncated dsDNA.

The *Sfi*I and *Xho*I digested library was ligated into the pACT2 plasmid and electroporated into BL21 *E. coli* cells (as described previously). Similar to the full-length library, ligations with an average insert size of 500 ng and a 2:1 ratio (insert: plasmid) yielded the highest transformation efficiency, i.e. $9,6 \times 10^6$ cfu/ μ g. The number of independent clones was

determined by PCR amplification of the inserts directly from the BL21 *E. coli* cells (Figure 4.16). DNA sequencing of various clones with similar molecular masses were performed to distinguish between them. It was clear that bands with similar molecular masses do contain different DNA sequences (Figure 4.17). Therefore, the percentage of independent clones exceeded 60% and the library was used during a two-hybrid screen (see section 4.5.1. for calculating the number of independent clones). Interestingly, only one clone (419 bp) contained a *SfiI*-*SfiI* fused insert. All of the other clones contained a *XhoI* site in the 3' UTR of the transcript indicating incomplete *SfiI* digestion in the 3' region.

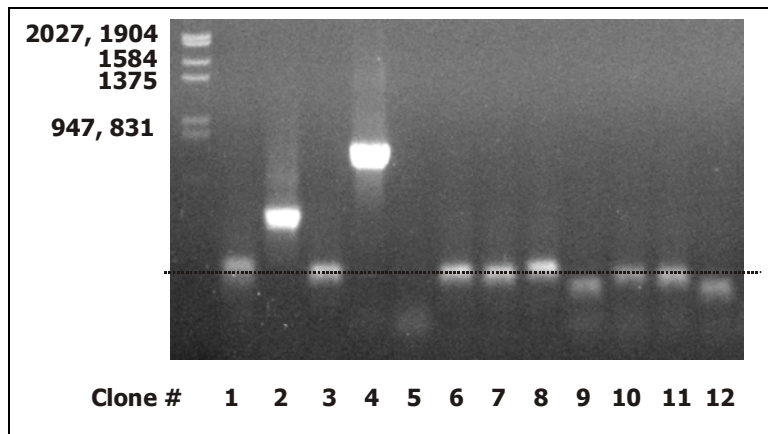


Figure 4.16. PCR screening of cloned inserts from transformed BL21 *E. coli* cells. PCR amplification of inserts was done directly from the BL21 *E. coli* cells using the forward and reverse nested primers. Each lane corresponds to a unique clone.

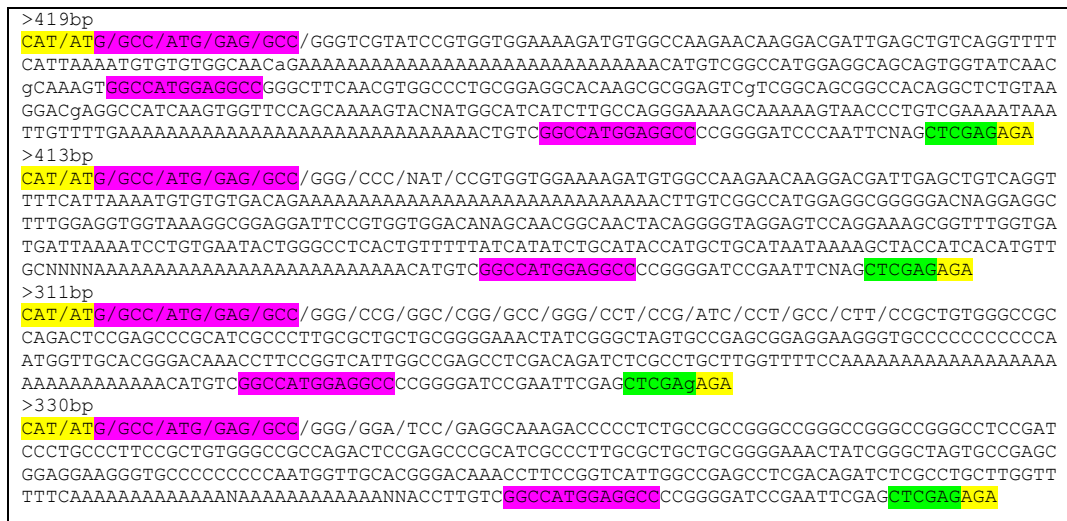


Figure 4.17. DNA sequence of four similar molecular mass clones from the fragmented *SfiI*/*XhoI* GAL4AD fusion library. The following is indicated: *pACT2* plasmid regions (yellow), *SfiI* restriction sites (purple), *XhoI* restriction site (green) and reading frame (/).

In order to amplify the library, the remaining cells (~ 2ml) from the 2:1 and 5:1 ratios were plated on 40 large plates (50 μ l per plate), grown overnight and the cells collected in 50 ml LB/glycerol. The titer of the library was determined as $3,5 \times 10^7$ cfu/ml. The cells were aliquoted into 1 ml fractions and stored at -70°C . To obtain sufficient plasmid for a library scale transformation, 500 μ l cells were diluted into 500 ml LB/Amp and grown until $A_{600} \sim 0.5 - 0.6$ before cells were collected by centrifugation, and a large-scale plasmid isolation (using the NucleoBond™ PC2000 system) performed. On average 800 -1000 μ g of plasmid was isolated from 2 liters of culture.

4.6.3. Bait construction

Full-length and truncated syntaxin

The full-length recombinant clone containing inserts encoding native rat brain syntaxin 1 (D45208) was a gift from Prof. Scheller. Primers were designed to PCR amplify and directionally clone (using the *Nde*I and *Pst*I sites) the entire coding sequence of syntaxin 1 in the correct reading frame. A second primer was used to create a truncated version of the insert. The latter coding sequence was also ligated directionally into the pAS2_1 plasmid using the *Nde*I and *Pst*I sites. Figure 4.18 shows the PCR amplified products obtained from the rat brain syntaxin plasmid. The bands were excised and purified using the NucleoSpin system. DNA sequencing of the obtained clones indicated that both the native and truncated bait constructs were correctly constructed in the pAS2_1 plasmid (Figure 4.19).

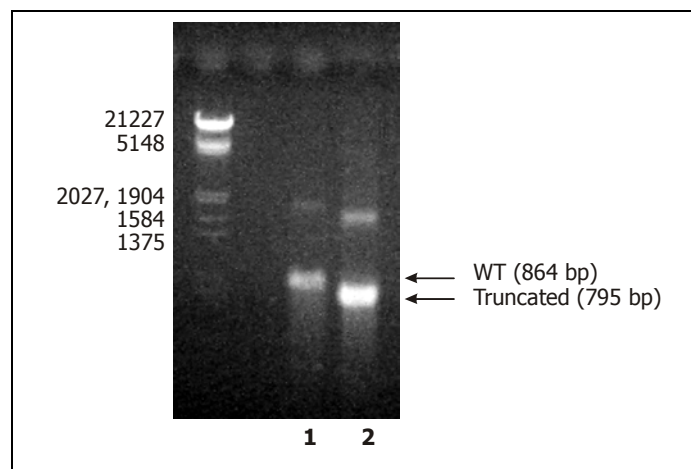


Figure 4.18. PCR amplification of syntaxin bait constructs. Native (lane 1) and truncated (lane 2) inserts from the rat brain syntaxin1A original construct are shown.

SynBait	MKDRTQELRTAKSDSDDDDDVTVTVDRDRFMDEFFEQVEEIRGFIDKIAENVVEEVKRKHA	60
TruncSyn	MKDRTQELRTAKSDSDDDDDVTVTVDRDRFMDEFFEQVEEIRGFIDKIAENVVEEVKRKHA	60
D45208	MKDRTQELRTAKSDSDDDDDVTVTVDRDRFMDEFFEQVEEIRGFIDKIAENVVEEVKRKHA	60

SynBait	ILASPNPDEKTKEELEELMSDIKKTANKVRSKLSIEQSIQEEGLNRSSADLRIRKTOH	120
TruncSyn	ILASPNPDEKTKEELEELMSDIKKTANKVRSKLSIEQSIQEEGLNRSSADLRIRKTOH	120
D45208	ILASPNPDEKTKEELEELMSDIKKTANKVRSKLSIEQSIQEEGLNRSSADLRIRKTOH	120

SynBait	STLSRKFVEVMSEYNATQSDYRERCKGRIQRQLEITGRTTTSEELEDMLSEGNPAIFASG	180
TruncSyn	STLSRKFVEVMSEYNATQSDYRERCKGRIQRQLEITGRTTTSEELEDMLSEGNPAIFASG	180
D45208	STLSRKFVEVMSEYNATQSDYRERCKGRIQRQLEITGRTTTSEELEDMLSEGNPAIFASG	180

SynBait	IIMDSSISKQALSEIETRHSSEIKLETSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHA	240
TruncSyn	IIMDSSISKQALSEIETRHSSEIKLETSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHA	240
D45208	IIMDSSISKQALSEIETRHSSEIKLETSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHA	240

SynBait	VDYVERAVSDTKKAVKYQSKARRKKIMIICCIVILGIIASTIGGIFG	288
TruncSyn	VDYVERAVSDTKKAVKYQSKARRKK	
D45208	VDYVERAVSDTKKAVKYQSKARRKKIMIICCIVILGIIASTIGGIFG	288

Figure 4.19. Amino acid sequence alignment of the syntaxin baits. The full-length rat brain syntaxin (SynBait), truncated syntaxin (TruncSyn) and native rat brain syntaxin (D45208) constructs are shown.

In order to confirm expression of the syntaxin bait proteins, total protein content of native and bait expression yeast cells were isolated and equal amounts of protein were coated onto an ELISA plate. Screening with an antibody against the DNA-BD was not performed, as this approach does not confirm expression of the correct reading frame and hence bait protein. In this approach, a polyclonal antibody directed at rat brain syntaxin 2 was used. We were able to confirm expression of the bait molecules (Figure 4.20). Significant signals were obtained for native AH109 cells, indicating cross-reactivity of the antibody with homologous yeast proteins. The signals are however 1,8 x and 3,6 x higher than the control (native AH109 cells) for AH109 cells expressing native syntaxin and truncated syntaxin, respectively.

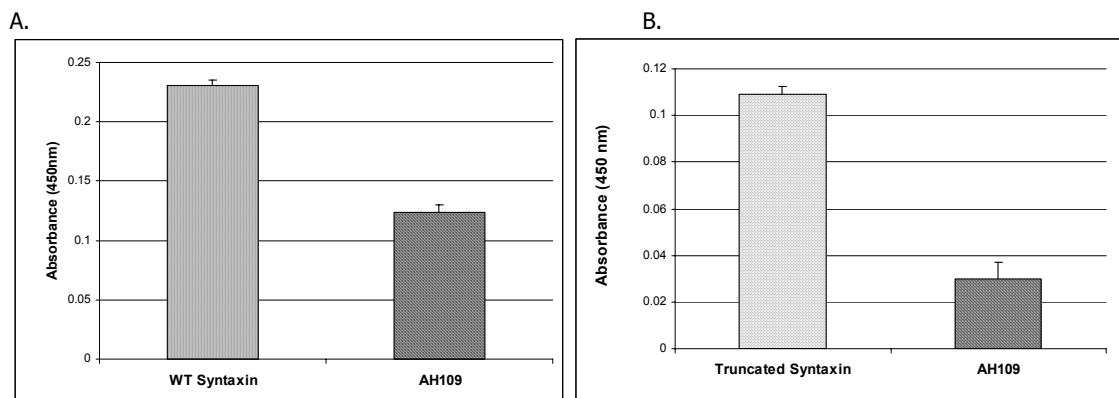


Figure 4.20. ELISA of syntaxin transformed AH109 cells with polyclonal anti-syntaxin 2 IgG. Equal amounts of total yeast protein extract from AH109 cells expressing (A) native syntaxin and (B) truncated syntaxin were investigated.

Native and GTPase deficient Rab3a baits

Similar to constructing the syntaxin baits, the native Rab3a bait was created from the mouse Rab3a construct that was a kind gift from Prof. Scheller. As described previously, Rab3a is a GTPase with different protein binding partners when in the GTP- and GDP-bound state. Therefore, a GTPase deficient isoform containing the T36N mutation was also constructed. Figure 4.21 indicates the band amplified from the obtained Rab3a construct. Figure 4.23 gives the DNA nucleotide sequence of the native and T36N mutated clones created in pAS2_1.

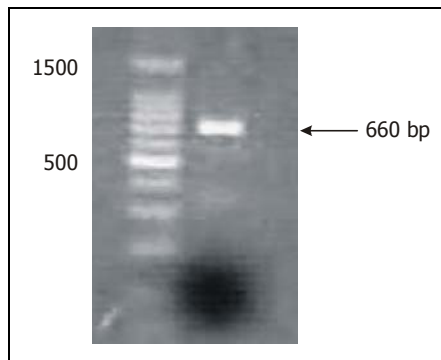


Figure 4.21. PCR amplification of the coding region of native mouse brain Rab3a.

To confirm expression of Rab3a T36N, ELISA of total yeast protein was performed using a polyclonal antibody against mouse brain Rab3a. The result indicates a 1,57 x fold higher signal for AH109 cells expressing the bait construct compared to native AH109 cells. Similar to the results obtained for syntaxin, AH109 cells also cross-reacted with the antibody (Figure 4.22).

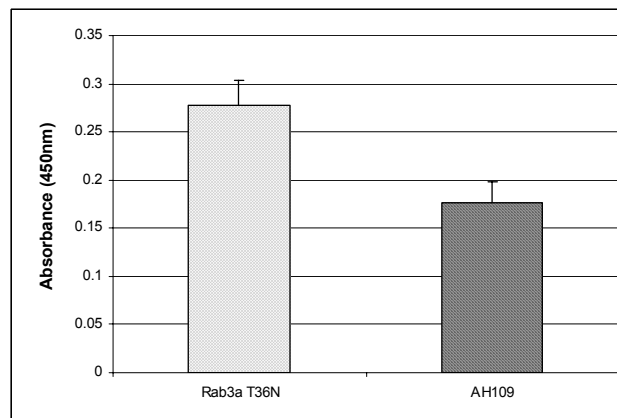


Figure 4.22. ELISA of Rab3a T36N transformed AH109 cells with polyclonal anti-Rab3a IgG. Equal amounts of total yeast protein extract from native AH109 cells and Rab3a T36N transformed cells were investigated.

BaitWT	ATGGCTTCCGCCACAGACTCTCGCTATGGGCAGAAGGAGTCTCTCAGACCAGAACTTCGAC	60
T36N	ATGGCTTCCGCCACAGACTCTCGCTATGGGCAGAAGGAGTCTCTCAGACCAGAACTTCGAC	60
NM_009001	ATGGCTTCCGCCACAGACTCTCGCTATGGGCAGAAGGAGTCTCTCAGACCAGAACTTCGAC	60

BaitWT	TATATGTTCAAGATCCTGATCATTGGGAACAGCAGCGTGGGCAAAACCTCGTTCCCTCTTC	120
T36N	TATATGTTCAAGATCCTGATCATTGGGAACAGCAGCGTGGGCAAAACCTCGTTCCCTCTTC	120
NM_009001	TATATGTTCAAGATCCTGATCATTGGGAACAGCAGCGTGGGCAAAACCTCGTTCCCTCTTC	120

BaitWT	CGCTACGCAGATGACTCCTTCACTCCAGCCTTTGTGTCAGCACCGTTGGCATAGACTTCAAG	180
T36N	CGCTACGCAGATGACTCCTTCACTCCAGCCTTTGTGTCAGCACCGTTGGCATAGACTTCAAG	180
NM_009001	CGCTACGCAGATGACTCCTTCACTCCAGCCTTTGTGTCAGCACCGTTGGCATAGACTTCAAG	180

BaitWT	GTCAAAACCATCTACCGCAACGACAAGAGGATCAAGCTGCAGATCTGGGACACAGCAGGG	240
T36N	GTCAAAACCATCTACCGCAACGACAAGAGGATCAAGCTGCAGATCTGGGACACAGCAGGG	240
NM_009001	GTCAAAACCATCTACCGCAACGACAAGAGGATCAAGCTGCAGATCTGGGACACAGCAGGG	240

BaitWT	CAAGAGCGGTACCGCACCATCACCACAGCCTATTACCGAGGCGCCATGGGCTTCAATCCTA	300
T36N	CAAGAGCGGTACCGCACCATCACCACAGCCTATTACCGAGGCGCCATGGGCTTCAATCCTA	300
NM_009001	CAAGAGCGGTACCGCACCATCACCACAGCCTATTACCGAGGCGCCATGGGCTTCAATCCTA	300

BaitWT	ATGTATGACATCACCAATGAGGAGTCATTTAATGCAGTGCAGGACTGGTCCACTCAGATC	360
T36N	ATGTATGACATCACCAATGAGGAGTCATTTAATGCAGTGCAGGACTGGTCCACTCAGATC	360
NM_009001	ATGTATGACATCACCAATGAGGAGTCATTTAATGCAGTGCAGGACTGGTCCACTCAGATC	360

BaitWT	AAAACCTACTCGTGGGACAATGCCAGGTGCTGCTGGTGGGAAACAAGTGTGACATGGAA	420
T36N	AAAACCTACTCGTGGGACAATGCCAGGTGCTGCTGGTGGGAAACAAGTGTGACATGGAA	420
NM_009001	AAAACCTACTCGTGGGACAATGCCAGGTGCTGCTGGTGGGAAACAAGTGTGACATGGAA	420

BaitWT	GATGAGCGAGTGGTGTCTCAGAGCGTGGCCGGCAGCTGGCTGACCACCTGGGCTTTGAG	480
T36N	GATGAGCGAGTGGTGTCTCAGAGCGTGGCCGGCAGCTGGCTGACCACCTGGGCTTTGAG	480
NM_009001	GATGAGCGAGTGGTGTCTCAGAGCGTGGCCGGCAGCTGGCTGACCACCTGGGCTTTGAG	480

BaitWT	TTCTTTGAGGCCAGCGCCAAGGACAACATTAATGTCAAGCAGACGTTTGAACGCTCTGGTG	540
T36N	TTCTTTGAGGCCAGCGCCAAGGACAACATTAATGTCAAGCAGACGTTTGAACGCTCTGGTG	540
NM_009001	TTCTTTGAGGCCAGCGCCAAGGACAACATTAATGTCAAGCAGACGTTTGAACGCTCTGGTG	540

BaitWT	GACGTGATCTGTGAGAAGATGTCAGAGTCCCTGGATACTGCAGACCTGCGGTACCCGGT	600
T36N	GACGTGATCTGTGAGAAGATGTCAGAGTCCCTGGATACTGCAGACCTGCGGTACCCGGT	600
NM_009001	GACGTGATCTGTGAGAAGATGTCAGAGTCCCTGGATACTGCAGACCTGCGGTACCCGGT	600

BaitWT	GCCAAGCAGGGCCCGCAGCTCACCAGCAGCAGGCGCCACCTCATCAGGATTGTGCCGTC	660
T36N	GCCAAGCAGGGCCCGCAGCTCACCAGCAGCAGGCGCCACCTCATCAGGATTGTGCCGTC	660
NM_009001	GCCAAGCAGGGCCCGCAGCTCACCAGCAGCAGGCGCCACCTCATCAGGATTGTGCCGTC	660

BaitWT	TGA	663
T36N	TGA	663
NM_009001	TGA	663

Figure 4.23. DNA nucleotide sequence alignment of the various Rab3a bait constructs. The native mouse Rab3a bait (BaitWT), GTPase deficient Rab3a (T36N) and original native mouse brain Rab3a construct (NM_009001) are shown. The mutation resulting in a T36N mutation is indicated in yellow.

Native α -SNAP bait

α -SNAP is a protein with an exceptionally crucial role in forming the 25S fusion complex. Upon recognizing the SNARE complex it induces a conformational change that renders it

competent for binding and stimulating the ATPase activity of the D1 domain of NSF (Burgoyne and Morgan 2003). From literature it is clear that cytosolic α -SNAP engage in protein-protein interactions with syntaxin, SNAP25, VAMP and NSF that makes it attractive for use in a two-hybrid screen (Table 4.2). The coding region from mouse brain α -SNAP was amplified from the construct obtained from Proff. Whiteheart and Rothman, and directionally cloned in frame into the pAS2_1 plasmid. Figure 4.24 indicates the band obtained after PCR amplification, while the coded sequences obtained from these clones (in frame with the DNA-BD) are indicated in Figure 4.25.

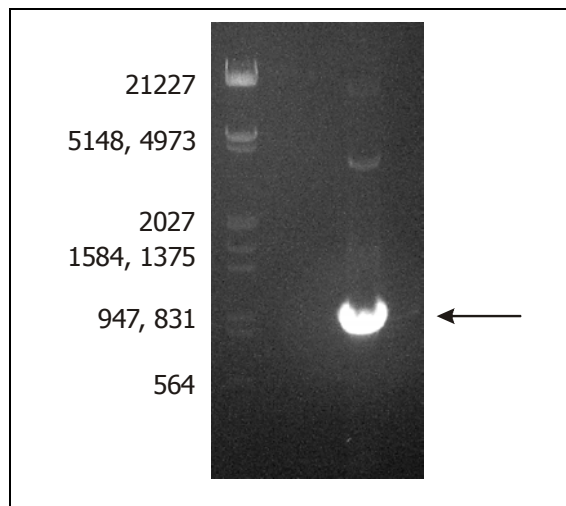


Figure 4.24. PCR amplification of the coding region of native mouse brain α -SNAP.



Figure 4.25. DNA nucleotide sequence alignments of α -SNAP bait constructs. α -SNAP baits constructed in pAS2_1 (Clones 2, 3 and 5) as well as native mouse brain α -SNAP (P5421) are indicated.

4.6.4. Transformation of bait/ GAL4 BD constructs into AH109

Using the small-scale yeast transformation protocol, the bait constructs were transformed into AH109 yeast cells. Since the pAS2_1 plasmid contains the *TRP1* reporter gene, the cells were plated on SD/-Trp. In order to confirm that the bait constructs themselves do not activate the two-hybrid reporter genes (*HIS3* and *ADE2*), the cells were also plated on SD/-Leu/-Trp/-His (TDO) and SD/-Leu/-Trp/-His/-Ade (QDO). Indicated in Figure 4.26 are six clones containing the truncated syntaxin bait plated on various selection media. It is evident that this bait does not activate the two-hybrid reporter genes. All baits were screened in this way (results not shown) and none activated the yeast reporter genes.

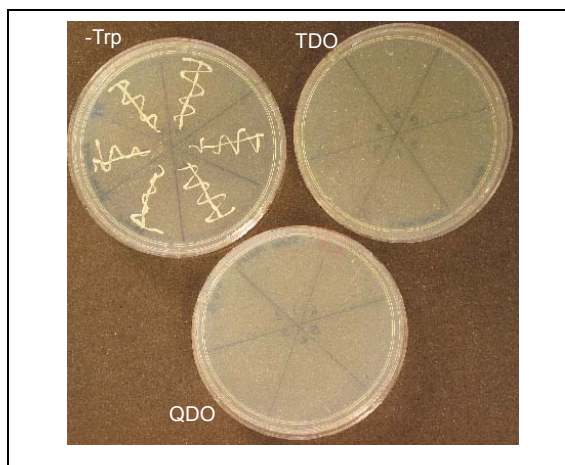


Figure 4.26. AH109 yeast cells containing the pAs2_1 truncated syntaxin bait construct. Cells are plated on *SD/-Trp (-Trp)*, *SD/-Leu/-Trp/-His (TDO)* and *SD/-Leu/-Trp/-His/-Ade (QDO)*

4.6.5. Library transformation and two-hybrid screening

Using the 30x scale described by the two-hybrid TRAFCO protocol (<http://tto.trends.com>) for library scale transformations, AH109 cells containing the various bait plasmids (truncated syntaxin, Rab3a, Rab3a T36N and α -SNAP, respectively) were transformed with 150 μ g of library plasmid. Co-transformed cells were directly plated on TDO plates and grown until colonies were visible (2-3 days). The cells were then scraped from the plates and re-plated at various dilutions on QDO plates (see Figure 4.27.i). From here single colonies were picked and plated in duplicate on master QDO plates (Figure 4.27.ii). Only the results of truncated syntaxin are shown, while the results for α -SNAP were identical. Both the truncated syntaxin and α -SNAP co-transformed cells were used for further two-hybrid screening.

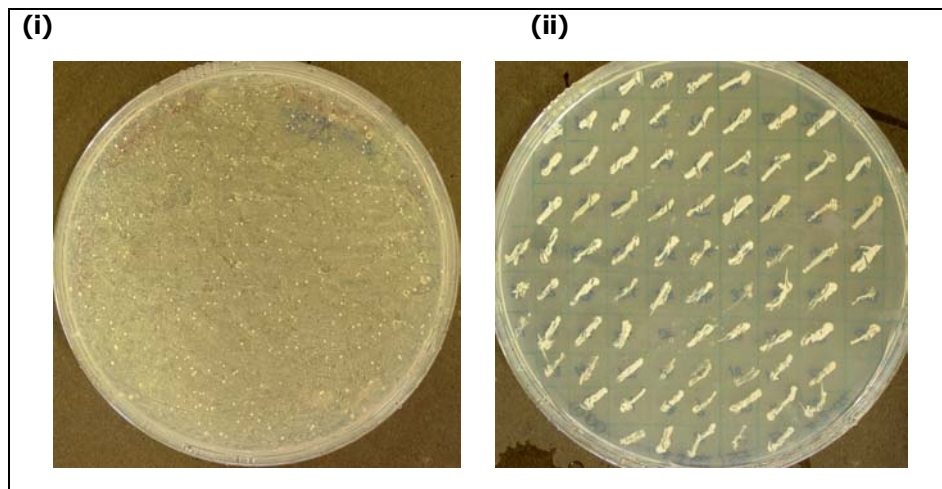


Figure 4.27. AH109 yeast cells co-transformed with truncated syntaxin bait and *SfiI/XhoI* truncated library. Co-transformed cells were plated on SD/-Leu/-Trp/-His/-Ade (QDO) and grown until single colonies were visible (i). The colonies were then streaked on a master QDO plate (ii) for subsequent storage and analysis.

When plating cells co-transformed with the Rab3a bait constructs and library, it was noted that the cells turned dark pink on both TDO- and QD-plates. It is known that Ade⁺ cells remain white to pale pink while Ade⁻ colonies gradually turn reddish-brown and stop growing. Therefore, stronger *ADE2* expression will be white while in contrast, weaker expression will become progressively more pink and red. In the case of AH109 cells containing the native Rab3a as well as the mutant Rab3a T36N bait constructs, the cells were extremely pink in colour indicating weak expression (Figure 4.28). Therefore, the Rab3a baits were not used during two-hybrid assays. Future studies could exploit use of the Rab3a Q81L mutation as described by Martincic *et al.* (Martincic *et al.* 1997).

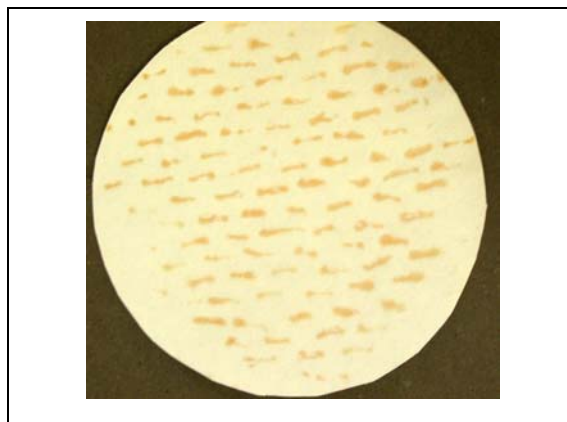


Figure 4.28. AH109 yeast cells containing the pAs2_1 native Rab3a bait construct. Co-transformed cells plated on SD/-Trp were transferred to filter paper to emphasize the pink color.

The number of clones screened can be calculated as (cfu/ μ g) x amount of library plasmid used. In the case of cells co-transformed with truncated syntaxin bait and library, the co-transformation efficiency was calculated at $5,3 \times 10^4$ cfu/ μ g and the number of clones screened $7,95 \times 10^6$. In the case of α -SNAP / library co-transformations the co-transformation efficiency was calculated at $3,4 \times 10^4$ cfu/ μ g and the number of clones screened $5,1 \times 10^6$.

4.6.6. Colony-lift β -galactosidase assay

Initial screening of the QDO positive clones was performed using the *lacZ* reporter gene, i.e. positive for β -galactosidase activity. The colony lift assay for β -galactosidase was used to screen large numbers of QDO positive clones in order to identify clones with elevated levels of β -galactosidase activity. Figure 4.29 indicates a typical result obtained using the colony-lift assay. Clones containing control plasmids turned blue within 30-60 minutes. Clones with elevated β -galactosidase activity was identified within 60 minutes and used for further screening with nested PCR.

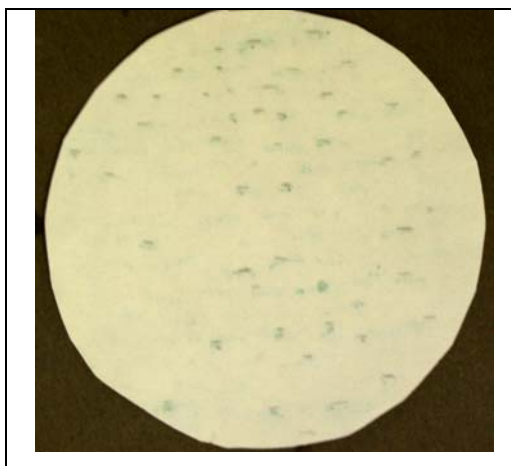


Figure 4.29. A typical β -galactosidase colony lift assay of AH109 yeast cells containing the pAS2_1 truncated syntaxin bait construct. *Co-transformed cells are plated on QDO, transferred to filter paper; lysed by freeze/thaw cycles and incubated with buffer containing X-gal. Blue colonies are positive for β -galactosidase activity.*

4.6.7. Nested-PCR screening of β -galactosidase positive clones

PCR screening directly from intact yeast cells requires nested PCR due to the high amount of chromosomal DNA present. Indicated in Figure 4.30 are the two sets of primers (indicated in blue and red, respectively) used to amplify the cloned inserts from the pACT2-AD/ library constructs.

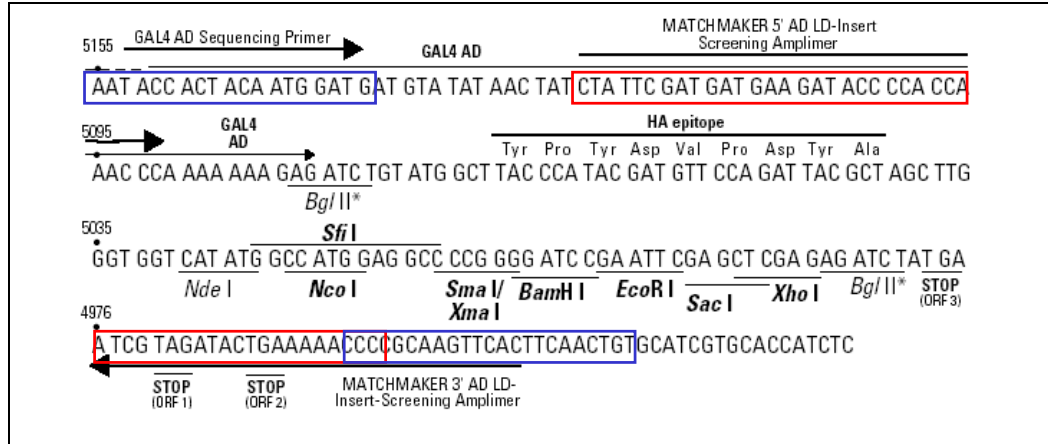


Figure 4.30. Partial sequence of the pACT2 plasmid. The first (blue) and second (red) set of primers used during nested PCR are indicated. The multiple cloning region, containing the *SfiI* and *XhoI* sites used for directional cloning of the library inserts, are also shown.

Cells (~1 μl) were suspended in PCR mixture containing the first set of nested primers (indicated in blue) and lysed by incubating the reaction mixture at 94°C for 7 minutes in a Perkin Elmer GeneAmp PCR system 9700. After cooling the mixture to 80°C, enzyme mix was added and PCR performed as described previously. Product obtained from this first PCR was used as template for the second (nested) PCR using the second set of primers (indicated in red, Figure 4.30). The final product was analyzed using agarose gel electrophoresis. As indicated in Figure 4.31, it is clear that one cannot distinguish between the various clones after only nested PCR. Therefore, restriction enzyme mapping was done using *BamHI* and *HindIII*. After restriction enzyme mapping, 7 unique clones could be identified from QDO positive clones using truncated syntaxin as bait (Figure 4.32).

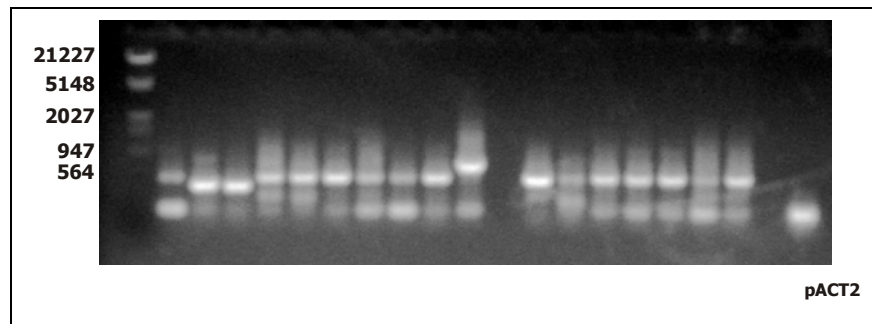


Figure 4.31. Typical agarose electrophoresis pattern obtained after nested PCR of QDO-positive clones containing truncated syntaxin as bait. Each lane corresponds to a different QDO β-galactosidase positive clone. The last lane indicates the multiple cloning site amplified from native pACT2 plasmid during nested PCR.

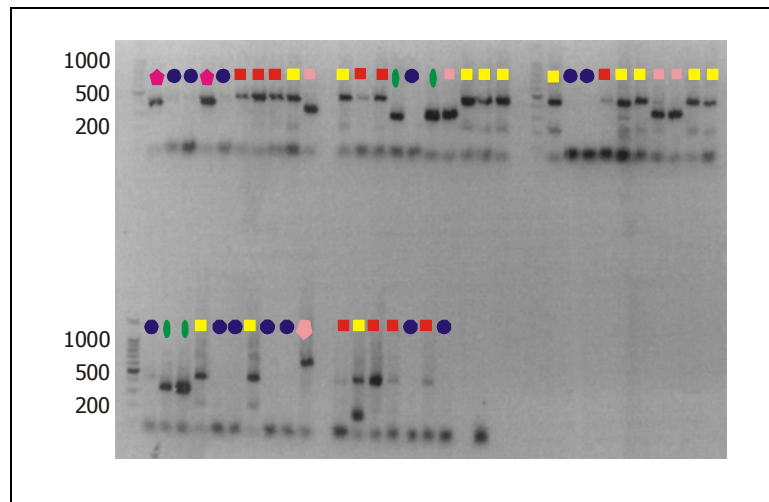


Figure 4.32. Agarose electrophoresis pattern obtained after *Bam*HI and *Hind*III digestion of nested PCR products obtained from QDO-positive clones containing truncated syntaxin as bait. Each lane corresponds to a different QDO β -galactosidase positive clone. Colored symbols are used to indicate unique clones.

Nested PCR and restriction enzyme digestion (*Bam*HI and *Hind*III) of the products obtained from positive clones using α -SNAP as bait resulted in the identification of only a single clone. Indicated in Figure 4.33 are the agarose electrophoresis results from 30 clones that were screened. All of the clones contained a single amplified band of \sim 500bp.

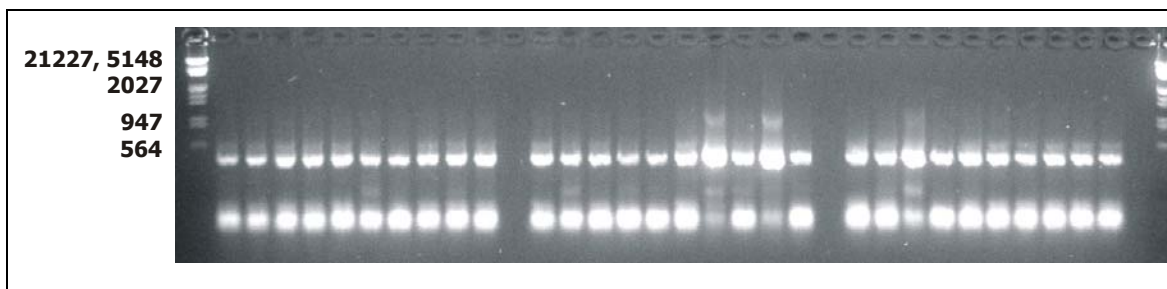


Figure 4.33. Agarose electrophoresis pattern obtained after *Bam*HI and *Hind*III digestion of nested PCR products obtained from QDO-positive clones containing α -SNAP as bait.

4.6.8. Sequencing and analysis of positive AD/library inserts

The various novel clones identified by both the β -galactosidase assay and nested PCR were identified on the master QDO plates. These clones, however, still contained both the pAS2_1 BD/Bait as well as the pACT2 AD/library plasmids. In order to isolate only the pACT2 AD/library plasmids for DNA sequencing of the interacting inserts, both plasmids were isolated from the clones and transformed into KC8 *E. coli* cells. These KC8 cells have a defect

in *leuB*, which can be complemented by *LEU2* from yeast and will hence only select the pACT2 AD/library plasmids.

The KC8 clones (plated on M9 minimal medium, with ampicillin, lacking leucine) were again screened for pACT2 AD/library plasmids and correct insert as detected during restriction enzyme analysis of QDO positive clones. Following High Pure plasmid isolation from the KC8 cells, the interacting inserts were subjected to DNA sequencing using the GAL4 AD sequencing primer.

Analysis of inserts interacting with truncated rat brain syntaxin1

The proteins encoded for the interacting inserts were deduced from the nucleotide sequences as encoded for by the open reading frame of the DNA activation domain. Two consensus domains that are shared between the five clones were identified. These are colored yellow (domain I) and green (domain II), respectively (Table 4.11).

Table 4.11. Prey molecules identified using truncated syntaxin and truncated library

Clone #	In frame translation
2 & 6	SYPWWKRCGQEQGRLSCQVFIKMCVAT
9	SYPWWKRCGQEQGRLSCQVFIKMCVTEKKKKKKKKKTCRPWRRGXRRLLWRW
10	PIPALPLWAARLRARIALALLRGNYRASAERRKGAPPNGCTGQTFRSLAEPRIQISPAWF
14	GGSEAKTPSAAGPGRAGPPIPALPLWAARLRARIALALLRGNYRASAERRKGAPPQWLHGTNLPVIGRASTDLACLVF
40	TLTGTTTRGVAGEAARNAGTSPGTVPGTAIPGGPDRRGTGAGESTVRRARVCVVRSHRITLSRTVAVRTEGDQCPAGPSCSYLDGNLGEI NLRVCKKKKKKKKKKHVGHGGRGTSSWWXQPPXYWQGLNSSKRCLRSRQGRSDCRQXDGSYPWWKRCGQEQG

Domain I (indicated in yellow, Table 4.11) was shared between clones 2, 6, 9 and partially in the C-terminal of clone 40. We were unable to ascribe an identity to domain I due to the limited length of the encoded peptides. Domain II (indicated in green, Table 4.11) was present in clone 10 and 14.

Since no significant identity could be attributed to clones 10 and 14 using normal BLAST-P, we used PSI-BLAST with the BLOSUM72 matrix (Position Specific Iterated) analysis since it is much more sensitive to detect weak but biologically relevant sequence similarities (Altschul *et al.* 1997). PSI-Blast analysis of only the consensus region (domain II) indicated various possible targets. Amongst these was the protein syntaphilin (gi_57104478), which shared a

44% identity with domain II (Figure 4.34). Syntaphilin has only been isolated from brain tissue to date, where it functions as a molecular clamp that controls free syntaxin-1 and dynamin-1 availability and thereby regulates synaptic vesicle exocytosis and endocytosis. The binding of syntaphilin to syntaxin is regulated via the phosphorylation of syntaphilin by a cAMP-dependent kinase. It is predicted that PKA phosphorylation acts as an “off” switch for syntaphilin, thus blocking its inhibitory function via the cAMP-dependent signal transduction pathway (Das *et al.* 2003; Boczan *et al.* 2004). The expect (E) value, which is a parameter that describes the number of hits one can expect to obtain just by chance when searching a database of particular size, indicated this similarity as not significant. We obtained an E-value of 33, while a lower E-value (closer to zero) indicates a more significant match. It must however be noted that searches with short sequences can be virtually identical and have relatively high E-value because calculation of the E-value also takes into account the length of the query sequence. This is because shorter sequences have a high probability of occurring in the database purely by chance.

Domain 2	1	PIPALPLWAARLRARIALALLRGNYRASAERRKGAP	36
		PIP L R R+ +A++L G+ RASA R G P	
Syntaphilin	346	PIPPL----TRTRSLMAMSL-PGSRRASAGSRSGGP	
	376		

Figure 4.34. Homology between domain I and syntaphilin using PSI-BLAST.

When the entire coding sequence of clone 10 was subjected to PSI-BLAST analysis, the data indicated similarity to casein kinase I, epsilon isoform from mouse (Q9JMK2) and human (P49674). In both cases 44% identity was observed with an e-value of 0.7, which indicates a greater degree of confidence. The alignments and identical residues are indicated in Figure 4.35. Literature indicates that syntaxin does interact with casein kinase I and gets phosphorylated by both casein kinase I and II (Dubois *et al.* 2002). In order to conclusively identify clone 10 one will have to obtain the entire open reading frame of the protein. Since a partial sequence is known, one can exploit the use of 3' and 5' RACE, or using the cloned insert as a probe for screening the full-length library. No identity could be ascribed for clones 14 and 40 using PSI-BLAST.

Clone 10	23	GNYRASAERRKGAPPNGCTGQTFRSLAEPQISPA	58
		G R SA R PP G T RS AEP +PA	
Q9JMK2	318	GQLRGSATRALPPGPPTGATANRLRSAAEPVASTPA	353
P49674	318	GQLRGSATRALPPGPPTGATANRLRSAAEPVASTPA	353

Figure 4.35. Homology between clone 10 and Casein kinase I epsilon isoform using PSI-BLAST.

Certain analysis programs (the so called threading programs) incorporate both PSI-BLAST, as well as structure prediction from a library of structures in order to identify possible identity. Using the threading programs 3D-PSSM Web Server V 2.6.0 (<http://www.igb.uci.edu/tools/scratch/>), both clones 10 and 14 were found to share 36% and 24% identity, respectively, to the voltage-gated potassium channel inactivation domain 2 (residues 1 - 75). Once again, the interaction between syntaxin and the voltage-gated potassium channel, in particular the inactivation domain, has been described in literature (Leung *et al.* 2003; Michaelevski *et al.* 2003). Again, no motifs or identity could be determined for clone 40.

Since syntaxin interacts with its binding partners via coiled-coiled regions (α -helical rich regions), we investigated the secondary structure of the identified peptides using the SCRATCH server of 3D-PSSM. This server uses 7 different servers (Sspro2, Sspro8, CONpro, ACCpro, CMAPpro, CCMAPpro and CMAP23Dpro) to assign the most likely secondary structure to an amino acid sequence (<http://www.igb.uci.edu/tools/scratch/>). From the data it is clear that all of the identified clones are rich in alpha helical structure (Figure 4.36). Domain I, clone 10 and clone 14 contain a single helix while clone 4 contain 2 helical domains as well as a helical C-terminal. Based on the helical content, one could postulate that these helical domains are most likely to be the ones involved in coil-coil interactions with the syntaxin bait.

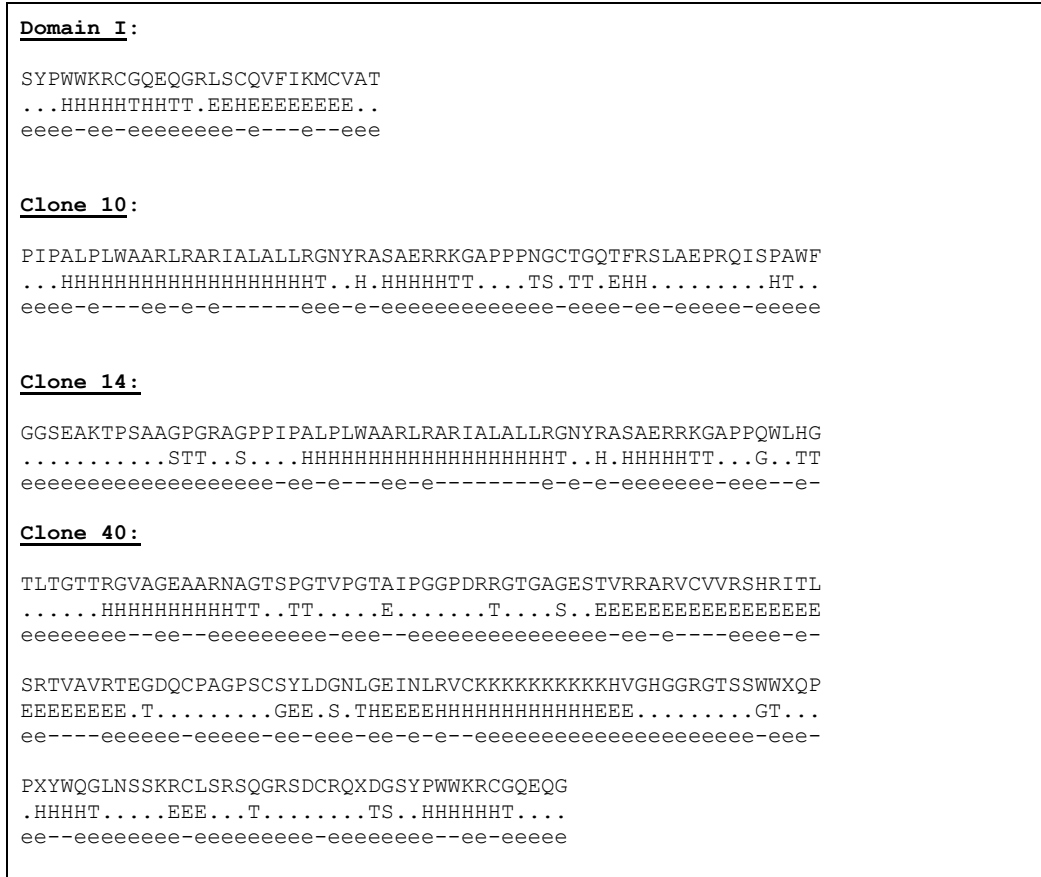


Figure 4.36. Structure prediction of syntaxin interacting peptides. Line 1: The 1-letter code of the protein primary sequence. Line 2: 8-class secondary structure prediction (H: alpha helix, E: extended strand, T: turn, S: bend, G: 310-helix). Line 3: Prediction of relative solvent accessibility (-: The residue is buried, e: The residue is exposed).

Analysis of inserts interacting with rat brain α -SNAP

All the interacting clones identified during two-hybrid assays using α -SNAP as bait contained an identical ~500 bp insert which encoded a 126 amino acid protein (Table 4.12, row 2). BLAST-P and PSI-BLAST analysis of the encoded protein detected no hits. The Predict protein database, which incorporates functional motifs, composition-bias, protein domains and threading (<http://cubic.bioc.columbia.edu/predictprotein>), indicated that the protein shares highest homology with the microtubule-associated protein 1B (also called neuraxin) from rat (P15205), various heat shock proteins and myosin (Table 4.12). In all of these cases the E-value was again not significant.

Table 4.12. Predict protein analysis of α -SNAP interacting protein.

α-SNAP interacting protein sequence:			
GLTTMHTNIKDIKSDLKTTENNISILSARAESIEANVASLGKVHEAVRDLTETSKNLSANVDFLKRKTDDFENMRRLVVFY GIPDDPAETWAQSELHVIKLCEQNLGKVVKPEEIERAHRSDL			
Predict protein analysis results:			
	Identity (%)	Accession number	Name
mapb_rat	26	P15205	LIGHT CHAIN LC1
dnak_brume	26	Q8YE76	HSP70
dnak_bruov	26	Q05981	DNAK PROTEIN (HEAT SHOCK)
tpm2_yeast	24	P40414	TROPOMYOSIN 2.
g160_human	23	Q08378	GOLGIN-160
dyna_neucr	22	Q01397	(DP-150)(DAP-150)
myh8_human	21	P13535	MYOSIN HEAVY CHAIN (SKELETAL MUSCLE)
tpm1_yeast	21	P17536	TROPOMYOSIN 1
smc3_yeast	21	P47037	CHROMOSOME SEGREGATION
ra50_sulso	21	Q97WH0	DNA DOUBLE-STRAND BREAK REPAIR PROTEIN
tpm1_chick	22	P04267	ALPHA-TROPOMYOSIN
mys2_dicdi	21	P08799	MYOSIN II HEAVY CHAIN
yhge_bacsu	21	P32399	HYPOTHETICAL 84.1 KDA PROTEIN

When subjecting the protein sequence to the 3D-PSSM threading web server V 2.6.0 it was found to share sequence and structural homology to syntaxin 1A N-terminal, syntaxin 6 and the neuronal synaptic fusion complex. Crystal structure data of syntaxin 1 indicates that the protein contains 4 α -helices linked via three loop regions (Figure 4.37). In order to further investigate possible similarity to the syntaxins, the secondary structure prediction of the α -SNAP binding protein was determined by using the analysis program 3D-PSSM. From the secondary structure prediction it is evident that the isolated protein contains three helical domains, similar to those found for syntaxin (Figure 4.38).

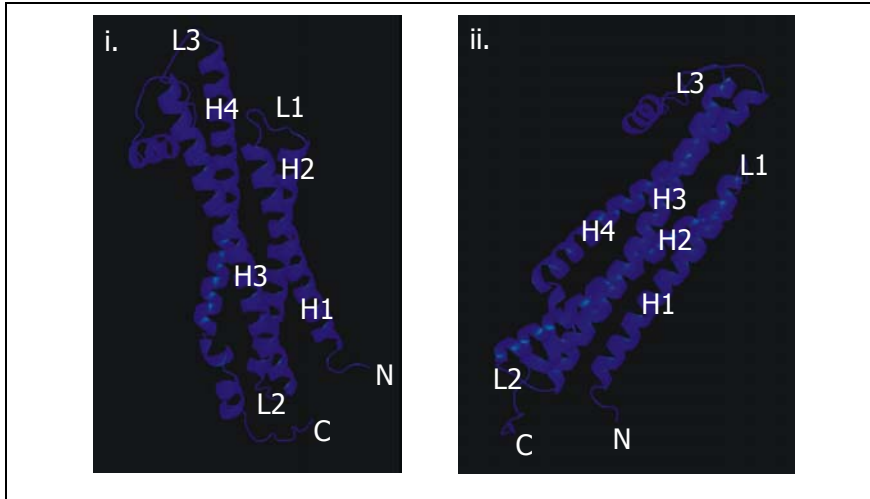


Figure 4.37. Crystal structure of syntaxin 1N. A vertical (i) and rotated (ii) view is shown with the *N*-terminal (*N*), *C*-terminal (*C*), helices 1-4 (*H1*-*H4*) and loops 1-3 (*L1*-*L3*) labelled.

	Helix 1																			
aSNAP_SS	C	C	E	E	E	E	E	E	E	E	C	C	C	C	C	C	C	C	C	C
aSNAP_Seq	G	L	T	T	M	H	T	N	I	K	D	I	K	S	D	.	L	K	T	T
Syn1A_Seq	R	D	R	F	M	D	E	F
Syn1A_SS	C	C	C	C	C	C	C	C

	Helix 2																			
aSNAP_SS
aSNAP_Seq	R	D	L	T	E	T	S	K	N	L	S	A	N	V	D
Syn1A_Seq	S	P	N	P	D	E	K	T	K	E	E	L	E	E	L	M	S	D	I	K
Syn1A_SS	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C

	Helix 3																			
aSNAP_SS	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
aSNAP_Seq	P	A	E	T	W	A	Q	S	E	L	H	V	I	K	L	C	E	Q	N	L
Syn1A_Seq	S	A	D	.	.	L	R	I	R	K	T	Q	H	S	T	L	S	R	K	F
Syn1A_SS	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C

Figure 4.38. Secondary structure prediction of the α -SNAP interacting protein. Data was obtained from 3D-PSSM Web Server V 2.6.0, (<http://www.igb.uci.edu/tools/scratch/>). aSNAP_SS indicates the predicted secondary Structure for the α -SNAP interacting protein, aSNAP_Seq indicates the α -SNAP interacting protein sequence, Syn1A_Seq indicates the sequence of human syntaxin 1A, Syn1A_SS represents the known secondary structure of the library sequence of syntaxin 1A (Assigned by STRIDE). Helical regions 1,2 and 3 are indicated (blue), as well as coils (C) and extended strands (E).

Alignment of various syntaxins and the α -SNAP interacting protein was performed using Pfam (<http://www.sanger.ac.uk/Software/Pfam/>), which is a comprehensive collection of protein domains and families, with a range of well-established uses including genome annotation. Each family in Pfam is represented by two multiple sequence alignments and two profile-Hidden Markov models (profile-HMMs) (Bateman *et al.* 2004). The results indicated that the sequences do share homology (Figure 4.39). Between the α -SNAP interacting protein (fragment) and the full length syntaxin 1A there is 14% identity and 40% similarity.

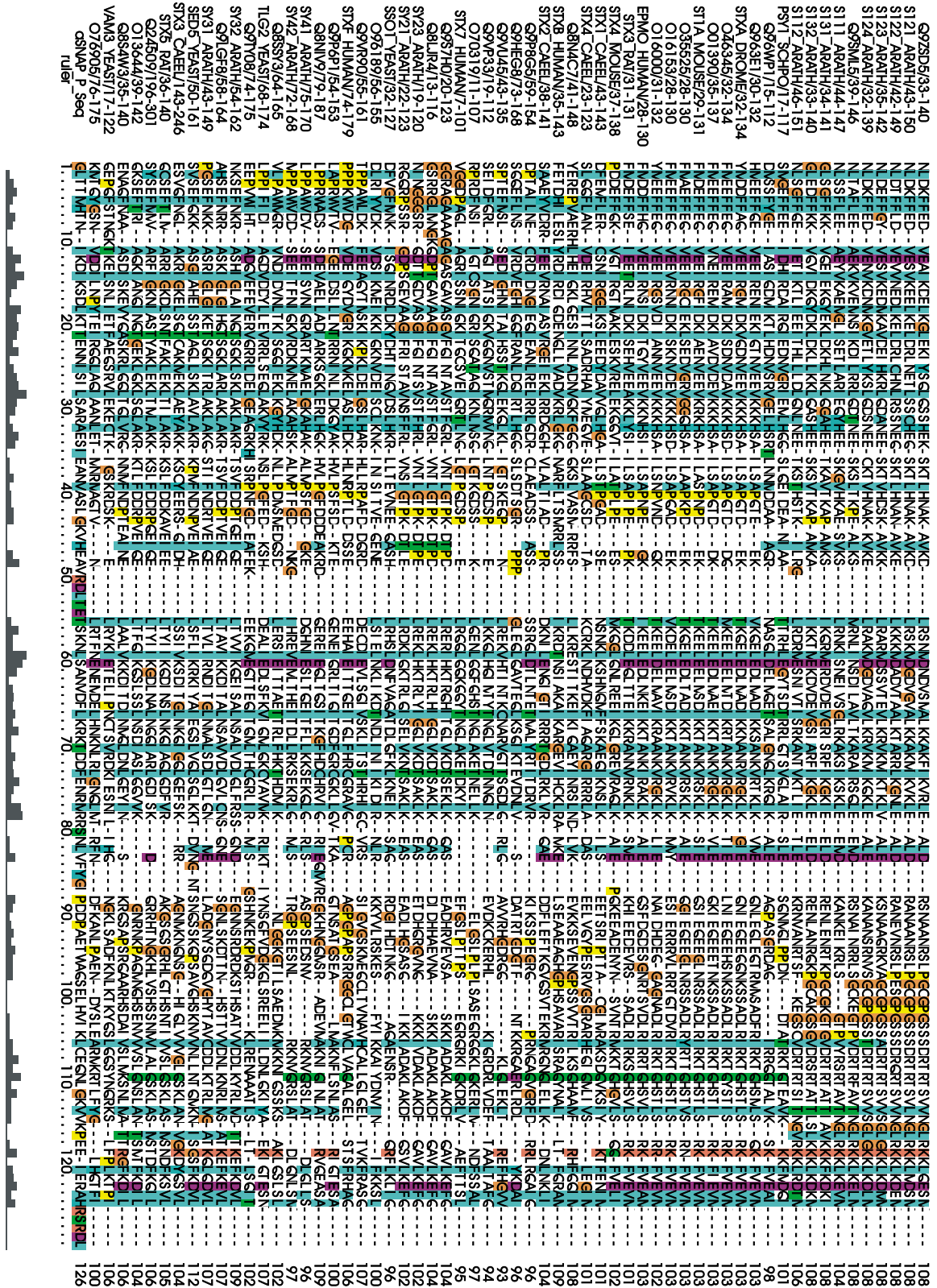


Figure 4.39. Multiple sequence alignment of syntaxins and α -SNAP interacting protein. Non-polar residues (blue), negative residues (purple), uncharged residues (green), positive residues (orange), and proline (yellow) are indicated. The identified α -SNAP interacting sequence aSNAP_P-Seq as well as a similarity scale (below sequences) is shown.

We continued with the alignments obtained from Pfam and compared the structure of the α -SNAP protein identified to the known crystal structure of syntaxin 1A using Modeller (Sali and Blundell 1993; Fiser and Sali 2000; Marti-Renom *et al.* 2000). Mr Tjaart de Beer from the Comparative Biology and Bioinformatics Unit, University of Pretoria performed all of the modeling studies. From the superimposed structures it is clear that the α -SNAP interacting protein shares structural homology to the N-terminal and three helices of syntaxin (Figure 4.40). In helix 2 we were unable to model the structure of the α -SNAP interacting protein due to lack of crystal structure data of syntaxin 1A (Indicated by an arrow, Figure 4.40.ii).

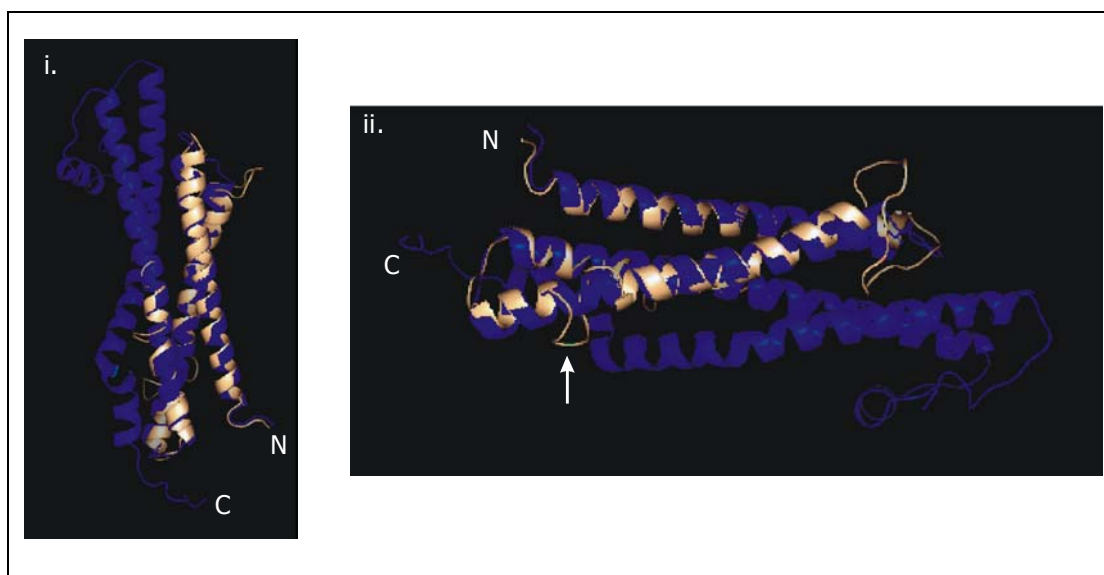


Figure 4.40. Modeled structure of the α -SNAP interacting protein. Superimposed model of the backbone structure of α -SNAP interacting protein (brown) on that of syntaxin 1A crystal structure (blue). The vertical view (i), horizontal view (ii), N-termini (N), C-terminal (C) and the single region for which no crystal structure data is available (arrow) is indicated. Structures were generated using Modeller and viewed using PyMol.

Based on the Pfam alignments, secondary structure prediction, threading and modeling results we suggest this protein to be a syntaxin. This finding is similar to that obtained from literature, where the use of α -SNAP as bait resulted in the identification of various syntaxin isoforms (see references in Table 4.2). In order to conclusively label the α -SNAP interacting protein a novel *O. savignyi* syntaxin, one will have to isolate and analyze the entire open reading frame of the protein.

4.7. CONCLUSION

The aim of this study was to investigate the possibility of exploiting protein-protein interactions across species and tissues in order to identify secretory proteins from *O. savignyi* able to interact with rat brain secretory proteins. This was attempted by using the two-hybrid system. We were able to use the two-hybrid system in detecting and isolating novel domains and proteins involved in binding known secretory proteins. Although our data is not conclusive, we hypothesize a model for the formation of a fusion complex between LDCVs and the plasma membrane in *O. savignyi* salivary glands (Figure 4.41). We hypothesize that fusion complex formation is inhibited by means of a protein similar to syntaphilin/munc/nsec bound to the N-terminal of syntaxin. The other two SNARE proteins, VAMP and SNAP25 are bound to the granule membrane (see Chapter 3) and do not interact with syntaxin prior to stimulation. Upon stimulation, the inhibitory protein is released from syntaxin and fusion complex formation is initiated. This recruits α -SNAP, which binds via syntaxin (as proposed by the two-hybrid results) to the fusion complex. Based on literature, binding of α -SNAP will result in recruiting the ATPase NSF. The possible interaction between syntaxin and the voltage-gated K^+ channel cannot be excluded based on the threading results obtained for clone 10 and 14 when truncated syntaxin was used as a bait molecule.

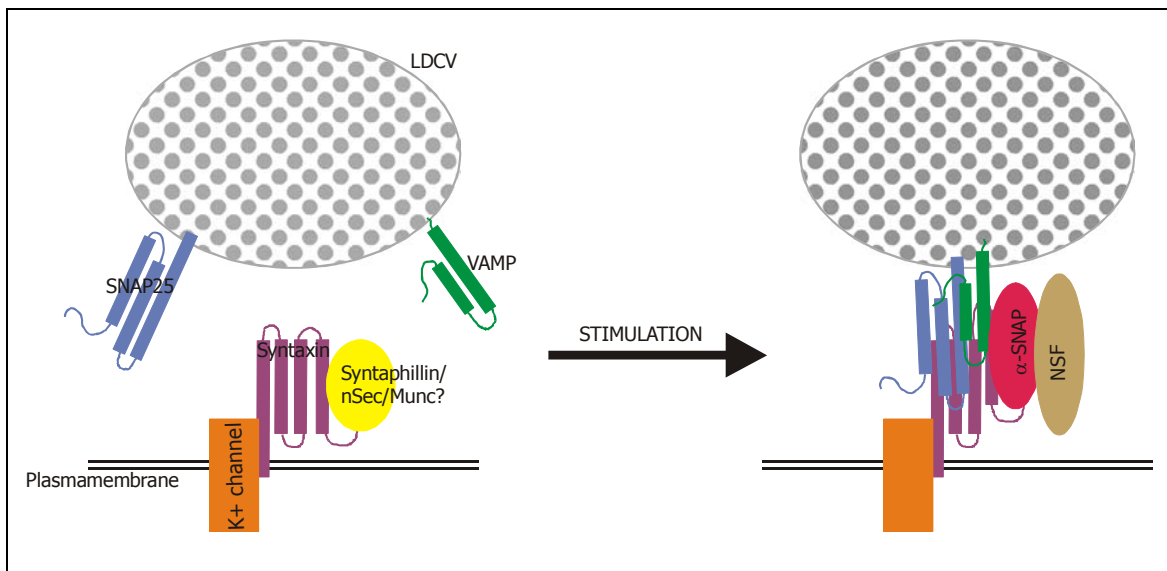


Figure 4.41. Schematic presentation of a possible model for fusion complex formation in the salivary glands of *O. savignyi*.

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