A yeast 2-hybrid screen to identify and characterize interaction partners of the cancer associated protein Retinoblastoma binding protein 6

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ABSTRACT

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Retinoblastoma binding protein 6 (RBBP6) is a 250 kDa protein that is implicated in mRNA processing and ubiquitination functions and has been shown to be highly up-regulated in a number of cancers. In humans and mice, RBBP6 interacts with both tumour suppressors p53 and pRb, suggesting that it is involved in regulation of transcription, induction of apoptosis and cell cycle control. Knock-out of an RBBP6 homologue PACT resulted in p53 dependent cell cycle arrest and apoptosis. Although the biological functions of RBBP6 remain largely unclear, it is possible that its functions are mediated through interaction with other cellular proteins. Since it is possible to unveil novel functions of a target protein through identifying its interacting protein partners, this study aims to further characterize the functions of RBBP6 through identifying novel protein interacting partners using a yeast 2-hybrid screen.

In order to identify interaction partners of RBBP6, two well characterized domains of RBBP6, the N-terminal ubiquitin-like DWNN domain and RING finger domain, were used as baits in a yeast 2-hybrid screen of a human testis cDNA library. Putative interactors were verified using *in vitro* and *in vivo*

immunoprecipitation assays. The RING finger domain was shown to interact with transcriptional factors Y-Box binding protein 1 (YB-1) and zinc finger and BTB containing protein 38 (zBTB38), resulting in their ubiquitination. In the case of YB-1 ubiquitination was correlated with a decrease in the intra-cellular levels of YB-1, suggesting that ubiquitination leads to degradation in the proteosome. The DWNN domain was shown to interact with a splicing associated small nuclear ribonucleoprotein polypeptide G (snRPG) and heat shock protein 70 (Hsp70).

The results of this work suggest that, at least in the case of YB-1 and zBTB38, RBBP6 plays a role in the regulation of gene expression by ubiquitination of transcription factors, causing them to be degraded in the proteosome. The study provides further evidence of RBBP6's involvement in mRNA splicing through its interaction with snRPG. The interaction with Hsp70 suggests a possible role in protein quality control similar to that played by other E3 ligases such as Parkin and CHIP.

Keywords: Retinoblastoma binding protein 6, RING finger, DWNN, yeast 2-hybrid, ubiquitination, mRNA splicing, protein-protein interaction, proteasome, apoptosis, co-immunoprecipitation

DECLARATION

I declare that "**A yeast 2-hybrid screen to identify and characterize interaction partners of the cancer associated protein Retinoblastoma binding protein 6"** is my own work that has not been submitted for any degree or examination in any other university and that all the sources I have used or quoted have been indicated and acknowledged by references

Moredreck Chibi June 2009

Signed:……

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LIST OF ABBREVIATIONS

aa	amino acids residues
AD	Activation domain
Ade	Adenosine
APS	Ammonium per sulphate
ATP	Adenosine triphosphate
BD	Binding domain
BLAST	Basic local alignment search tool
BLASTN	Basic local alignment search tool (nucleotide)
BLASTP	Basic local alignment search tool (protein)
BLASTX	Basic local alignment search tool (translated)
bp	Base pair
BPB	Bromophenol blue
BSA	Bovine serum albumin
cDNA	Complimentary deoxyribonucleic acid
°C	Degrees Celsius
C-terminal	Carboxyl-terminal
CHO	Chinese hamster ovary
Co-IP	Co-immunoprecipitation
CTL	Cytotoxic T lymphocyte
ddH2O	Double distilled water
DDO	Double dropout
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate

DWNN	Domain With No Name
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraaceticacid
EST	Expressed sequence tag
GFP	Green fluorescent protein
GST	Glutathione-*S*-transferase
His	Histidine
Hsp	Heat shock protein
IP	Immunoprecipitation
kDa	Kilo Dalton
kb	Kilobase pairs
LB	Luria broth
Leu	Leucine
MAb	Monoclonal Antibody
MCS	Multiple Cloning Site
MHC	Major Histocompatibility Complex
mRNA	messenger RNA
MW	Molecular Weight
NCBI	National Centre for Biotechnology Information
NMR	Nuclear Magnetic Resonance spectroscopy
N-terminal	Amino terminal
ORF	Open reading frame
PAb	Polyclonal Antibody
PACT	p53-associated cellular protein-testis derived
PBS	Phosphate Buffered Saline

PCI	Phenol chloroform isoamyl
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PMSF	Phenylmethylsulphonyl fluoride
QDO	Quadruple dropout
Rb	Retinoblastoma
RFP	Red fluorescent protein
Rluc	*Renilla* luciferase
RING	Really interesting new gene
RNA	Ribonucleic acid
SD	Synthetic dropout
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
snRPG	small nuclear ribonucleoprotein polypeptide G
TAE	Tris acetic acid and EDTA buffer
TBE	Tris, boric acid and EDTA buffer
TBS	Tris-buffered saline
TDO	Triple dropout
TEMED	N,N,N',N'-tetramethylethylethylenediamine
Trp	Triptophan
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
Tris-HCl	Tris (containing HCl)
Ub	Ubiqutin
Ura	Uracil
UPS	Ubiquitin-Proteasomal system

UV	Ultraviolet
WB	Western blot
YB-1	Y-Box binding protein 1
Y1H	Yeast 1-hybrid
Y2H	Yeast 2-hybrid
Y3H	Yeast 3-hybrid
zBTB38	zinc finger and BTB binding protein 38

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Above everything, all honour and praise be to my heavenly father for bringing me this far. Even during the darkest hours, God would always whispers through my ears and says “I am with you”.

DEDICATION

Tino, knowledge is power and enthusiasm

pulls the switch

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CHAPTER 1: LITERATURE REVIEW

1. Introduction

Retinoblastoma binding protein 6 (RBBP6) is a multi-functional protein whose function is still poorly understood. Complete characterization of its function may give important insights into the understanding of biological processes in both normal and disease states. Previous studies have suggested that RBBP6 may be involved in mRNA processing and ubiquitination related functions[1,2]. The interaction of RBBP6 with tumour suppressor proteins pRB and p53 suggest that RBBP6 plays a role in cell proliferation and tumourigenesis. This chapter begins with a description of previous experiments leading to the isolation and identification of RBBP6.

A number of studies have demonstrated that RBBP6 forms part of an interaction network that is associated with intracellular signaling pathways as well cancer genesis. Because of RBBP6's involvement in these crucial cellular processes it is possible that RBBP6, as reviewed in this chapter, may be useful as a potential target for immunotherapy.

RBBP6 contains a number of discrete conserved domains**.** Protein domains are structural and/or functional units of proteins[3] and different proteins sharing related domain architecture may play similar roles in cellular processes[4]. In the same light, as described in this chapter, RBBP6 may share similar functions with other proteins containing similar domains.

Because of the presence of RING finger domain as well as the ubiquitin-like DWNN domain, it is likely that RBBP6 is an E3 ligase whose possible biochemical role involves ubiquitination related mechanisms. This chapter reviews in detail the ubiquitination process and mechanism of protein degradation through the ubiquitin-proteasome system.

Since the function of RBBP6 appears to be mediated primarily through protein-protein interaction, this chapter also reviews a number of tools that have been developed to study protein-protein interactions. In particular this chapter reviews the yeast 2-hybrid (Y2H) system and co-immunoprecipitation methods as they were intensively applied to this study for identifying and characterizing the protein interactors of RBBP6.

1.1 The RBBP6 family of proteins

Retinoblastoma binding protein 6 is a 250-kDa multidomain protein that has been strongly implicated in various cellular activities although its mechanism remains largely unclear[5]. Analysis of the RBBP6 locus on human 16p12.2 suggests that three major transcripts of 6.1, 6.0 and 1.1 kb occur by a combination of alternative splicing and alternative poly-adenylation. These transcripts encode proteins of 1792, 1758 and 118 amino acids, which have been designated RBBP6 isoforms 1, 2 and 3 respectively (Genbank: NP_008841, Genbank: NP_061173, Genbank: NP_116015).

Previous three different studies have isolated and sequenced three partial cDNA's from the full length RBBP6 transcript. First, using purified pRB (retinoblastoma protein) as a probe, a 140 kDa truncated protein was isolated

from a small lung carcinoma H69c expression library that was named retinoblastoma binding Q protein 1 (RBQ-1)[6], corresponding to residues 150–1146 of the full length RBBP6 human protein. Two other protein designated RBQ-2 and RBQ-3 were also identified in the study. It was observed that all three proteins bind to the hypophosphorylated form of retinoblastoma protein, pRb.

Second, using purified wild type p53 protein as a probe to screen a mouse testis expression library, a cDNA encoding amino acid residues corresponding to 207–1792 of the RBBP6 protein was subsequently isolated and denoted PACT (p53 Associated Cellular protein-Testis-derived)[7]. The DNA sequence encoding the PACT was also found to contain a 437 bp 3' non-coding region with a polyA signal and tail[7].

The third study identified a proliferation potential protein-related (P2P-R)[8], which was shown to be another truncated version of the RBBP6 corresponding to residues 199–1792 that was isolated based on its recognition by two antibodies specific for heterogeneous nuclear ribonucleoproteins (hnRNPs)[8]. Further analysis indicated that P2P-R is the alternatively spliced form of RBBP6, lacking the 34 amino acid exon and also that P2P-R appears to be the dominant product expressed in multiple cell lines[9]. Using cDNA microarray analysis, a human homologue of P2P-R, called proliferation potential-related protein (PP-RP) was identified strongly expressed in esophageal cancer cells, in normal testis and placenta and weakly expressed in some normal tissues[10].

Studies that first characterized these 3 truncated versions of RBBP6 have demonstrated involvement of RBBP6 in functions mediated through interacting with other proteins. For instance, the RBQ-1 was observed to bind to hypophosphorylated but not to phosphorylated pRB, and the binding could be disrupted by an adenovirus protein E1a, suggesting physiological relevance of the interaction[11].

P2P-R fusion protein derived from a region of the P2P-R cDNA coding for hnRNP association was able to bind single-stranded DNA with P2P-R expression markedly repressed during terminal differentiation[8]. In addition, P2P-R was also shown to interact with tumour suppressor, pRb by precipitating pRb from cellular extracts using GST-P2P-R fusion protein and the interaction could be reduced by competition with the adenovirus E1a protein in the same way as RBQ-1[8]. Furthermore, P2P-R was identified as one of the proteins that contribute to genome stability[9]. P2P-R strongly localizes to chromosomes during mitosis and to nuclear speckles, which are believed to be the main sites of activity for pre-mRNA splicing and processing, during interphase[12]. Over-expression P2P-R has been shown to lead to cell cycle arrest and apoptosis[9,13-16]

Li and co-workers demonstrated that the PACT interacts with p53 via Hdm2 and the interaction was shown to play a critical role in embryonic development and tumourogenesis and knockdown of PACT gene in mice leads to early embryonic lethality before embryonic day 7.5 (E7.5), accompanied by an accumulation of p53 and widespread apoptosis[2]. In another study, PACT

was observed co-precipitating with Sm splicing factor[1] and also that it had an N-terminal serine/arginine (SR) rich domain, shared by many pre-mRNA splicing factors. These observations were suggested having a possible role for PACT in pre-mRNA splicing[1].

1.2. Association of RBBP6 in the regulation of intracellular pathways

Recent study by Wang and colleagues implicated involvement of RBBP6 in the regulation of Ras-MAPK and PI3K-AKT signaling pathways[17]. Yeast 2-hybrid screen was carried out to identify the protein interaction network associated with RBBP6 in Ras-MAPK and PI3K-AKT signaling pathways. Ras-MAPK and PI3K pathways regulate various cellular processes such as cell proliferation, survival, and differentiation [18,19] and these processes are facilitated through extensive cross-talk and co-operation that occurs between the MAPK and PI3K signal transduction pathways[19]. RBBP6 was speculated to be associated with Ras-MAPK and PI3K-AKT signaling pathways because of its direct interaction with a tumour suppressor, p53[1,2]. Activation of p53 is a downstream effect as a result of activated Ras-MAPK and PI3K-AKT signaling pathways that lead to cell proliferation and prevention of apoptosis as shown in Fig 1.1.

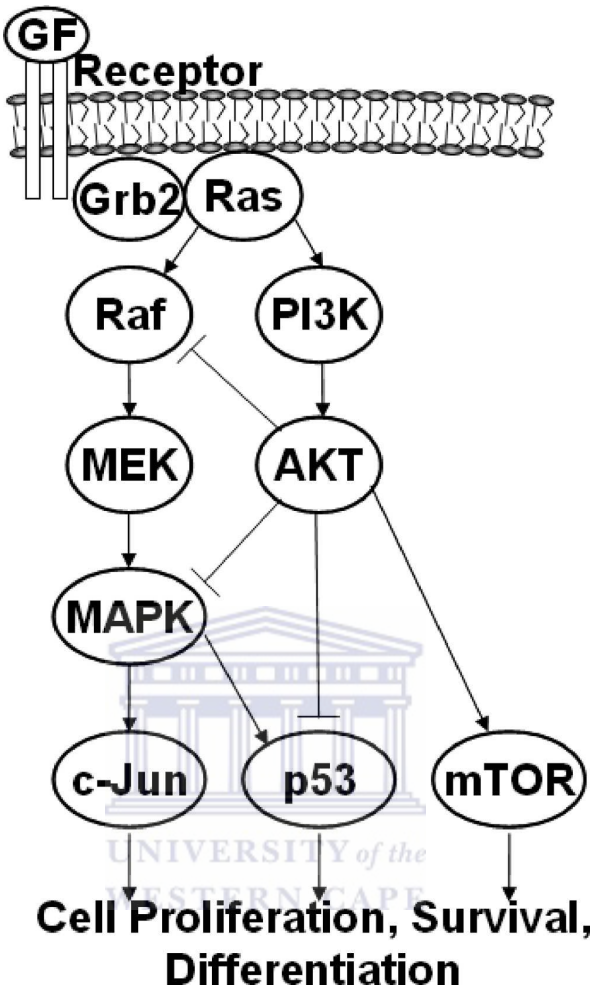


Figure 1.1. Schematic representation of Ras-MAPK/PI3K signalling pathways.

An extracellular signal such as a growth factor (GF) interacts with its receptor and induces receptor dimerization and activation. The growth-factor-receptor-bound protein 2 (Grb2) is then recruited to the receptor. The Ras-family GTPases change from an inactive state to an active state. Activated Ras binds to the Raf serine/threonine kinases and PI3K kinases. Activated Raf activates the MEK-MAPK signaling pathway, and PI3K activates the AKT-mTOR (mammalian target of rapamycin) downstream signaling cascade. Finally, transcription factors such as c-Jun and p53 are activated, which results in cell proliferation and prevention of apoptosis.

The Drosophila homologue of RBBP6, Mnm was also shown to play a role in hedgehog signaling in the developing eye[20]. Hedgehog signaling is involved in many human congenital diseases and many human cancers[21]. A catalogue of pathological conditions that involve the hedgehog pathway lists abnormalities in the central and peripheral nervous systems, the circulatory system, the gut, the kidney and many bone related abnormalities[22]. It is therefore possible that involvement of RBBP6 protein in the hedgehog pathway may be associated with a number of pathological conditions such as human tumors and developmental abnormalities[23]. Consistent with this speculation, RBBP6 has been identified associated with development of pancreatic ductal adenocarcinoma[24] an important condition in which hedgehog signaling is found deregulated[25].

1.3 Association of RBBP6 with cancer

It is becoming more apparent that RBBP6 is a cancer associated protein that can serve as a target for immunotherapy. Its association with tumour suppressor proteins pRB and p53 is evident to the involvement of RBBP6 in cancer genesis[1,2,26]. It has been reported that RBBP6 is strongly up-regulated in oesophageal cancer cells[10,27] and high levels of expression correlate with higher rates of proliferation in cultured oesophageal cancer cell and low survival rates in cancer patients. Cytotoxic T cells specific for RBBP6-derived peptides were able to lyse oesophageal cancer cells in culture and to produce regression of oesophageal tumours in mice xenograft models[10].

The expression profile of RBBP6 in the A549 cell line **(**carcinomic human alveolar basal epithelial cells) was investigated and found up-regulated, hence

giving an indication that RBBP6 may play role in lung cancer[28]. RBBP6 is widely expressed in many tumor cell lines and its expression is found to be increased in tumors like breast cancer[2]. However, the correlation of PACT overexpression and tumourogenesis remains unclear. Moreover, RBBP6 was identified as one of the protein interactions mediated by multi-SH3 domain-containing proteins associated with the formation of dynamic protein complexes that function in pancreatic cancer cell signaling[24].

1.4. RBBP6 as a therapeutic target

The critical roles of RBBP6 appear to be in cell cycle regulation, cell growth and in transcriptional and translational regulation. Moreover, the molecular mechanism of RBBP6 functions are coming to light, further illustrating its relevance as a potential target for immunotherapy. In addition, the regulatory effect that the RBBP6 family of proteins have on key tumor suppressors, p53 and pRB, suggest that they represent a noteworthy class of potential targets for anticancer therapy[7]. Inhibitors of RBBP6 protein should prevent p53 degradation and increase apoptosis in tumour cells. Small molecule inhibitors of the E3 ubiquitin ligase called nutlins have also been tried in retinoblastoma cells and found to induce p53-mediated cell death[29]. Antisense oligonucleotides have also been used to inhibit expression of the Mdm2 gene[30] and in another study small-molecule benzodiazepinedione inhibitors of the Hdm2:p53 interaction were developed for the treatment of wild type p53-expressing tumors[31]. In the same context, similar approaches could be designed to target the p53-RBBP6 interface.

Another interesting observation has been the establishment of cross-talk between RAS/MAPK and hedgehog signaling pathways in pancreatic ductal adenocarcinoma[25]. Because RBBP6 is speculated to be involved in both pathways[17,20], it is rational to suggest that targeting the RAS/MARK and hedgehog pathways through RBBP6 protein may represent a new therapeutic strategy for pancreatic ductal adenocarcinoma.

1.5. RBBP6 functions through its domain motifs

As a multidomain protein as shown schematically in Figure 1.1, RBBP6 is speculated to have its structural and functional properties influenced through conserved domains that form part of its structure[5]. Moreover, since domains are considered elementary units of molecular function, and proteins related by domain architecture may play similar roles in cellular processes[4], it is therefore possible to predict the functional role of RBBP6 through inferring to other proteins containing similar domains.

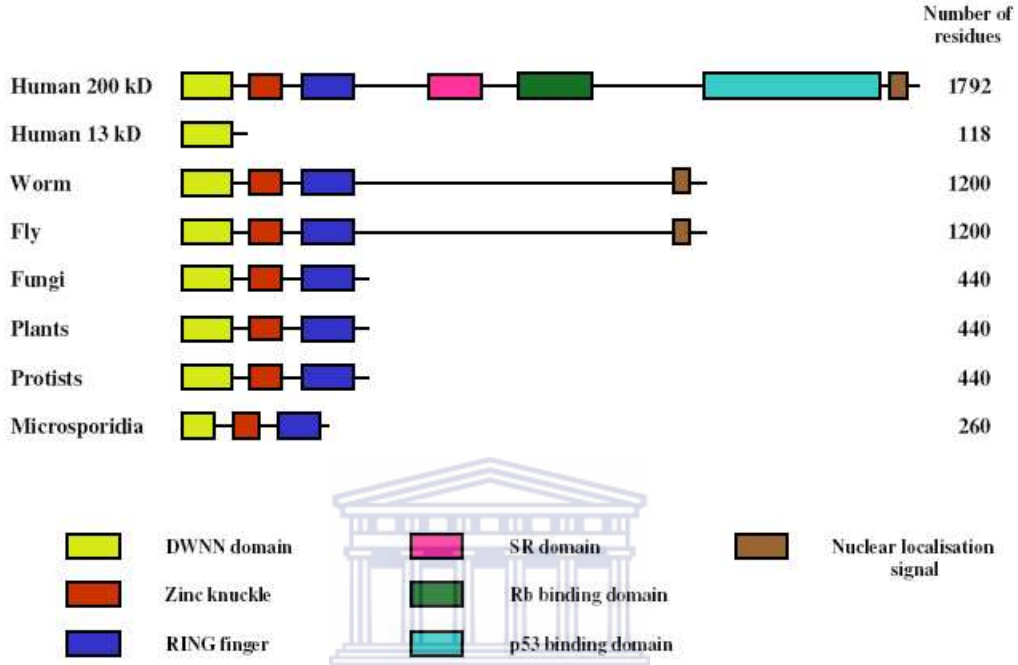


Figure 1.2. The domain structure of the RBBP6 family of proteins

RBBP6 homologues containing a DWNN domain, a zinc knuckle and a RING finger are found in all complete eukaryotic genomes analyzed to date, including the single celled parasite *E. cuniculi*, in which it is very much reduced in size. In vertebrates and insects, the protein includes a long C-terminal extension containing p53 and Rb-interaction domains in human and mouse. A short form consisting of the DWNN domain and a poorly conserved C-terminal tail is also found in vertebrates.

1.5.1. The RING finger domain

Many proteins containing RING finger domain characterized so far have been shown to be involved in a range of cellular processes, including development, oncogenesis, apoptosis, and viral replication[32]. By 1999, the function of the RING finger domain was clarified, with the observation that the RING finger domain of c-Cbl mediates a protein-protein interaction with proteins known to be involved in the protein ubiquitination and 26S proteasome degradation pathways[33]. Thereafter, a similar function was deduced for a number of RING finger containing proteins[34].

The RING finger domain of RBBP6 was implicated in playing a role in ubiquitination pathways. A recent study by Li and colleagues showed that RBBP6/PACT interacts with p53 resulting in p53 ubiquitination via an E3 ligase protein, Hdm2. After generating RING finger mutant of PACT that lacks the RING finger domain, it was observed the PACT mutant did not cause any effect on p53 ubiquitination and degradation, although in the presence of overexpressed Hdm2[2].

In unpublished data by Pugh and colleagues, it was observed that RING finger of RBBP6 adopts the same structural fold as U-box domain from CHIP (carboxyl terminus of Hsc70-interacting protein). CHIP is an E3 ligase protein that was implicated in many biological roles including cellular protein quality control through ubiquitination of misfolded protein. The CHIP protein was shown to complex with an ubiquitin-like protein, BAG-1 to facilitate chaperone dependent ubiquitination and degradation of misfolded protein[35,36]. Because of the presence of RING finger domain and an ubiquitin-like DWNN,

it is however, possible that RBBP6 may play a similar role in protein quality control, but in a context dependent manner.

1.5.2. The DWNN domain

Following the discovery of protein modification by the small, highly conserved ubiquitin polypeptide, a number of distinct ubiquitin-like proteins (Ubls) have been found to function as protein modifiers as well. As reviewed by Schwartz and Hochstrasser, these Ubls that include SUMO, Interferon-stimulated gene 15, NEDD8, Atg8, AUT7 and APG12 function as critical regulators of many cellular processes, including transcription, DNA repair, signal transduction, autophagy, and cell-cycle control[37]. A growing body of data also implicates the dysregulation of Ubl-substrate modification and mutations in the Ubl-conjugation machinery in the etiology and progression of a number of human diseases[38].

Pugh and colleagues (2006) described RBBP6 DWNN domain as an ubiquitin-like protein domain that can be found independently expressed in higher vertebrates[5]. The DWNN consists of 76 amino acid residues that constitute a phylogenetically conserved domain. Using the promoter-trap mutagenesis technique, DWNN was shown to play a role in CTL-killing and apoptosis [George, DPhil thesis, Oxford, 1995].

Because of the presence of DWNN, RBBP6 may be involved in protein modification in a similar fashion like other ubiquitin-like proteins such as SUMO and NEDD8[39-41]. Moreover, DWNN domain protein was consistently found to resolve at a higher molecular weight on SDS-page

[Seameco T, MSc thesis, UWC, 2004] and further suggesting its covalent attachment to other proteins.

1.5.3. The SR domain

Simons *et al*, 1997[7] confirmed that RBBP6 contains the SR domain by using a well documented protocol developed by Zahler *et al*, 1992[42], for precipitating out SR domain-containing proteins in 20 mM $MgCl_2$. Further evidence was gathered when RBBP6 was shown to be strongly localized to chromosomes during mitosis and to nuclear speckles, which are believed to be the main sites of activity for pre-mRNA splicing and processing, during interphase. Because of the presence of the SR domain on RBBP6, this may facilitate its splicing speculated role.

Protein splicing factors with an SR rich domain can be classified into two groups. The first group is called the 'classical' SR protein family and the criteria used to define the family members are structural similarity, dual function in constitutive and alternative splicing, the presence of a phosphoepitope recognized by mAb104 antibodies and finally their purification using magnesium chloride[42]. The second group is called the SR related proteins[43] containing an RS domain but lacking a defined RNA recognition motif (RRM). It is speculated that the RBBP6 may be a member of the latter group, since its SR domain is present at the amino terminus, and it is not recognized by MAb104 antibodies[7]. A genome-wide survey in metazoans identified a large number of RS domain-containing proteins with a role not only in splicing but also in other cellular processes such as chromatin remodeling, transcription and cell cycle progression[44]. The SR related

proteins may bind to RNA through other domains such as the PWI motif found in the splicing activator SRm160[45,46].

Previously characterized SR-containing proteins are implicated in splicing activity. It was observed that binding of SR proteins to their target pre-mRNAs and protein–protein interactions of SR proteins with both regulatory and general splicing factors are crucial interactions in determining splice sites. Therefore, it is rational to speculate that RBBP6 may be involved in pre-mRNA splicing. This speculation is further supported by the presence of the Zinc knuckle domain on RBBP6 structure (Figure 1.2). The Zinc knuckle domain[47] occurs in a number of mRNA-associated proteins, including the splicing factors SLU7, h9G8 and hSF1[48] and has been shown to be involved in protein-protein and protein-DNA interactions[49]. It is therefore possible that the Zinc knuckle domain as part of RBBP6 structure serves as an RNA binding motif during splicing.

1.5.4. The RB binding domain

The retinoblastoma gene (*RB-1*) is one of the best studied tumor suppressor genes. Its characterization and cloning were made possible by the frequent mutation of *RB-1* in the development of retinoblastoma and osteosarcoma[50]. All retinoblastomas studied to date contain mutations in both RB-1 alleles, and these mutations lead to the loss or functional inactivation of the gene product pRB protein[51]. Subsequent studies have identified *RB-1* mutations in a wide variety of other tumors, including small-cell lung carcinomas, breast carcinomas, prostate carcinomas and bladder carcinomas[52].

Based on retinoblastoma protein's prominent and ubiquitous role in cancer, many investigators have focused their efforts on determining its biochemical function by identifying interacting protein partners. Using purified pRB as a probe, Sakai *et al,* (1995)[6] isolated clones for cellular proteins that bind to the pRB protein by direct screening of cDNA expression libraries. RBBP6/RBQ-1 was identified from the library screen. Further investigations confirmed that RBQ-1 binds to hypophosphorylated pRB and the binding could be disrupted by E1A protein, raising speculation that the binding could be physiologically relevant. Understanding the consequences of the loss of one type of pRB interaction in isolation holds enormous promise for characterizing how pRB works in controlling proliferation or other functions that makes it a tumor suppressor.

Interestingly, other binding assays to study pRB interactions with E2Fs, chromatin regulators, and other binding partners have revealed an almost uniform preference for binding to the hypophosphorylated form of pRB[53]. This indicates that pRB binds to its interacting partners in G1 when it is unphosphorylated, implicating this as the active form. Phosphorylation at the beginning of S-phase then prevents pRB from interacting with other proteins until the end of mitosis when it is dephosphorylated[54].

1.5.5. The p53 binding domain

Cells have evolved various sophisticated pathways to sense and overcome DNA damage as a mechanism to preserve the integrity of the genome. Environmental attacks like radiation or toxins, as well as spontaneous DNA

lesions, trigger checkpoint activation and consequent cell cycle arrest and/or apoptosis. One key protein that coordinates DNA repair with cell cycle progression and apoptosis is the tumor suppressor protein p53, which in humans is encoded by the *TP53* gene[55-57].

Using p53 as a probe to screen an expression library, Simons *et al* (1997)[7] isolated a cDNA encoding a 250 kDa protein which was confirmed to be RBBP6/PACT protein. Recombinant forms of this protein, designated PACT, bind to wild type p53 while two mutant p53 proteins identified in human tumors abolish this interaction suggesting that binding is dependent on p53 conformation. In another experimental setup, using gel retardation analysis of p53 and a specific DNA binding oligonucleotide together with increasing amounts of PACT-GST, it was observed that PACT protein interferes with p53 specific DNA binding[7]. The characteristics of this interaction argue that RBBP6 may be involved in some aspect of p53-mediated tumor suppression.

A plethora of other proteins have been found to bind various regions of p53 in order to regulate the specificity of its activity. Cellular negative regulation of p53 is principally mediated by certain ubiquitin ligases, such as MDM2. MDM2 forms a tight negative feedback loop with p53: active p53 stimulates MDM2 gene expression, and the resulting MDM2 protein binds to p53, exports it out of the nucleus and targets it for ubiquitin-mediated degradation[58]. Other RING finger proteins, COP1 and Pirh2, were found to be negative p53 regulators by working in a similar manner as MDM2[59],[60]. In addition, c-*Jun*-NH_2-kinase (JNK), a p53 activator in stress conditions, targets p53 for ubiquitination and degradation in nonstressed cells[61].

1.6. Ubiquitination

Ubiquitination is a post-translational modification of a protein substrate through covalent attachment of an ubiquitin (Ub) moiety and this process is facilitated by a set of three enzymes, E1 (Ub-activating enzymes), E2 (Ub-conjugating enzymes) and E3 (Ub ligases). Ubiquitin is an evolutionary highly-conserved 8.5 kDa regulatory protein that is ubiquitously expressed in eukaryotes and found either free or covalently attached to other cellular proteins. The most prominent function of ubiquitin is tagging proteins for proteasomal degradation via the ubiquitin-proteasome system. Besides this function, ubiquitination also controls the stability, function, and intracellular localization of a wide variety of proteins[62].

Because ubiquitination has several possible consequences, the manner in which a covalent ubiquitin signal is interpreted must depend in some cases on additional factors, such as the subcellular localization of the substrate or the number the ubiquitins that are covalently added to the substrate[63]. Mono-ubiquitination is the attachment of a single ubiquitin to a substrate lysine residue. Multiple lysine residues may be modified within a single substrate. Mono-ubiquitination is involved in at least three distinct cellular functions: histone regulation, endocytosis, and the budding of retroviruses from the plasma membrane[64-66]. Poly-ubiquitination is the synthesis of a multi-ubiquitin chain on the substrate, where an ubiquitin attached during one round of the ubiquitination cascade becomes the substrate for the next ubiquitin transfer. There are seven surface exposed lysines in ubiquitin, each with the potential to be utilized for poly-ubiquitin chain linkage sites. The two most reported poly-ubiquitin chain linkages are formed *via* Lys48 or Lys63 of

ubiquitin. A chain of at least four ubiquitin subunits linked at Lys48 is the primary signal for proteasome mediated degradation[67-69]. Lys63-linked chains target a substrate for non-proteasomal processes such as involvement in post-replicative repair of DNA damage and in activation of NF-κB [70-73].

1.6.1. Ubiquitin-activating enzyme (E1)

In most organisms, including humans and the yeast *Saccharomyces cerevisiae*, a single E1 enzyme activates ubiquitin for the entire array of downstream conjugating enzymes[74,75]. The chemistry of the E1 reaction is well understood[76]. The reaction begins with the ordered binding of MgATP and then of ubiquitin, leading to the formation of a ubiquitin adenylate intermediate that serves as the donor of ubiquitin to a cysteine in the E1 active site. Each fully loaded E1 molecule carries two molecules of activated ubiquitin with one as a thiol ester, the other as an adenylate that serves as the donor of ubiquitin to the active cysteine in E1[76]. The thiol-linked ubiquitin is transferred to the next enzyme in the conjugating cascade, the E2.

A recent study identified PYR-41 as a first cell permeable inhibitor of the ubiquitin E1, thereby representing an important step forward in developing leads for preclinical evaluation of inhibitors of E1 in cancer and potentially other diseases. PYR-41 was also shown to increase the level of a cell cycle inhibitor and tumour suppressor p53 and inhibits NF-κB activation[74].

1.6.2. Ubiquitin-conjugating enzymes (E2)

The *S. cerevisiae* genome encodes a total of 13 E2-like proteins (Ubc1-Ubc13). Two of these, Ubc9 and Ubc12, are E2s for SUMO and Nedd8,

respectively, rather than for ubiquitin[77]. On the other hand, mammalian genomes include over 30 E2 enzymes[78]. All E2s are recognizable by their conserved catalytic domains (referred to as Ubc) which contain the active cysteine residue that accepts ubiquitin from E1 and three-dimensional structures solved for dozens of E2 Ubcs reveal a conserved architecture, which is the hallmark of E2s[79]. Some E2s have substantial amino- or carboxyl-terminal extensions and some have insertions in the Ubc[68]. These sequences may either facilitate or preclude interactions with specific E3s. It was recently shown that certain E2s show preference either for attaching the first ubiquitin to a substrate lysine or for attaching ubiquitin to itself (chain elongation), suggesting that an E2 may play a role in dictating product formation[78].

Additionally, E2s are categorized into four classes, depending on whether they consist of only a Ubc (Class I), or have additional sequences N-terminal, C-terminal, or both to the Ubc domain (Classes II, III, and IV, respectively). Although several co-crystal structures and NMR mapping studies of E2/E3 complexes confirm that Ubcs interact directly with E3s, there are examples of non-Class I E2s that use elements outside the Ubc in their E3 interaction (UbcH10[80] and UbcM2[81]. Of the approximately thirty human E2s, eleven are Class I[79]. Thus, although the functions of non-Ubc regions remain to be determined for most E2s, strategies that utilize only the Ubc may miss important interactions or features.

1.6.3. Ub ligases (E3)

Ubiquitin ligase (E3) functions are at the cross-road between ubiquitin-activation and attachment of ubiquitin to protein substrates. During this process the E3 interacts with both a substrate and a ubiquitin-conjugating enzyme (E2). Specificity for ubiquitination is believed to be mediated primarily by an E3, which interacts directly with both an E2 and a substrate to meditate the transfer of ubiquitin from E2 to a lysine residue (*via* an isopeptide bond) on the E3-bound substrate. Most E3 enzymes belong to either the RING finder domain or HECT domain families of proteins and they are classified according to how they interact with the target protein.

The RING domain-type E3s and structurally-related U-box enzymes constitute the first group[34,82,83]. The RING finger domain-type E3s bind to both the ubiquitin conjugated E2 and the protein substrate and this in turn facilitates transfer of the ubiquitin moiety from ubiquitin-conjugated E2 to the protein substrate through the nucleophilic lysine residue of the substrate[84,85]. The RING-domain E3s are by far the largest family of ubiquitin ligases, with more than 600 RING-containing proteins encoded in the human genome[86]. RING-domain E3s come in four molecular architectures: single-chain, homodimeric, heterodimeric, and multi-component. In all known cases, a RING domain interacts directly with an E2. However, in an increasing number of RING and U-box E3s, additional structural elements contribute significantly to the interaction. Elements proximal to the RING are responsible for either homo-or heterodimerization of the RING domains[87-90], which modulate their activity and substrate specificity.

Members of the HECT domain protein family are characterized by sequence similarity of their C-terminal regions to the C-terminus of E6-AP, an E3 ubiquitin-protein ligase[91]. E6-AP, as its name implies, is a human cellular protein that interacts with the human papillomavirus E6 protein. E6 binding enables E6AP to ubiquitinate the p53 tumor suppressor. Moreover, an abnormality of the E6AP E3 activity has been linked to a neurological disorder, Angelman's syndrome[92]. An essential intermediate step in E6-AP-dependent ubiquitination is the formation of a thioester complex between E6-AP and ubiquitin in the presence of distinct E2 ubiquitin-conjugating enzymes including human UbcH5, a member of the UBC4/UBC5 subfamily of E2s[93]. Similarly, several HECT domain proteins, including *Saccharomyces cerevisiae* RSP5, form ubiquitin thioester complexes, indicating that HECT domain proteins in general have E3 activity[93]. HECT domain E3s contain a cysteine residue that, similar to the E1 and E2 enzymes, forms a thiolester intermediate with the C-terminus of activated ubiquitin. In this case, ubiquitin is transferred from an E2 to an E3 and finally to a lysine side chain of a substrate protein[92].

1.6.4. The ubiquitn-proteasome system

The 26S proteasome is a conserved chambered protease complex that is present in both the cytoplasm and the nucleus[94]. As reviewed by Groll and colleagues[95] the 26S proteasome is formed by a cylinder-shaped multimeric complex referred to as the 20S proteasome (core particle, CP), capped at each end by another multimeric component called the 19S complex as regulatory particles/complexes that bind to ubiquitinated substrates, cleave off

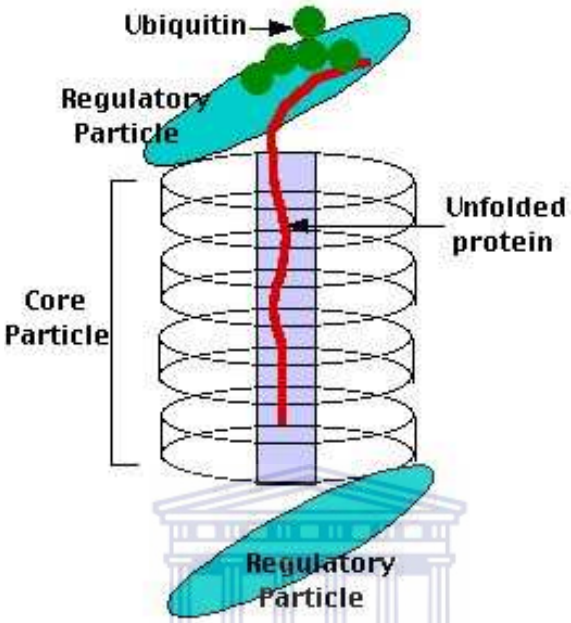


Figure 1.3. A schematic representation of the 26S proteasome

A 26S proteasome complex showing cylinder-shaped multimeric complex referred to as the 20S proteasome (core particle), capped at each end by another multimeric component called the 19S complex (regulatory particle).

ubiquitin, and then unfold and translocate the substrate into the 20S core[96] (see Figure 1.3).

The ubiquitin-proteasome system is part of the cellular proteolytic machinery that has been identified as a key regulatory mechanism in many eukaryotic cells. Accumulating evidence revealed that the ubiquitin-proteasome system is involved in the regulation of fundamental processes in mammalian stem and progenitor cells of embryonic, neural, hematopoietic and mesenchymal origin[97]. Degradation of proteins via the ubiquitin-proteasome system involves two distinct steps that occur sequentially. The first step involves the covalent attachment of multiple ubiquitin molecules to the target proteins. In this process, the ubiquitin activating protein, E1, utilizes ATP to form a high energy ubiquitin-thiol ester and then transfers the activated ubiquitin to an E2 (ubiquitin carrier protein), forming an E2-ubiquitin-thiol ester. The ubiquitin is then linked to the substrate in a reaction requiring E3, an ubiquitn-protein ligase.

The second step involves the degradation of the tagged or poly-ubiquitinated proteins to small peptides by a 2500 KDa complex, the 26S proteasome. The 20S proteasome contains the proteolytic enzymes, and the 19S complex contributes multiple functions to the 26S proteasome, including subunits able to bind poly-ubiquitin chains, isopeptidases that catalyze the release of free ubiquitin, and six essential ATPase subunits[98].

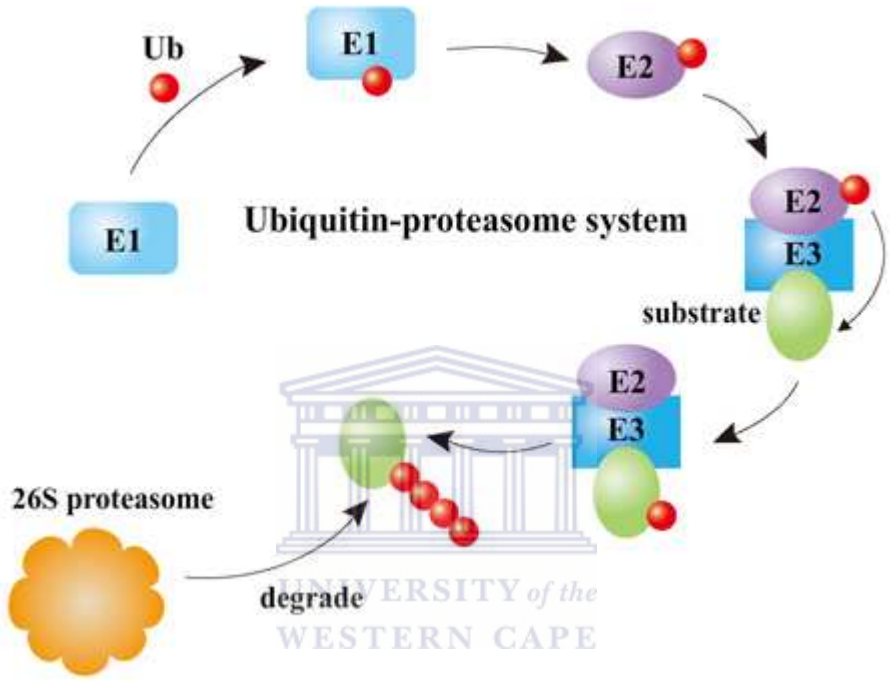


Figure 1.4. Schematic representation of ubiquitin-proteasome system

Protein substrates for ubiquitination are covalently attached to ubiquitin moiety through series of ubiquitinating enzymes, E1, E2 and E3 and subsequent degradation by 26S proteasome. E1 activates the C-terminus of ubiquitin by forming a thioester bond with the terminal carboxyl group of ubiquitin. The activated ubiquitin is transferred from E1 to the active site cysteine of an E2 enzyme, preserving the thioester linkage. E3 interact with both the substrates and E2, facilitating transfer of ubiquitin to lysine residues of substrate proteins. 26s proteasome degrades the substrate by recognizing attached ubiquitins.

1.7. Yeast 2-hybrid (Y2H) system and protein-protein interaction

Completion of the Human Genome project has been one of the most important endeavours for identifying genetic variants that are associated with complex human diseases[99-102]. However, elucidation of the genome sequence merely sets the stage for a still more challenging task, and that is the assignment of biological function to the tens of thousands of newly discovered proteins. The identification of protein-protein interactions can help to determine the biological function of novel proteins, by associating them relative to other proteins in known cellular pathways or functional classes[103].

The approximately 30,000 genes of the human genome are speculated to give rise to at least a million proteins through a series of post-translational modifications and gene splicing mechanisms[104]. Although a small population of these proteins can be expected to work in relative isolation, the majority are expected to operate in concert with other proteins in complexes and networks to orchestrate the myriad of processes that impact cellular structure and function. These processes include DNA replication, transcription, translation, splicing, cell cycle control, signal transduction and many more [105-110]. However, the detailed characterization of such protein interaction networks has established a comprehensive approach in identifying the defective pathways of tissues in pathological state and the mechanisms of pathogens. Moreover, this knowledge is applicable to designing more effective therapeutic approaches for both infectious and non-infectious diseases.

Numerous techniques have been developed to study protein-protein interactions, from biochemical approaches such as coimmunoprecipitation and affinity chromatography, to molecular genetic approaches such as the Y2H system. The Y2H system is proving itself to be a powerful tool for proteomic-based investigations. The technology has already been employed to investigate the protein-protein interactions between many of the full-length open reading frames predicted from the yeast (*Saccharomyces cerevisiae*) genome sequencing initiative[111-113]. A similar approach has also been taken for the large-scale mapping of protein-protein interactions in *Caenorhabditis elegans*[106,114] and *Helicobacter pylori*[115].

1.7.1. Yeast 2-hybrid system

The Y2H system remains a preferred large scale method because it offers a number of advantages over many of the biochemical procedures often used for the analysis of protein-protein interactions. Relative low costs and high sensitivity are among the advantages conferred by Y2H technology as an ideal method for identification of protein-protein interactions. Moreover, the basic premise of Y2H has undergone modifications, variations and extensions. These adaptations enable the application of Y2H method to a variety of diverse scientific questions that include studying of DNA-protein interactions and RNA-protein interactions.

1.7.2. The principle of Y2H system

The Y2H system is a simple robust assay for protein-protein identifying interactions that was first developed by Fields and Song in 1989[116] and was later developed for high-throughput screening during the early 1990s[117].

The basic principle behind the Y2H system relies on the yeast gene product GAL4, a transcriptional factor protein with two functional domains, DNA binding domain (BD) and activation domain (AD), that activates transcription of genes involved in galactose metabolism[118]. In a GAL4-based Y2H assay, a gene of interest, referred to as 'bait', is cloned fused in-frame with the BD into a yeast expression vector such as pGBKT7. On the other hand, cDNA library encoding unknown genes, also referred as 'prey' genes, are cloned fused to the AD into a different yeast expression plasmid e.g. pACT2. The bait and prey containing plasmid can be co-transformed into a yeast strain, for instance, AH109. When bait and library prey fusion proteins interact, the BD and AD are brought into proximity, thus activating transcription of reporter genes such as *lacZ* gene (an *E. coli* gene responsible for galactose metabolism), resulting in selection for blue yeast colonies indicating presence of interacting proteins (Fig 1.3).

1.7.3. Application of Y2H

The Y2H technology can be used to identify novel protein interactions, confirm suspected protein interactions, define domains or amino acids critical for an interaction and screen libraries for proteins that bind to a target protein[119]. The residues required for the physical interaction of a given pair of proteins can be mapped easily by generating deletion constructs of the genes of interest and assaying reporter-gene activation both qualitatively and quantitatively. The most powerful application of the system is the ability to isolate novel genes encoding proteins that associate with a known protein of interest rapidly[120].

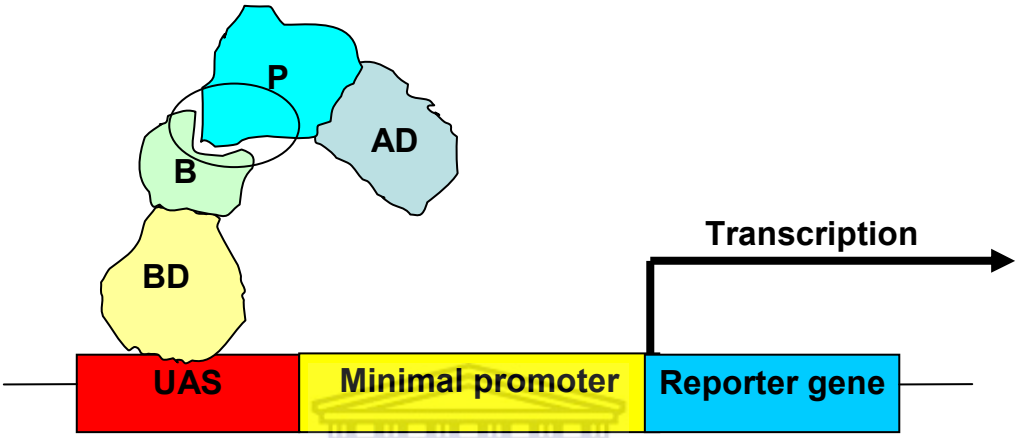


Figure 1.5. The principle of Y2H system

A schematic representation of the principle of Y2H system. The Y2H screening utilizes yeast mating, in which expression plasmids initially in two different haploid yeast strains are brought together. In the first strain, protein (B) is fused to a DNA-binding domain (BD) and will bind at an engineered site upstream of the reporter gene. In the second strain, protein (P) is fused to a transcription activation domain (AD). To conduct the assay, the two strains are mated and the reporter activity measured in the resulting diploids. If B and P interact, AD activates transcription of a reporter gene, leading to selection.

Several industrially significant applications of Y2H systems have emerged. One application is the identification of new protein targets for pharmaceutical research. Another industrial significance of the Y2H is to find compounds that modulate protein interactions. Interaction technology has already had a large impact on basic and applied biological research. In industry, it is being used to isolate and characterize new targets for drug development[121].

The Y2H system has contributed tremendously to our understanding of basic cellular processes, and to the way these processes contribute to disease. A recent high throughput Y2H screen focused on proteins involved in inherited neurodegenerative disorders[122]. The screen resulted in a map with 770 mostly novel interactions centered on 20 ataxia-related proteins. The map linked many of the poorly characterized disease proteins to each other and to proteins with known functions, providing new clues about the pathways involved in the ataxia diseases. This study also illustrates the continued value of Y2H screens that focus on specific diseases or pathways[122].

1.7.3.1. Y2H in directed molecular evolution of proteins

Directed molecular evolution of functional proteins has emerged as an alternative to traditional forms of protein engineering, such as structure-based site-directed mutagenesis[123]. Directed evolution involves multiple cycles of random gene mutagenesis and/or DNA recombination followed by screening or selection. The advantage of this technique is that knowledge of structural data, relationship between sequence, structure and mechanism is not required for a fast generation of a huge number of mutants[124].

Recently, Bichet and colleagues improved the protein-protein interactions by directed evolution by developing a new *in vivo* selection system based on the Y2H system. The system enabled screening for increased protein-protein interactions between stable and functional species including cofactor-containing proteins (FAD, [2Fe–2S], heme). The method was successfully applied for the directed evolution of Adx and selected variants[124].

1.7.4. Variants of the yeast 2-hybrid system

Other important modifications of the two-hybrid system have been developed to address a growing list of molecular interactions and these include yeast 1-hybrid (Y1H)[125] and yeast 3-hybrid (Y3H)[126,127] systems.

1.7.4.1. The yeast 1-hybrid system

The yeast 1-hybrid variation is designed to investigate protein-DNA interactions. Unlike in yeast 2-hybrid system, the binding domain plasmid is eliminated and a hybrid expression library is constructed by fusion of a transcriptional AD to a cDNA library such that expression of a reporter gene is induced when the hybrid protein recognizes the binding site. The library is screened against the desired target sequence which is inserted in the promoter region of the reporter gene construct[128]. Several novel protein-promoter interactions were identified using the yeast 1-hybrid system[129]. For example, Y1H assay was applied to identify multifunctional zinc-finger transcription factor, YY1 as a binding factor for a proacrosin promoter element and drives transcription of proacrosin[130]. Proacrosin gene is a gene that is specifically expressed in the testis and encodes an acrosomal enzyme, an enzyme is associated with sterility[131].

1.7.4.2 The yeast 3-hybrid system

Because of the limitation of the currently used form of Y2H as it is restricted to bipartite interactions, another Y2H variant was developed that enables identification of proteins that interacts as a complex or through shared binding to RNA[126,127]. For example, signaling pathways often require a third molecule to mediate association or interactions of proteins. The principle of Y3H strategy as shown in Fig 1.4 allows the study of such ternary complexes. The BD domain fused to the 'bait' protein and AD fused to the 'prey' protein cannot activate reporter gene expression and require a third molecule, either RNA or a protein, to bring them into close proximity. For the effective interaction to be observed, the molecule that links the 'bait' and 'prey' proteins should contain shared binding site to the proteins.

The Y3H system can be applied to identifying cDNAs encoding receptors of a ligand of interest and to screen for new ligands that bind to a specific receptor. In one example, Zhang and Lautar used Y3H to verify that, after epidermal growth factor (EGF) stimulation, EGF receptor, and C-terminal region of Sos, a guanine-nucleotide exchange factor for Ras proteins need an adaptor protein, Grb2, for interaction[127]. The Y3H was also successfully used for screening and identification of glucocorticoid receptor interacting proteins[132].

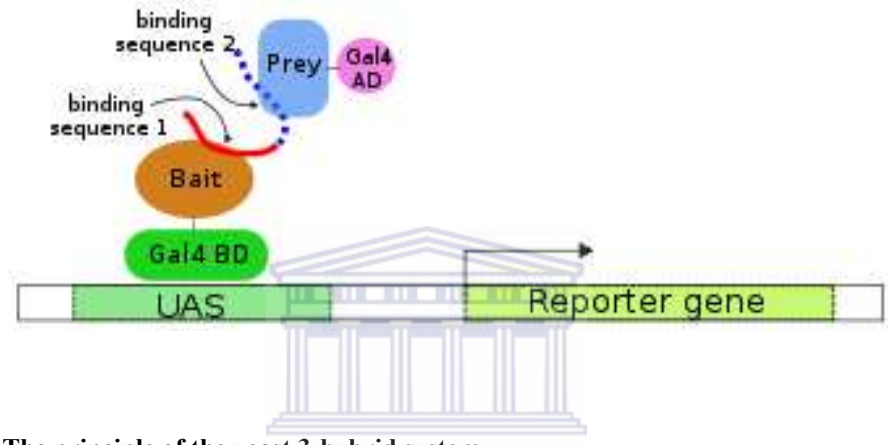


Figure 1.6. The principle of the yeast 3-hybrid system

Binding domain is fused to the bait protein and the AD fused to the prey protein. Both bait and prey protein interacts through shared binding to the RNA as shown thereby bringing into proximity the BD and the AD to drive transcription of the reporter gene.

1.7.4.3. Reverse yeast 2-hybrid

Reverse Y2H is another modification of Y2H that uses counter selection by employing a reporter gene, whose product is either toxic or cytostatic to growing cells such that only the cells that do not express it can grow[133]. Reverse Y2H system enables genetic selection against a specific protein/protein interaction[134]. Once a positive interaction has been identified, mutation of these interacting proteins may result in decreased ability to interact. Therefore, these mutants that result in abolishment of interaction can be identified using the reverse Y2H as a way of following up of the biological significance of the interaction and in defining the residues involved in the interaction. Furthermore, the reverse Y2H, in conjunction with suitable yeast expression libraries, might facilitate the identification of genes which encode proteins that interfere with a particular protein/protein interaction[134]. Several studies have used reverse Y2H in identifying protein mutants resulted in loss of interaction with respective partner. In one study a reverse Y2H was used to identify loss-of-interaction mutations of the catalytic subunit of the *Escherichia coli* heat-labile toxin (LTA1) that showed decreased binding to the active (GTP-bound) form of human ARF3, its protein cofactor[135].

1.8. Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) is a one of the biochemical techniques used to verify protein-protein interactions identified from Y2H library screen. The technique was first developed in 1974 to resolve the immunoprecipitated proteins on slab gels[136] and has since then become one of the most important techniques to study protein-protein interactions.

In a typical Co-IP experiment, specific IP antibody is directed towards a target protein known as 'bait' from a sample containing other proteins, *e.g.*, a cell lysate. The antibody is then immobilized using either protein A or protein G covalently attached to sepharose beads[137]. After washing of the beads, the antibody, the bait and proteins associated to the bait are eluted by boiling and analyzed using gel electrophoresis, mass spectrometry, western blotting, and other methods for identifying constituents in the complex[138].

1.8.1. Different ways of preparing for Co-IP experiment

There are generally 3 different ways of performing Co-IPs that are classified as traditional (classical) method, oriented affinity method and direct affinity method. The traditional method entails incubating the IP antibody with the sample and sequentially binding it to Protein A or G agarose beads to facilitate target antigen recovery. However, this approach results in the target protein becoming non-covalently bound with the IP antibody, and this can interfere with downstream analyses. The orientated affinity method uses Protein A or G beads to serve as an anchor to which the IP antibody is crosslinked, thereby preventing the antibody from co-eluting with the target protein. Similarly, the direct affinity method also immobilizes the IP antibody, except that in this case, it is directly attached to a chemically activated support. Both methods prevent co-elution of the IP antibody, enabling reuse of the immunomatrix[139].

1.8.2. Different ways of performing a Co-IP experiment

Several approaches to co-immunoprecipitation experiments have been adopted and these include: first, co-immunoprecipitate from cell-lines or

tissues expressing endogenous proteins. This approach studies the interaction of endogenous protein complexes as specific antibody for the endogenous bait protein is used for the pull down[140]. The advantage of this approach is that endogenous protein complexes are studied. Therefore, any artificial effects of affinity tags or overexpression are avoided. The disadvantage is that highly specific antibodies are required.

Second, co-immunoprecipitation from cells transfected with a plasmid encoding a tagged bait protein. An antibody directed against the tag (instead of against the bait protein) can then be used in the co-IP experiments. An advantage of this approach is that one can be relatively confident that the antibody directed against the tag is specific and does not cross react with other proteins. Furthermore, epitope-tagged proteins can often be eluted by incubation with competing peptides, or other small molecules, instead of boiling. Such specific elution often reduces the amount of contaminating proteins in the eluate.

Third, performing Co-IP experiments using cells transfected with tagged versions of two putative interaction partners such as c-Myc tagged and HA tagged protein. The obvious advantage of this method is that the co-immunoprecipitated protein can be easily detected since both proteins are over expressed through transfections[141].

1.8.3. Other applications of co-immunoprecipitation

Besides being a golden standard for verifying putative protein-protein interactions, Co-IP can be applied for different purposes that include

determination of the molecular weight and isoelectric point of immunoprecipitated proteins by one-dimensional or two-dimensional SDS-PAGE, and verification that an antigen of interest is synthesized by a specific tissue. This is achieved by directing specific antibody against the antigen then followed by immunoprecipitation[142]. Immunoprecipitation can also be applied in the determination of whether a protein contains carbohydrate residues by evaluating whether immunoprecipitated antigen from cells cultured with radioactive monosaccharides is radiolabelled. Suriano and colleagues applied immunoprecipitation techniques to characterize differences in glycosylation patterns of heat shock protein, gp96[143]. Moreover, the technique can also be applied to quantify the rate of synthesis of proteins in culture by determining the quantity of immunoprecipitated, radiolabelled protein.

1.9. Rationale of the study

A number of functions imputed on RBBP6 protein appeared to be primarily mediated through interaction with other proteins via its domains. Observation of RBBP6 interaction with tumor suppressor proteins p53 and pRB[6,7] suggested that the protein plays a significant role in a number of cellular mechanisms that are linked to cell cycle regulation. Because of the presence of several domains as part of the RBBP6 structure, it is possible that the biochemical functions of RBBP6 are not only limited to its interaction with these tumour suppressor proteins but through other cellular proteins that are not yet unveiled. Therefore, identification of proteins that interact with RBBP6 may provide information about other novel functions of RBBP6.

1.10. The aims and objectives

The study aims to identify protein interaction partners of RBBP6. Two well characterized domains of RBBP6, the N-terminal ubiquitin-like DWNN domain and RING finger domain, shall be used as baits in a Y2H screen of a human testis cDNA library. Putative interactors would be further verified using *in vitro and in vivo* immunoprecipitation assays as well as co-localisation experiments. Moreover, depending on the feasibility of the experiments, assays to test the functional relevance of the interactions would be carried out.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials and Suppliers

2.2 Commercial kits used

2.3 General stock solutions, buffers and media

2.3.1. Electrophoresis solutions

2.3.2. Buffer and media for bacterial cultures

2.3.4. Yeast Media and Buffers

2.3.5. Co-IP buffer

2.3.6. Mammalian cell lysis RIPA buffer

2.4. Plasmids

2.4.1. pGBKT7 (Clontech, USA)

2.4.2. pACT2 (Clontech, USA)

2.4.3. pCMV-HA (Clontech, USA)

2.4.4. pCMV-Myc (Clontech, USA)

2.4.5. pEGFP-C1 (Clontech, USA)

2.4.6. pDsRed1-C1 (Clontech, USA)

2.4.7. pALUC

2.4.8. pRL-SV40 (Promega)

2.5. Bacterial culture

2.5.1. Strain phenotype

2.5.2. Preparation of bacterial competent cells (*E. coli* strain DH5α)

2.5.3. Bacterial transformation with plasmid DNA

2.5.4. Extraction of plasmid DNA

2.6. High fidelity PCR

2.7. Agarose gel electrophoresis of DNA

2.8. Purification of PCR products

2.9. Restriction enzyme digestion for cloning purposes

2.10. Ligation reactions

2.11. Colony PCR

2.12. Yeast 2-hybrid methods

2.12.1. Yeast strains used

2.12.2. Yeast transformation with plasmid DNA

2.12.3. Construction of Yeast cDNA library

2.12.4. Yeast library mating procedure

2.12.5. PCR of bait and prey inserts for *in vitro* transcription/translation

2.12.6. *In vitro* synthesis of bait and prey proteins

2.12.7. Co-immunoprecipitation of translated bait and prey proteins

2.13. Mammalian cell culture

2.13.1. Transfection of HEK293 cells

2.13.2. Cell lysis and preparation

2.14. *In vivo* co-immunoprecipitation

2.15. SDS PAGE

2.16. Western blotting

2.17. *In vitro* ubiquitination assay

2.18. Luciferase Assays

2.19. Flourescence microscopy

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials and Suppliers

40 % 37.5:1 acrylamide:bis-acrylamide	Promega
Agarose	Promega
Ampicillin	Roche
APS (Ammonium persulphate)	Merck
Bacteriological Agar	Merck
Bacto tryptone	Fluka analytical
Boric Acid	Merck
BSA (Bovine serum albumin)	Roche
Bromophenol blue	Roche
Calcium chloride	BDH AnalaR
Cell culture media and reagents	Gibco Life Technologies
Coomasie Brilliant Blue R 250	Sigma
DEPC (Diethyl Pyrocarbonate)	Sigma
DMSO (Dimethyl sulphoxide)	Sigma
DTT (Dithiothreitol)	Roche
EDTA (Ethylene diamine tetra-acetic acid)	Merck
Ethanol	BDH
Ethidium bromide	Sigma
Glucose	Saarchem UniVAR
Glycerol	Merck
Glycine	Saarchem UniVAR

Herring sperm DNA	Promega
Hoechst H-33342	Sigma
Hydrochloric acid	Merck
Kanamycin monophosphate	Roche
Lithium Acetate	Sigma
Lipofectamine 2000 transfection reagent	Invitrogen
Magnesium Chloride	Merck
Metafectene^{TM} transfection reagent	Biontex
MG132 (proteasome inhibitor)	Sigma
PBS (Phosphate-buffered saline)	Life Technologies
PEG 8000	Sigma
PMSF (Phenylmethylsulphonyl fluoride)	Sigma
PVDF (Polyvinylidene difluoride) membrane	Roche diagnostics
Restriction enzymes	Fermentas
RNase A	Roche
SDS (Sodium dodecyl sulphate)	Roche
SD (Synthetic Dropout) suppliments	Clontech
Sodium Chloride	Merck
TEMED (N, N, N', N'-tetra methlethylene-diamine)	Promega
T4 ligase	Inqaba Biotechnologies
Tris (hydroxymethyl) aminomethane	BDH
Triton X-100	Merck
Tryptone	Merck
Tween 20	Merck
Yeast extract	Merck

Yeast nitrogen base	Clontech

2.2 Commercial kits used

GeneElute™ PCR Clean-up kit	Fermentas
GeneJET™ Plasmid Miniprep Kit	Fermentas
TNT® Quick Coupled system kit	Promega
ECL plus™ Western Blotting Detection System	Amersham Pharmacia
S-100 *HeLa* Conjugation Kit	Boston Biochem
Dual-Luciferase Reporter Assay System	Promega

List of commercial antibodies used

Rabbit Anti-HA MAb antibody (sc-805)	Santa Cruz
Mouse Anti-cMyc MAb antibody (sc-40)	Santa Cruz
Mouse Anti-β-actin MAb (4967)	Cell signaling
Goat Anti-YB-1 PAb antibody (sc-18057)	Santa Cruz
Mouse Anti-GFP MAb antibody **(**sc-9996)	Santa Cruz
Mouse Anti-RFP (DsRed) MAb antibody (632392)	Clontech
Rat Anti-Hsp70 MAb antibody (SPA-815F)	Stressgen
Donkey anti-goat IgG MAb antibody (sc-2020)	Santa Cruz
Goat anti-mouse IgG MAb antibody (sc-2005)	Santa Cruz
Goat anti-rat IgG MAb antibody (sc-2006)	Santa Cruz

2.3 General stock solutions, buffers and media

All the general solutions listed below were prepared in double distilled water and stored at room temperature unless otherwise stated.

2.3.1. Electrophoresis solutions

10 % Ammonium persulphate

Ammonium persulphate	10 % (w/v)
Make up in double distilled water	100 ml

Mix well and store at 4 °C

10x TBE stock solution

Tris base	108 g
Boric Acid	58 g
EDTA	9.3 g
Make up in double distilled water	1000 ml

Ethidium bromide

Ethidium bromide	1 % (w/v)
Make up in double distilled water	50 ml

Stir well on magnetic stirrer, and store in a dark container at room temperature

Bromophenol blue loading dye

Gycerol	30 % (v/v)
EDTA (pH 8.0)	15 mM
Bromophenol blue	0.5 % (w/v)

10x SDS-PAGE running buffer

Tris base	25 mM
Glycine	192 mM
SDS	0.1 % (w/v)
Make up in double distilled water	1000 ml

SDS-PAGE separating buffer

Tris-HCl, pH 8.8	1.5 M
SDS	0.1 %
Make up in double distilled water	500 ml

SDS PAGE stacking buffer

Tris-HCl, pH 6.8	0.5 M
SDS	0.1 % (w/v)
Make up in double distilled water	500 ml

Transfer Buffer

Tris-HCl, pH 8.8	25 mM
Glycine	192 mM
Methanol	20 % (v/v)
Make up in double distilled water	1000 ml

TBST

NaCl	150 mM
Tris-HCl, pH 8.0	20 mM
Tween-20	0.1 %
Make up in double distilled water	1000 ml

Western blotting blocking solution

BSA (Bovine serum albumin Fraction V)	5 % (w/v)
Make up in TBST	100 ml

2x Sample buffer

Glycerol	25 % (v/v)
Bromophenol blue	0.01 %
DTT	50 mM
Make up in 100mM Tris buffer, pH 6.8	50 ml

Storage -20 °C

1x PBS, pH7.4

NaCl	8.0 g
KCl	0.2 g
$Na_2HPO_4.2H_2O$	1.42 g
KH_2PO_4	0.2 g
Make up in double distilled water	1000 ml

2.3.2. Buffer and media for bacterial cultures

CAP buffer (pH 7.0)

$CaCl_2$	2.21 g
Glycerol	37.5 ml
PIPES	0.76 g
Make up in double distilled water	250 ml

Storage 4 °C

LB liquid media

Bacto tryptone	1 % (w/v)
Yeast extract	0.5 % (w/v)
NaCl	1 % (w/v)
Make up in double distilled water	500 ml

Autoclave at 121 °C for 15 minutes

LB agar

Bacto tryptone	1 % (w/v)
Yeast extract	0.5 (w/v)
NaCl	1 % (w/v)
Bacteriological agar	1.5 % (w/v)
Make up in double distilled water	500 ml

Autoclave at 121 °C for 15 minutes and add appropriate antibiotic to media when the temperature is approximately 55 °C and pour out onto Petri dishes

2.3.4. Yeast Media and Buffers

YPDA media

Difco peptone	2 % (w/v)
Yeast extract	2 % (w/v)
Glucose	2 % (w/v)
L-adenine hemisulphate (0.2 % w/v stock solution)	7.5 ml
Make up in double distilled water	500 ml

Autoclave at 121 °C for 15 minutes

YPDA agar

Difco peptone	2 % (w/v)
Yeast extract	2 % (w/v)
Glucose	2 % (w/v)
Bacteriological agar	2 % (w/v)
L-adenine hemisulphate (0.2 % w/v stock solution)	7.5 ml
Make up in double distilled water	500 ml

Autoclave at 121 °C for 15 minutes, allow to cool down to approximately 55 °C and pour out onto Petri dishes

SD/-W media

Glucose	2 % (w/v)
Yeast nitrogen base	0.67 % (w/v)
SD/-W amino acid supplement	0.067 % (w/v)
NaOH (4M stock solution)	160 µl
Make up in double distilled water	600 ml

Autoclave at 121 °C for 15 minutes

SD/-W agar media

Glucose	2 % (w/v)
Yeast nitrogen base	0.67 % (w/v)
SD/-W amino acid supplement	0.067 (w/v)
Bacteriological agar	2 % (w/v)
NaOH (4M stock solution)	160 µl
Make up in double distilled water	600 ml

Autoclave at 121 °C for 15 minutes, allow to cool down to approximately 55 °C and pour out onto Petri dishes

SD/-L liquid media

Glucose	2 % (w/v)
Yeast nitrogen base	0.67 % (w/v)
SD/-L amino acid supplement	0.067 % (w/v)
NaOH (4M stock solution)	160 µl
Make up in double distilled water	600 ml

Autoclave at 121 °C for 15 minutes

SD/-L agar media

Glucose	2 % (w/v)
Yeast nitrogen base	0.67 % (w/v)
SD/-L amino acid supplement	0.067 % (w/v)
Bacteriological agar	2 % (w/v)
NaOH (4M stock solution)	160 µl
Make up in double distilled water	600 ml

Autoclave at 121 °C for 15 minutes, allow to cool down to approximately 55 °C and pour out onto Petri dishes

SD/-H agar media

Glucose	2 % (w/v)
Yeast nitrogen base	0.67 % (w/v)
SD/-H amino acid supplement	0.067 % (w/v)
Bacteriological agar	2 % (w/v)
NaOH (4M stock solution)	160 µl
Make up in double distilled water	600 ml

Autoclave at 121 °C for 15 minutes, allow to cool down to approximately 55 °C and pour out onto Petri dishes

SD/-Ade agar media

Glucose	2 % (w/v)
Yeast nitrogen base	0.67 % (w/v)
SD/-Ade amino acid supplement	0.067 % (w/v)
Bacteriological agar	2 % (w/v)
NaOH (4M stock solution)	160 µl
Make up in double distilled water	600 ml

Autoclave at 121 °C for 15 minutes, allow to cool down to approximately 55 °C and pour out onto Petri dishes

SD/-L-W (DDO) liquid media

Glucose	2 % (w/v)
Yeast nitrogen base	0.67 (w/v)
SD/-L-W amino acid supplement	0.067 (w/v)
NaOH (4M stock solution)	160 µl
Make up in double distilled water	600 ml

Autoclave at 121 °C for 15 minutes

SD/-L-W (DDO) agar media

Glucose	2 % (w/v)
Yeast nitrogen base	0.67 % (w/v)
SD/-L-W amino acid supplement	0.067 % (w/v)
Bacteriological agar	2 % (w/v)
NaOH (4M stock solution)	160 µl
Make up in double distilled water	600 ml

Autoclave at 121 °C for 15 minutes, allow to cool down to approximately 55 °C and pour out onto Petri dishes

SD/-L-W-H (TDO) liquid media

Glucose	2 % (w/v)
Yeast nitrogen base	0.67 % (w/v)
SD/-L-W-H amino acid supplement	0.067 % (w/v)
NaOH (4M stock solution)	160 µl
Make up in double distilled water	600 ml

Autoclave at 121 °C for 15 minutes

SD/-L-W-H (TDO) agar media

Glucose	2 % (w/v)
Yeast nitrogen base	0.67 % (w/v)
SD/-L-W-H amino acid supplement	0.067 % (w/v)
Bacteriological agar	2 % (w/v)
NaOH (4M stock solution)	160 µl
Make up in double distilled water	600 ml

Autoclave at 121 °C for 15 minutes, allow to cool down to approximately 55 °C and pour out onto Petri dishes

SD/-L-W-H-Ade (QDO) liquid media

Glucose	2 % (w/v)
Yeast nitrogen base	0.67 % (w/v)
SD/-L-W-H-Ade amino acid supplement	0.067 % (w/v)
NaOH (4M stock solution)	160 µl
Make up in double distilled water	600 ml

Autoclave at 121 °C for 15 minutes

SD/-L-W-H-Ade (QDO) agar media

Glucose	2 % (w/v)
Yeast nitrogen base	0.67 % (w/v)
SD/-L-W-H-Ade amino acid supplement	0.067 % (w/v)
Bacteriological agar	2 % (w/v)
NaOH (4M stock solution)	160 µl
Make up in double distilled water	600 ml

Autoclave at 121 °C for 15 minutes, allow to cool down to approximately 55 °C and pour out onto Petri dishes

Yeast lysis buffer

SDS	1 %
Triton X-100	2 %
NaCl	100 mM
Tris-HCl (pH 8)	10 mM
EDTA	I mM

2.3.5. Co-IP buffer

Aprotinin (2mg/ml stock solution)	12.5 µl
DTT (100mM stock solution)	50 µl
PMSF (50mM stock solution)	50 µl
Tween-20	5 µl
Make up in 1x PBS	5 ml

2.3.6. Mammalian cell lysis RIPA buffer

NP-40	1 % (w/v)
Na-deoxycholate	0.25 % (w/v)
NaCl	150 mM
EDTA	1 mM
PMSF	1 mM
Aprotinin, leupeptin, pepstatin	1 µg/ ml each
Na_3VO_4	1 mM
NaF	1 mM
Make up in 50mM Tris-HCl, pH 7.4	100 ml

2.4. Plasmids

A variety of plasmids for use in bacterial, yeast and mammalian cells were used in the study.

2.4.1. pGBKT7 (Clontech, USA)

pGBKT7 is a yeast expression vector that expresses proteins fused to the GAL4 DNA binding domain (BD). Fusion proteins are expressed at high levels from the constitutive *ADH1* promoter (P_{ADH1}) and transcription is terminated by the T7 and *ADH1* transcription termination signals ($T_{T7\ \&\ ADH1}$). pGBKT7 also contains a T7 promoter for transcription of proteins *in vitro* and a c-Myc epitope tag upstream of the MCS. pGBKT7 is a shuttle vector that replicates autonomously in both *E. coli* and *S. cerevisiae* and carries the *Kan*^{r} gene, which confers kanamycin resistance in *E. coli,* and the *TRP1* nutritional gene marker that allows yeast auxotrophs to grow on synthetic drop-out media lacking tryptophan (Figure 2.1).

2.4.2. pACT2 (Clontech, USA)

pACT2 a yeast expression vector that expresses proteins fused to the GAL4 activation domain (AD), an HA epitope tag upstream of the MCS. The hybrid protein is expressed at high levels in yeast host cells from the constitutive ADH1 promoter (P_{ADH1}); transcription is terminated at the ADH1 transcription termination signal (T_{ADH1}). pACT2 is a shuttle vector that replicates autonomously in both *E. coli* and *S. cerevisiae* and carries the *Amp*^{r} gene, which confers ampicillin resistance in *E. coli.* pACT2 also contains the *LEU2* nutritional gene that allows yeast auxotrophs to grow on synthetic drop-out media lacking leucine (Figure 2.2).

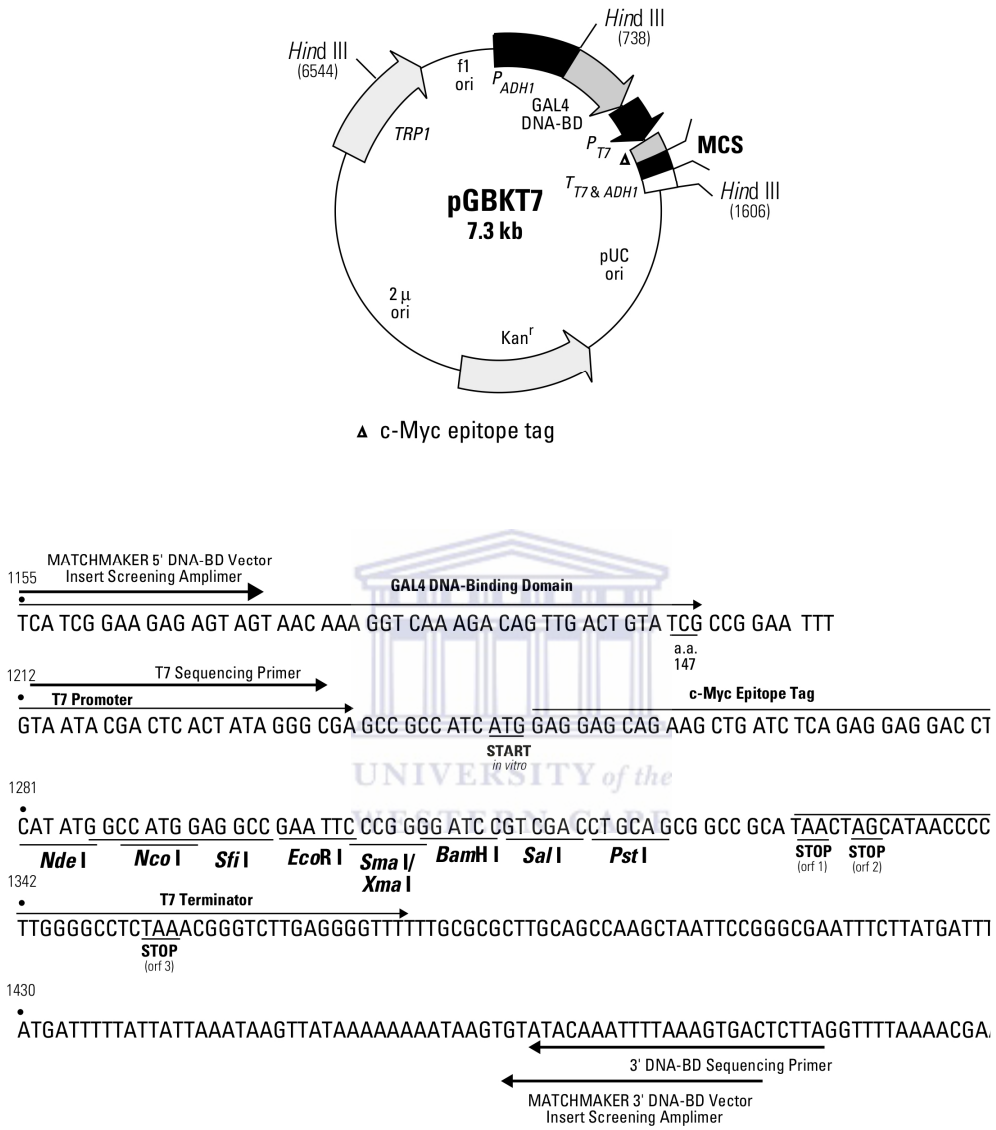


Figure 2.1. Restriction map and multiple cloning site (MCS) of pGBKT7

Protein encoding sequences cloned in frame into the MCS of pGBKT7 are expressed as fusions to the GAL4 DNA-BD and a cMyc eppitope tag. The vector contains a kanamycin resistance gene and origins for replication in both yeast and bacteria. The T7 promoter is used for *in vitro* transcription and translation of the epitope tagged fusion protein (not including the GAL4 DNA-BD).

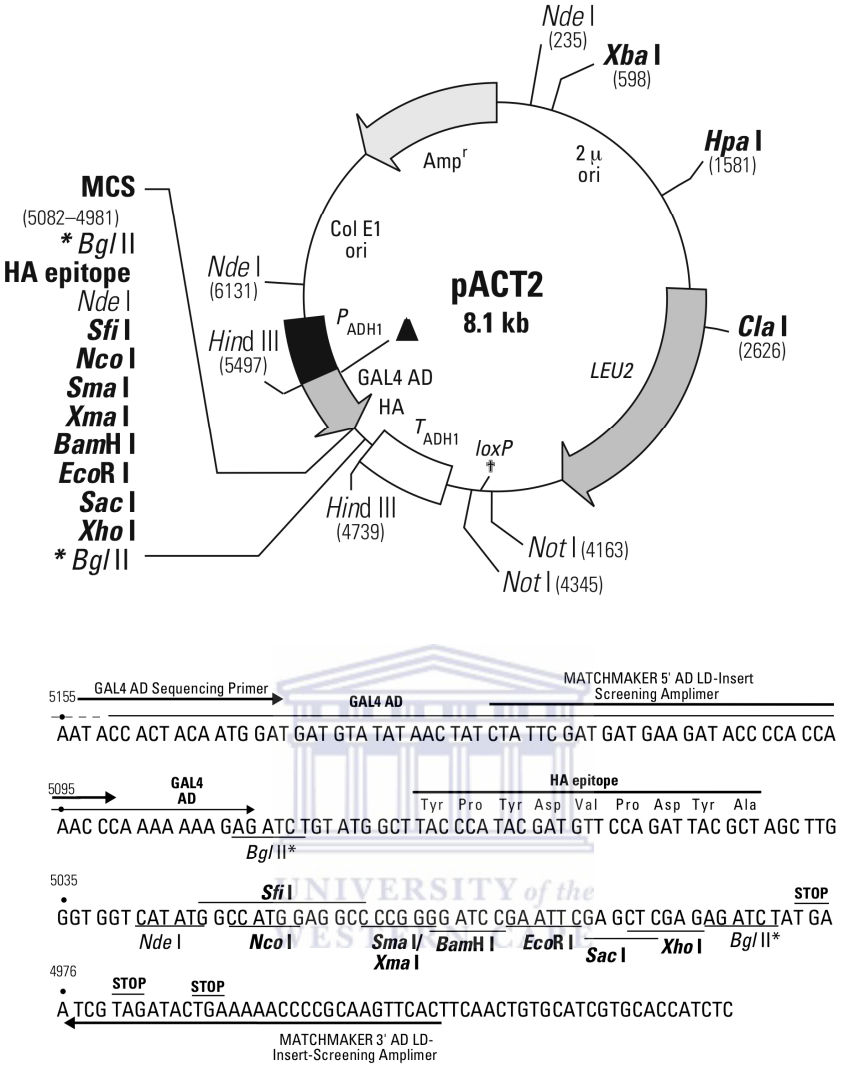


Figure 2.2. Restriction map and multiple cloning site (MCS) of pACT2

Protein encoding sequences cloned in frame into the MCS of pACT2 are expressed as fusions to the GAL4 AD and an HA eppitope tag. The vector contains an ampicilin resistance gene and origins of replication in both yeast and bacteria.

2.4.3. pCMV-HA (Clontech, USA)

pCMV-HA is a mammalian expression vector that expresses proteins containing N-terminal hemagglutinin (HA) epitope tag. The HA epitope tag is well-characterized and highly immunoreactive which facilitates detection of fusion proteins using commercial anti-HA antibodies. High level expression in mammalian cells is driven from the human cytomegalovirus immediate early promoter/enhancer ($P_{CMV\ IE}$). The vector contains both an intron (splice donor/splice acceptor) and polyadenylation signal from SV40 to enhance expression of the fusion proteins[144]. Presence of the Amp^r gene confers ampicillin resistance in *E. coli*. The desired constructs are cloned in the multiple cloning site (MCS) in frame with the HA epitope tag. The pCMV-HA plasmid can be used in partner with pCMV-Myc plasmid in *in vivo* co-immunoprecipitation assays of differently tagged exogenous proteins (Figure 2.3).

2.4.4. pCMV-Myc (Clontech, USA)

pCMV-Myc is a mammalian expression vector that expresses proteins containing an N-terminal c-Myc epitope tag. The c-Myc epitope tag is well-characterized and highly immunoreactive and this facilitates detection of fusion proteins using commercial anti-Myc antibodies. High level expression in mammalian cells is driven from the human cytomegalovirus immediate early promoter/enhancer ($P_{CMV\ IE}$). The vector contains both an intron (splice donor/splice acceptor) and polyadenylation signal from SV40 to enhance expression of the fusion proteins[144]. This vector also carries the Amp^r gene, which confers ampicillin resistance in E. coli. The desired constructs for

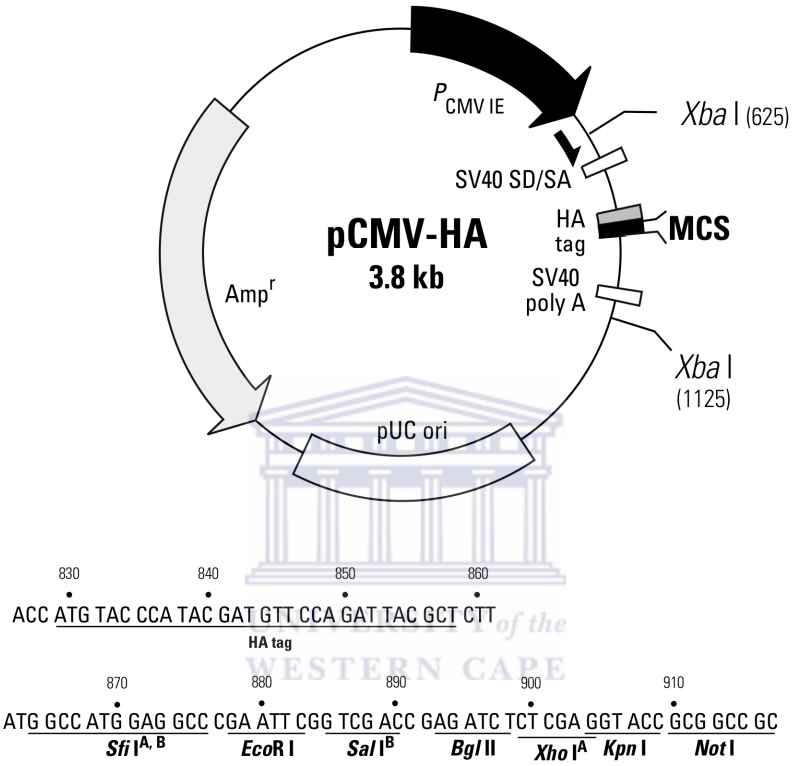


Figure 2.3. Restriction map and multiple cloning site (MCS) of pCMV-HA

Protein encoding sequences cloned in frame into the MCS of pCMV-HA are expressed in mammalian cells fused to the HA epitope tag. The expression of protein is driven by the CMV promoter and can be detected using commercial antibodies raised against the HA epitope tag. The vector also carries the amplicilin resistance gene, which confers ampicillin resistance in *E. coli.*

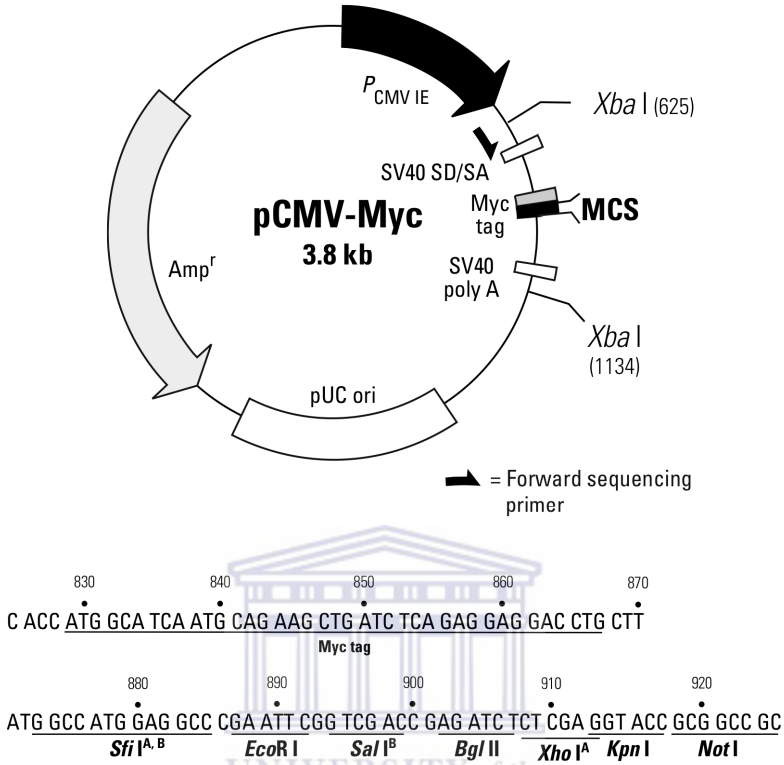


Figure 2.4. Restriction map and multiple cloning site (MCS) of pCMV-Myc

Protein encoding sequences cloned in frame into the MCS of pCMV-Myc are expressed in mammalian cells fused to the c-Myc epitope tag. The expression of protein is driven by the CMV promoter and can be detected using commercial antibodies raised against the c-Myc epitope tag. The vector also carries the amplicilin resistance gene, which confers ampicillin resistance in *E. coli.*

expression as fusion proteins are cloned in the downstream MCS in frame with the cMyc epitope tag.

2.4.5. pEGFP-C1 (Clontech, USA)

The pEGFP-C1 encodes a red-shifted variant of wild-type GFP which has been pEGFP-C1 encodes the GFP mut1 variant which contains the double-amino-acid substitution of Phe64 to Leu and Ser65 to Thr. It also incorporates more than 190 silent nucleotide changes corresponding to human codon-usage preferences. Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site to further increase the translation efficiency in eukaryotic cells. Genes cloned into the MCS are expressed as fusions in frame to the C-terminus of EGFP. An SV40 polyadenylation signal downstream of the MCS directs proper processing of the 3' end of the transcribed mRNA.

The vector backbone contains an SV40 origin for replication in mammalian cells expressing the SV40 T-antigen. A neomycin resistance cassette, consisting of the SV40 early promoter, the *Neo*^{r}/*Kan*^{r} resistance gene of Tn5, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells to be selected using an aminoglycoside antibiotic, G418. A bacterial promoter upstream of this cassette expresses kanamycin resistance in *E. coli*. The pEGFP-C1 backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single stranded DNA production (Figure 2.5).

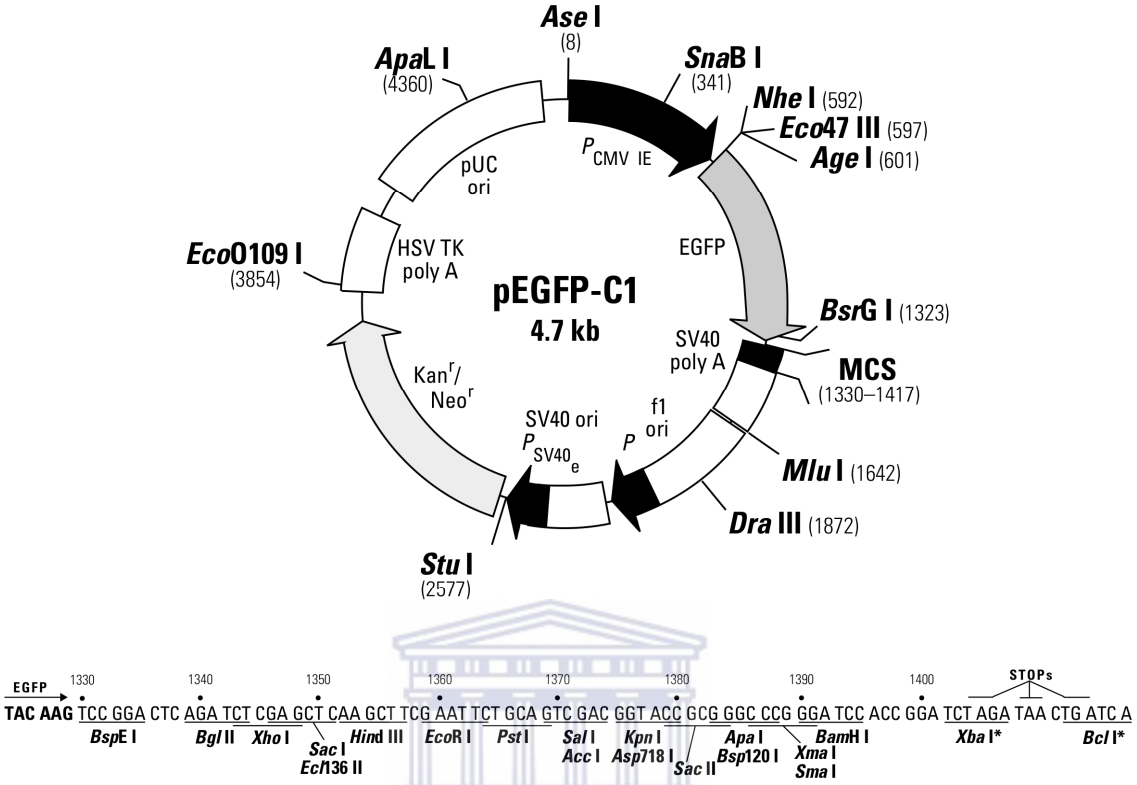


Figure 2.5. Restriction map and multiple cloning site (MCS) of pEGFP-C1

Protein encoding sequences are cloned in frame into MCS of pEGFP-C1 fused to the C-terminus of EGFP. The expression of the fused protein is driven under the control of CMV promoter. The fluorescent properties of the EGFP facilitate observation of localized fusion protein *in vivo*. The vector contains both *Kan*^{r} and *Neo*^{r} resistance genes, which to confer kanamycin and neomycin resistance in *E. coli* and mammalian cells respectively.

2.4.6. pDsRed1-C1 (Clontech, USA)

The pDsRed1-C1 encodes a novel red fluorescent protein (DsRed1) that has been optimized for high expression in mammalian cells. Red fluorescent protein was originally isolated from a relative of the IndoPacific sea anemone, *Discosoma sp*. DsRed1's coding sequence incorporates 144 silent nucleotide changes corresponding to human codon usage preferences, facilitating high expression in mammalian cells. A nucleotide sequence upstream of DsRed1 has been converted to a Kozak consensus translation initiation site to further increase the translation efficiency in eukaryotic cells. Genes cloned into the MCS will be expressed as fusions in frame to the C-terminus of DsRed1. SV40 polyadenylation signal downstream of the MCS direct proper processing of the 3' end of mRNA transcripts (Figure 2.6).

The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T-antigen. A Neomycin resistance cassette, consisting of the SV40 early promoter, the *Neo*^{r}/*Kan*^{r} resistance gene of Tn5, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette expresses Kanamycin resistance in *E. coli*. The pDsRed1-C1 backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

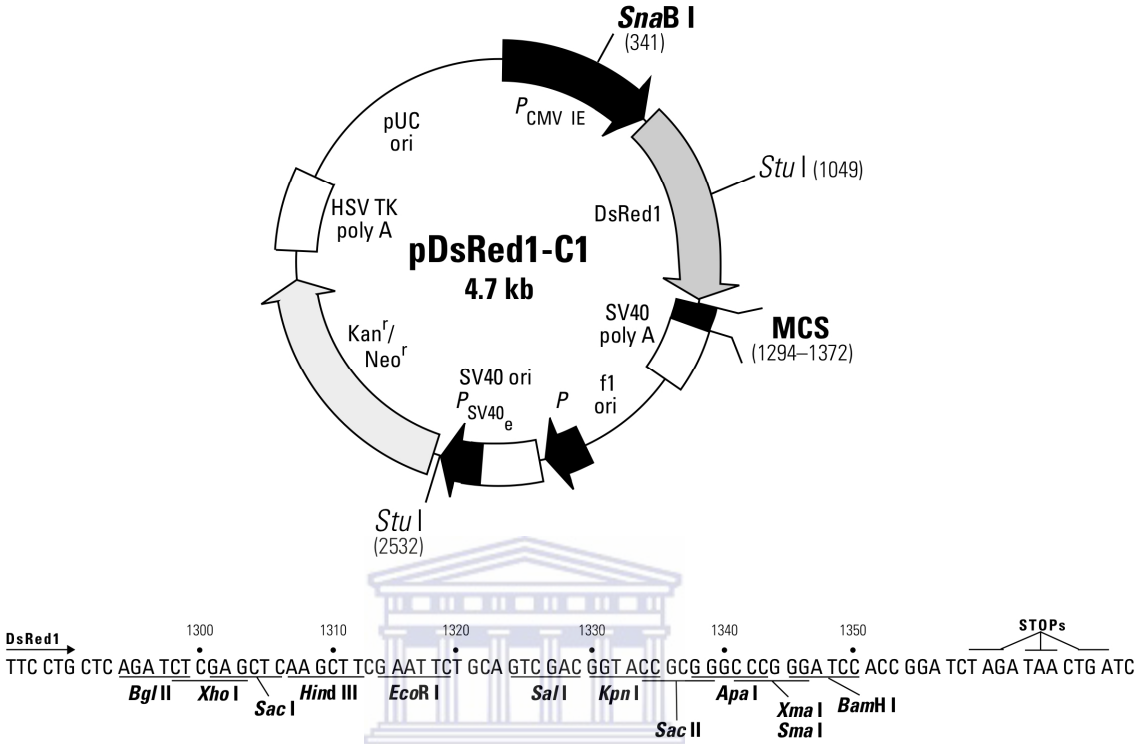


Figure 2.6. Restriction map and multiple cloning site (MCS) of pDsRed1-C1

Protein encoding sequences are cloned in frame into MCS of pDsRed1-C1 fused to the C-terminus of DsRed1. The expression of the fused protein is driven under the control of CMV promoter. The fluorescent properties of the DsRed1 facilitate observation of localized fusion protein *in vivo*. The vector contains both *Kan*^{r} and *Neo*^{r} resistance genes, which to confer kanamycin and neomycin resistance in *E. coli* and mammalian cells respectively.

2.4.7. pALUC

pALUC, which was a kind donation from Dr B Henglein (Institut Curie, Paris, France**)** is a mammalian expression vector containing the gene coding for luciferase from the North American firefly *Photinus pyralis*[145,146] under the control of a 7 kb cyclin A promoter region[147]. Y-box binding protein 1 (YB-1) is an example of a transcriptional factor that induces the cyclin A promoter activity[148], therefore making it useful as a reporter construct for investigating YB-1 induced expression.

2.4.8. pRL-SV40 (Promega)

pRL-SV40 is a mammalian expression vector containing the gene coding for *Renilla* luciferase gene (*Rluc*), which was originally cloned from the marine organism *Renilla reniformis*[149]. The presence of SV40 early enhancer/promoter region provides strong, constitutive expression of R*luc* in a variety of mammalian cell types. Immediately downstream of the promoter is a chimeric intron that provides enhanced expression of *Renilla* luciferase. The intron is a composite of the 5´-donor splice site of human β-globin intron 1 and the branch and 3´-acceptor splice site from an intron derived from the heavy chain variable region of an immunoglobulin gene. For optimal splicing, the sequences of the donor and acceptor splice sites, along with the branch point site, have been altered to match the consensus sequences. pRL-SV-40 is intended for use as an internal control reporter and may be used in combination with any experimental reporter vector e.g. pALUC in co-transfected mammalian cells (Figure 2.7).

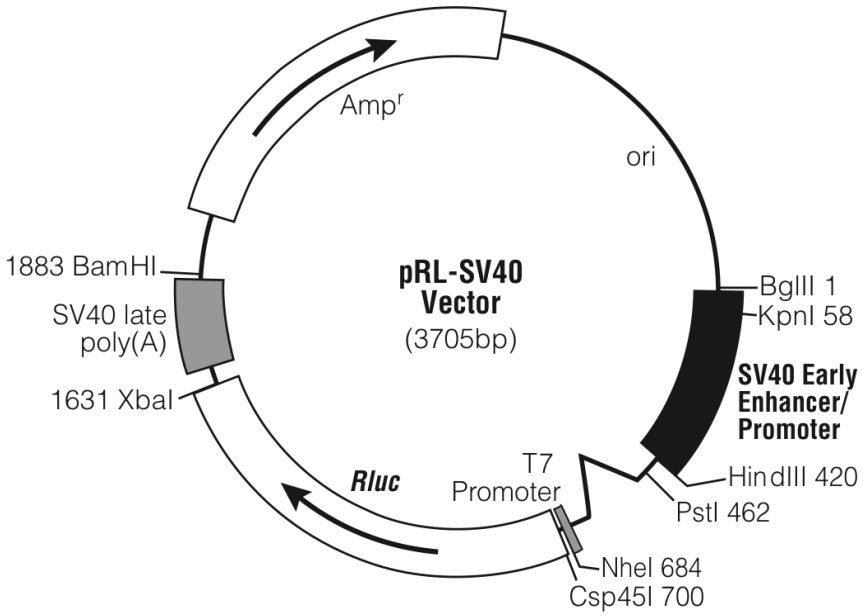


Figure 2.7. The vector map of pRL-SV40 (Promega)

pRL-SV40 contains *Rluc,* the gene encoding the *Renilla* luciferase enzyme, under the control of the SV40 early enhancer/promoter region, an optimized chimeric intron and the SV40 late polyadenylation signal. These three elements combine to yield strong, constitutive expression of the cloned *Renilla* luciferase gene in mammalian cells. The vector plasmid also contains SV40 origin of replication and *Amp*^{r} gene, which confers ampicillin resistance in *E. coli*.

2.5. Bacterial culture

2.5.1. Strain phenotype

E. coli strain DH5α

Φ80d *lacZΔM*15 *recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *deoR* Δ(lacZYA-*argF*)U169[150]

2.5.2. Preparation of bacterial competent cells (*E. coli* strain DH5α)

Bacterial cultures (200µl) were overgrown in 10 ml LB media overnight in a Gallenkamp Orbital incubator (Rhys International Ltd, UK) at 37 °C with shaking at 300 xg. Fifty microliters of the overnight culture was inoculated in 200 ml LB contained in a sterile 2 liter Erlenmeyer flask and incubated at room temperature with shaking at 150 xg on a Labcon shaker (Advanced African Technology, South Africa) until the optical density at 600 nm (OD_{600}) was between 0.4 to 0.6. The bacterial culture was partitioned into 4x 50 ml polypropylene tubes and then centrifuged for 10 minutes at 3000 xg in a Beckman model TJ-6 centrifuge (Beckman Coulter, Scotland, UK). The supernatant was discarded and the pellet gently resuspended in 16 ml of ice cold CAP buffer and again centrifuged for 10 minutes at 3000 xg in a Beckman model TJ-6 centrifuge (Beckman Coulter, Scotland, UK). Following centrifugation, the pellet from each 50 ml tube was gently resuspended in 4 ml ice cold CAP buffer and dispensed into 200 µl aliquots in 2 ml Epperndorf tubes and stored -80 °C.

2.5.3. Bacterial transformation with plasmid DNA

Bacterial transformation refers to genetic alteration of a bacterial cell as a result of taking up foreign DNA. Vials containing competent cells were thawed on ice for 15 minutes after which 1.0-10 ng of plasmid DNA was added. The transformation mixture was gently mixed and left on ice for 20 minutes after which it was heat shocked by immersing vials in a waterbath (Lasec model 102 circulating waterbath, Lasec Laboratory and Scientific Company (Pty) Ltd, South Africa) at 42 °C for 45 seconds. The transformed bacteria were left at room temperature for 2 minutes, after which 1 ml of LB media was added and the mixture incubated at 37 °C with shaking at 300 xg in a Gallenkamp Orbital Incubator (Rhys International Ltd, UK) for another hour to allow the expression of the antibiotic resistance gene. Thereafter, 100-200 µl of the transformed cells were plated onto pre-warmed LB agar plates containing the appropriate antibiotic and incubated in a Scientific Series 9000 incubator (Scientific Engineering Co, Cape Town, South Africa) at 37 °C for 16 hours.

2.5.4. Extraction of plasmid DNA

Extraction of plasmid DNA from transformed *E. coli* cells was carried out using GeneJET™ Plasmid Miniprep Kit (Fermentas Inc, Canada). A single bacterial colony grown on LB agar was picked and inoculated into a 50 ml polypropylene tube containing 10 ml LB medium supplemented with the appropriate antibiotic selection. The inoculum was incubated for 16 hrs at 37 °C while shaking at 300 xg in a Gallenkamp Orbital Incubator (Rhys International Ltd, UK). The bacterial culture was harvested by centrifugation at 3000 xg for 10 minutes in a Beckman model TJ-6 centrifuge (Beckman Coulter, Scotland, UK) after which the supernatant was discarded. The

pelleted cells were resuspended completely in 250 µl of the Resuspension Solution and transferred to a 1.5 ml Eppendorf tube. 250 µl Lysis Solution was added and mixed thoroughly by inverting the tube 4-6 times. 350 µl of the Neutralisation Solution was then added and the solution immediately mixed by inverting the tube 4-6 times, after which it was centrifuged for 5 minutes at 10000 xg using a Beckman Microfuge Lite (Beckman Instruments Inc, USA) to pellet the cell debris and chromosomal DNA. The resultant supernatant was loaded onto the GeneJET^{TM} spin column mounted in a 1.5 ml Epperndorf tube and centrifuged for 1 minute at 10000 xg in a Beckman Microfuge Lite. The column was washed by adding 500 µl Wash Solution and centrifuged for another minute at 10000 xg. The wash was repeated after which 50 µl of double distilled water was added and the column centrifuged for another minute at 10000 xg. The eluted plasmid DNA was stored at -20 °C for downstream applications.

2.6. High fidelity PCR

The polymerase chain reaction (PCR) was used extensively in this study. The typical PCR reaction mixture was prepared as follows: 150 ng of each primer, 4 µl of an equimolar 2.5 mM dNTP stock solution (Takara Shuzo Co.Ltd, Japan), 5µl of 10x Ex Taq™ $MgCl_2$ containing buffer (Takara Shuzo Co.Ltd, Japan), 1 unit of Ex Taq™ (Takara Shuzo Co.Ltd, Japan), 20ng DNA template and double distilled water to a final volume of 50 µl.

Thermal cycling was performed in a GeneAmp PCR system 9700 (PE Biosystems, USA) for 25 cycles. The cycling profile was 30 seconds at 94 °C (for DNA denaturation), 30 seconds at Tm-5 °C (for primer annealing), and 2

minutes at 72 °C (for primer extension). Following the amplification, 5µl aliquot of each PCR product was subjected to electrophoresis on 1.0 -1.5 % agarose gels to verify the success of the amplification.

2.7. Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was used to analyse the results of (i) PCR products and (ii) plasmid DNA extractions from both bacteria and yeasts. In both cases, 5 µl of DNA sample was mixed with 5 µl of Bromophenol blue loading dye and loaded onto a 1-1.5 % horizontal agarose gel containing 1 µg/ml ethidium bromide. The gel was placed in a tank containing in 1xTBE buffer and an electric field of 3.5 V/cm applied. After the dye front reached the end of the gel, the gel was removed from the tank and DNA bands visualized using a long wave 3UV transilluminator (UVP, Inc, CA, USA. Photographic records were obtained using an ITC Polaroid camera and Sony video-graphic printer.

2.8. Purification of PCR products

Prior to cloning, the PCR products were cleaned up using the GeneElute™ PCR Clean-up kit (Sigma, USA). PCR products were mixed with 5x volume of the Binding solution provided and the mixture transferred to a Gene Elute Binding Miniprep Column and centrifuged for 1 minute at 10000 xg in a Beckman Microfuge lite (Beckman Instruments Inc, USA). The eluate was discarded and the column washed by addition of 0.5 ml of Wash Solution and centrifugation for 1 minute at 10000 xg in a Beckman Microfuge lite (Beckman Instruments Inc, USA). The column was further centrifuged for another minute at 10000 xg in a Beckman Microfuge lite (Beckman Instruments Inc, USA)

without additional wash solution to remove excess ethanol. Finally, the column was transferred to a fresh 1.5 ml Eppendorf tube after which 50 µl of double distilled water was added to elute the PCR product by centrifuging for 1 minute at 10000 xg in a Beckman Microfuge lite (Beckman Instruments Inc, USA).

2.9. Restriction enzyme digestion for cloning purposes

Appropriate restriction enzymes were used according to the manufacturer's instructions (Fermentas International Inc, Canada) to sequentially double digest both PCR amplified product and plasmid DNA. Generally, 30 µl of either the PCR amplified product or the plasmid DNA were added to 5 µl 10x digestion buffer and 2 µl appropriate restriction enzyme. The reaction volume was made up to 50 µl per reaction using double distilled water after which the reaction tubes were incubated at 37 °C for 2 hours in a Scientific Series 9000 incubator (Scientific Engineering Co, Cape Town, South Africa). Following digestion with the first enzyme, the DNA were cleaned using the GeneElute™ PCR clean up kit as described above and again digested with the second restriction enzyme in the appropriate buffer. The double digested DNA was again purified using the GeneElute™ PCR clean up kit prior to use in the ligation reactions.

2.10. Ligation reactions

For cloning of PCR products into plasmid DNA, plasmid DNA and a 3-fold excess of PCR product was added to 1 µl of T4 DNA ligase and 1 µl of 10x ligation buffer (Promega) and the volume made up to 10 µl with double distilled water. The ligation mixture was incubated at 4 °C for 16 hours,

following which 5 µl of the ligation mixture was used to transform *E. coli* DH5α cell as described in Section 2.5.3.

2.11. Colony PCR

Colony PCR was used to distinguish recombinant from non recombinant bacteria. 150 ng of each primer, 4 µl of an equimolar 2.5 mM dNTP stock solution (Takara Shuzo Co.Ltd, Japan), 5 µl of 10x Ex Taq™ $MgCl_2$ containing buffer (Takara Shuzo Co.Ltd, Japan), 1 unit of Ex Taq™ (Takara Shuzo Co.Ltd, Japan) were added to a sterile 500 µl Eppendorf tube and the volume made up to 50 µl with double distilled water. Using a sterile pipette tip, a tiny portion of the bacterial colony was picked and directly re-suspended in the PCR mix to serve as a DNA template. Thermal cycling was performed in a GeneAmp PCR system 9700 (PE Biosystems, USA) for 25 cycles as described in section 2.7, after which 5 ul of the PCR reaction mixture was subjected to agarose gel electrophoresis and visualized using a long wave 3UV transilluminator.

2.12. Yeast 2-hybrid methods

2.12.1. Yeast strains used

Yeast strain AH109 phenotype

MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4D, gal80D, LYS2 : : GAL1$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2, URA3 : : MEL1*$_{UAS}$*-MEL1* $_{TATA}$*-lacZ*[151]

Yeast strain Y187 phenotype

MATa, ura3-52, his3-200, ADE2-101, trp1-901, leu2-3, 112, gal4D, met^{-}*, gal80D, URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ*[152]

2.12.2. Yeast transformation with plasmid DNA

Yeast strains AH109 and Y187 were used in the study. The yeasts were first plated out onto YPDA agar plates and incubated for three to five days at 30 °C in a ventilated incubator (Sanyo MIR262, Sanyo Electronic Co, Japan). Following incubation, yeast cells representing an approximate volume of 20 to 50 µl was picked and resuspended in 1 ml of double distilled water in a 2 ml Eppendorf tubes. The cells were pelleted down by centrifuging at 10000 xg for 30 seconds using a Beckman Microfuge lite (Beckman Instruments Inc, USA). After removal of the supernatant, the cell pellet was resuspended in 1 ml 100mM lithium acetate (LiAc), and incubated for 5 minutes at 30 °C in Sanyo MIR262 ventilated incubator. Following the incubation, the cells were again centrifuged and the retained pellet had the following reagents added on sequentially; 240 µl 50 % polyethylene glycol (PEG), 36 µl 1M LiAc, 25µl of 2 mg/ml stock of sonicated and heat denatured Herring sperm DNA (Promega, Madison, USA) and 50 ng plasmid DNA. The final volume was adjusted to

350 µl with double distilled water. The transformation mixture was vortexed at high speed for 1 minute and subsequently incubated at 42 °C for 25 minutes in a lasec model 102 circulating waterbath (Lasec Laboratory and scientific Company (Pty) Ltd., Cape Town, RSA). Following the incubation, the transformation mixture was centrifuged down again at 10000 xg for 30 seconds as above. The supernatant was again discarded and the pellet was resuspended in 200 ul sterile double distilled water, after which 150 µl of the resuspension was plated out onto appropriate nutritionally selective agar plates and incubated at 30 °C for three to five days in a Sanyo MIR262 ventilated incubator.

2.12.3. Construction of Yeast cDNA library

The Yeast library used in the study was a Human Testis MATCHMAKER cDNA obtained from Clontech (Clontech Laboratories, Inc, CA, USA). The library was constructed by generating cDNA from total mRNA pooled from eleven Caucasians testis with age group of 10 to 61 years. The library was cloned into the Xho1 and EcoR1 sites of yeast plasmid pACT2 and amplified in E. coli strain BNN132 before transforming into yeast strain Y187. Quality control procedures carried out showed that the library resulted in 290 x 106 independent yeast colonies and that the cDNA constructed had size ranging between 0.4-4.0 kb with an averaged size of 2.0 kb (Clontech product package).

2.12.4. Yeast library mating procedure

The Yeast library encoding the prey proteins was obtained already pre-transformed in yeast strain Y187. The library transformed Y187 was mated with yeast strain AH109 transformed with bait plasmid encoding the bait protein. The mating procedure was done following a series of procedures summarized as follows:

2.12.4.1. Preparation of bait transformed yeast AH109 for library mating

Four yeast colonies transformed with the bait of interest, were inoculated into four separate 500 ml Erlenmeyer flasks, each containing 50 ml SD/-W media and incubated for 24 hours at 30 °C with shaking at 200g in a YIH DER model LM-510R shaking incubator (SCILAB Instrument Co Ltd., Taipei Taiwan). Following incubation, the cultures were transferred to 50 ml polypropylene tubes (Greiner Labortechnik GmbH, Frickenhausen, German) for centrifugation at 3000g for 10 minutes at room temperature using a Beckman model TJ-6 centrifuge (Beckman Scotland, UK). The pellets were pooled together and resuspended to a final volume of 5 ml SD/-W medium to represent the bait culture, from which the titer of bait culture was determined by means of a haemocytometer cell count. In order to proceed with library mating, the titer was expected to represent > $1x10^{10}$ cells.

2.12.4.2. Yeast matings

Pre-transformed Human Testis MATCHMAKER cDNA library was received from Clontech as 5x1.0 ml library culture. One vial containing the library culture was thawed at room temperature water bath and added to 5 ml bait culture after which the volume of the mixture was adjusted to 50 ml by adding

44 ml of 2x YPDA media containing 10ug/ml Kanamycin, in a 2 liter Erlenmeyer flask. The mating culture was then incubated at 30 °C for 24 hours with shaking at 30 g in a YIH DER model LM-510R shaking incubator (SCILAB instrument Co Ltd., Taipei, Taiwan).

After incubation, the mating culture was transferred to 50 ml polypropylene tube and centrifuged for 10 minutes at 3000g in a Beckman model TJ-6 centrifuge (Beckman Coulter, Scotland, UK). The 2 liter Erlenmeyer flask used for mating was rinsed twice using with 40 ml 2x YPDA and the rinsing mixture was centrifuged as above to recover the left over mating culture. The centrifuged mating culture was resuspended in 10 ml liquid 0.5x YPDA media containing 10 µg/ml Kanamycin after which a 100 µl of the culture was set aside for control matings to determine the mating efficiency as described in Section 2.13.4.3. The rest of the mating culture suspension was plated onto 150 mm TDO plates using 200 µl of the suspension per plate. The TDO plates were incubated at 30 °C for up to two weeks in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan.

2.12.4.3. Determination of mating efficiency

Small-scale yeast matings were performed to determine the effect the baits had on the mating efficiency of AH109. In these mating experiments, the AH109 singly transformed with pGBKT7 containing a bait insert was mated with the prey host strain Y187, transformed with the non-recombinant prey vector pACT2. Concurrently, control mating was also performed where yeast strain AH109 transformed with non-recombinant pGBKT7 was mated with the prey host strain Y187, transformed with non-recombinant prey vectors pACT2.

Yeast mating mixtures were incubated in YPDA liquid media overnight at 30 °C in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan). The overnight culture was spun down at 10000 xg for 30 seconds in a Beckman Microfuge lite (Beckman Instruments Inc., CA, USA). The resultant pellet was resuspended in 100 µl double distilled water from which three sets of serial dilutions of 1/10, 1/100, 1/1000 and 1/10000 prepared and from each serial dilution set 100 µl was plated onto three separate agar plates containing SD/-L, SD/-W and SD/-L-W for each mating. The plates were incubated for four days at 30 °C in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan). The colonies that appeared on the plates were counted and used to calculate the mating efficiency. The mating efficiency was calculated according to the recommendations by the manufacturer of Y2H systems (BD Matchmaker™ Pretransformed Library User Manual, Clontech) (see Appendix I).

Similarly for control matings (Section 2.13.4.2), a 100 ul set aside after the library mating was serially diluted to 1/10, 1/100, 1/1000 and 1/10000 after which 100 ul of each serial dilution was plated onto three different agar plates containing SD/-L, SD/-W and SD/-L-W and incubated for four days in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan). Following incubation, yeast colonies were also counted and used to calculate the library mating efficiency (see Appendix I).

2.12.4.4. Screening for protein interaction

Plating the library mating mixture onto TDO plates was the first step for screening for diploid yeast containing interacting proteins. Growth of yeast clones on TDO plate was counted to give an initial number of clones screened for interaction. These clones were transferred to QDO plates (a more stringent nutritionally selective medium) using a sterile loop and incubated at 30 °C for 4 to 6 days in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan). The appearance of the same yeast clone on both TDO and QDO were assessed and scored in terms of the size and coloration of the colony.

2.12.4.5. X-α-Galactosidase (X-α-Gal) assays

X-α-Gal is a chromogenic substrate for α-galactosidase (also known as melibiase or alpha-D-galactoside galactohydrolase, EC 3.2.1.22), an enzyme which enables yeast to use the disaccharide melibiose as a carbon source during growth or fermentation. In the yeast *Saccharomyces cerevisiae* this enzyme is encoded by the *MEL1* gene which is regulated by several *GAL* genes. Secretion of this enzyme in response to *GAL4* activation leads to hydrolysis of X-α-Gal in the medium causing yeast colonies to develop a blue color[153].

The yeast colonies that survived on QDO plates were again transferred using a sterile loop onto QDO plates previously spread with a 200 µl of 20mg/ml X-α-Gal solution (Clontech Laboratories, Inc, CA, USA). These plates were incubated for 16 to 48 hrs at 30 °C in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan). Following the

incubation, the appearance of the yeast colonies were assessed and recorded for blue color development and scored according to the intensity of the color.

2.12.4.6. Extraction of library plasmids from yeast

The library plasmids were extracted from yeast colonies that were positive for interaction assays. The positive yeast colonies were picked from QDO plates and suspended in 15 ml polypropylene tubes (Greiner Labortechnik GmbH, Frickenhausen, German) containing 5 ml of SD/-L liquid medium for overnight at 30 °C with shaking at 200 g in a YIH DER model LM-510R shaking incubator (SCILAB Instrument Co Ltd., Taipei Taiwan). Following incubation, the overnight culture was centrifuged down by centrifugation for 10 minutes at 3000 g in a Beckman model TJ-6 centrifuge (Beckman Coulter, Scotland, UK). The pellet was resupended in sterile double distilled water and transferred to a 1.5 ml Eppendorf tube after which, its was centrifuged at 10000 xg for 30 seconds using a Beckman Microfuge lite (Beckman Instruments Inc, USA). The supernatant was discarded and the pellet was briefly vortexed after which the following reagents were added; 200 ul smash and grab buffer (2 % Triton X-100, 1 % SDS, 100 mM NaCl, 100 mM Tris-Cl, pH 8.0 and 1 mM EDTA), 200µl Phenol/Chloroform/ Isoamyl Alcohol (25:25:1) solution and 0.3 g of acid washed Sigma glass beads (425-600 µm) (Sigma-Aldrich, Inc, St Louis, USA). The mixture was vortexed at high speed for 2 minutes before centrifuging at 10000 xg for 5 minutes using a Beckman Microfuge lite (Beckman Instruments Inc, USA). The supernatant (200 µl) was transferred to another 1.5 ml Eppendorf tube for further clean up using Sigma GenElute PCR clean-up kit according to the manufacturer's protocol (Sigma-Aldrich, Inc, St Louis, USA).

2.12.4.7. Transformation of *E. coli* and isolation of library prey plasmids

The bait constructs were cloned in pGBKT7, a plasmid that contains a Kanamycin resistance gene but the library prey cDNA was cloned in pACT2 which contains a different antibiotic resistance gene, ampicillin. The prey plasmids isolated from the bait plasmids by transforming *E. coli* strain DH5α with the mixture of bait and prey plasmids and 100 µg/ml ampicillin was used for selecting pACT2 or prey plasmids transformed bacteria. The bacteria transformation and plasmid preparation procedures were carried as indicated in sections 2.5.3 and 2.5.4.

2.12.5. PCR of bait and prey inserts for *in vitro* transcription/translation

The bait and prey inserts contained in bait vector pGBKT7 and prey vector pACT2 respectively were PCR amplified in an RNase free environment. Preys were PCR amplified using the pACT2 primers as shown in Table 2.1 to generate PCR products that incorporated bacteriophage T7 promoter sequences and HA-epitope tag encoding sequence. On the other hand, the bait encoding sequences were PCR amplified using the pGBKT7 primers as shown in Table 2.1 to generate PCR products incorporating bacteriophage T7 promoter sequences and Myc-epitope tag encoding sequence. Incorporating bacteriophage T7 promoter sequences as part of the PCR products provides the promoter site to facilitate the *in vitro* transcription/translation of the respective proteins. Myc and HA epitope tags in bait and prey proteins makes immunoprecipitation of prey and bait protein possible using anti-Myc and anti-HA antibodies respectively. Primer sequences were obtained from Clontech MATCHMAKER Vectors Handbook.

Table 2.1. Primers to amplify bait and prey inserts respectively cloned into pGBKT7 and pACT2

Primer name	Primer sequence	Ta (°C)
pGBKT7 Forward	5′-AATAAAATTGTAATACGACTCACTATAGGGCGAGCCGCCACCATGGAGGAGCAGAAGCTGATGTCA-3′	65
pGBKT7 Reverse	5′-TCACTTTAAAATTTGTATACAC-3′	44
pACT2 Forward	5′-AATAAAATTGTAATACGACTCACTATAGGGCGAGCCGCCACCATGTACCCATACGACGTTCCAGAT-3′	61
pACT2 Reverse	5′-GGGGTTTTTCAGTATCTACGAT-3′	52

Ta is the primer annealing temperature in degrees Celsius

2.12.6. *In vitro* synthesis of bait and prey proteins

The baits and the respective prey proteins were *in-vitro* synthesized using the TNT Quick Coupled transcription/translation system kit according to the manufacturer's instructions (Promega). Generally, 40 µl of the TNT T7 Quick Master Mix was mixed with 7µl of the PCR-generated DNA template, 1 µl TNT T7 PCR enhancer and 2 µl ^{35}S-methionine (Amersham) and the mixture was incubated at 30 °C for 90 minutes using a dry block heater HB2 (Hagar designs, South Africa). Following the incubation, the translated products were run on an SDS-polyacrylamide gel and visualized using autoradiography to check for successful translation.

2.12.7. Co-immunoprecipitation of translated bait and prey proteins

Five microlitres of each bait and prey translated products were mixed together in a sterile 500µl Eppendorf tube and incubated for 1 hour at room temperature. Following incubation, 10µl agarose conjugated Myc antibody (Santa Cruz) was added to the mixture and the volume adjusted to 250 µl using Co-IP buffer. The sample was incubated for 1 hour at 4 °C on a rotating Labnet rotor (Labnet Inc, USA) at 10 g. The sample was subsequently centrifuged for 30 seconds at 10000 xg in a Beckman Microfuge lite (Beckman Instruments Inc, USA). After discarding the supernatant, the pellet was washed 5 times using TBST after which it was mixed with 10 µl sample buffer and boiled for 5 minutes before loading and running on a 12.5 % SDS polyacrylamide gel and subsequently visualized using autoradiography.

2.13. Mammalian cell culture

HEK293 cells were used is this study. HEK293 is a human embryonic kidney cell line that was first developed by Graham *et al* in 1977[154] after transforming cells by exposing them to sheared fragments of adenovirus type 5 DNA. The HEK293 cells were cultured in 6-well cell culture plates and maintained in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10 % fetal calf serum, 100 µg/ml penicillin and 100 µg/ml streptomycin at 37 °C in a 5 % CO_2 humidified cell culture incubator (Farma International, Miami, Florida, USA).

2.13.1. Transfection of HEK293 cells

Transfection is a method by which experimental cDNA construct is exogenously introduced into a cultured mammalian cell, after which the gene product is monitored by different biochemical methods. Extensive transfections were carried out for pull down assays, co-localisation studies and gene functional experiments. Genejuice (Merck) and Metafectene^{TM} (Biontex) were the transfection reagents mostly used in this study and they were used according to the manufacturers' instructions. Generally, 6 µl of both the transfection reagents and 2 µg plasmid DNA were prediluted in 50 µl PBS after which they are gently mixed and incubated at room temperature for 25 minutes to allow lipid/DNA complexes to form. Following the incubation, the transfection mixture was added evenly directly to the respective cells cultured in 6 well culture plates. The transfected cells were incubated for 48 hours at 37 °C in a 5 % CO_2 humidified cell culture incubator (Farma International, Miami, Florida, USA).

2.13.2. Cell lysis and preparation

Generally, the cultured cells were harvested after 48 hours of transfection reactions. The cells were resuspended in the culture media by pipetting up and down until all the adhering cells were in suspension. The cell suspension was transferred to a 15 ml tube and centrifuged at 5000g for 5 minutes in a Beckman model TJ-6 centrifuge (Beckman Coulter, UK). The supernatant was discarded and the pellet resuspended in 10 ml pre-warmed PBS and centrifuged again at 5000g for another 5 minutes. The pellet was resuspended in 500 µl of RIPA buffer and transferred to a sterile 1.5 ml Eppendorf tube and incubated for 20 minutes on ice. Following incubation, the

cell suspension was vortexed at high speed for 1 minute and further sonicated for 10 seconds before centrifuging the lysates at 10000 xg for 15minutes in a pre-cooled Beckman Microfuge Lite. The supernatant was stored at -80 °C as cell lysates for downstream analysis. For RNAse treatment of lysates where indicated, a final concentration of 50 ng/ul RNAse A[155] (Roche) was dissolved in autoclaved 2x RNase Buffer (20 mM Tris (pH 7.5), 10 mM EDTA, 0.6 M NaCl, in DEPC (Diethylpyrocarbonate) treated water) was added to lysates and incubated on ice for 10 minutes prior immunoprecipitation procedure.

2.14. *In vivo* co-immunoprecipitation

After cell lysis, appropriate sample volume of cell lysates were incubated with 5 µg of the appropriate antibodies, either directed against the exogenous protein through respective epitope tags or endogenous proteins, for 2 hours at 4 °C on a rotating Labnet rotor (Labnet Inc, USA) at 10 g. The samples were subsequently centrifuged for 30 seconds at 10000 xg in a Beckman Microfuge lite (Beckman Instruments Inc, USA). After discarding the supernatant, the resultant pellets were washed 5 times using PBS before re-suspended in SDS loading buffer and boiled prior to SDS-PAGE and Western blotting.

2.15. SDS PAGE

SDS polyacrylamide gel electrophoresis was carried out using Laemmli's protocol[156] which uses two phase gel: a staking gel above the resolving gel. Depending on the sizes of the proteins to be resolved, different gels were prepared containing different acrylamide percentage. For example, a 12 % resolving gel and a 4 % staking gel were prepared as indicated in Table 2.2.

Table 2.2. Preparation of a 12 % resolving gel and a 4 % staking gel

	12 % Resolving gel	4 % Stacking gel
Double distilled water	4.5 ml	6.5 ml
1.5M Tris-HCl, pH8.8	2.5 ml	-
0.5M Tris-HCL, pH6.8	-	2.5 ml
40 % Acrylamide	3.0 ml	1.0 ml
10 % SDS	100µl	100µl
10 % APS	80 µl	80µl
TEMED	6.0µl	6.0µl

Proteins were separated on SDS-PAGE gels that were prepared from a 40 % of pre-mix acrylamide: bisacrylamide (37.5:1) (Bio-Rad). The samples were mixed with an equal volume of 2× Sample Buffer, boiled for 5 min, centrifuged for 30 seconds at 10000 xg in a Beckman model TJ-6 centrifuge (Beckman Coulter, UK), and electrophoresed using 1x Running buffer at 100 V/cm (constant voltage) using a Hoefer Mighty Small II Gel electrophoresis system (Amersham Pharmacia). Electrophoresis was stopped when the bromophenol blue dye front had reached the bottom of the gel.

2.16. Western blotting

Following SDS gel electrophoresis, the proteins were transferred from the gel onto a PVDF-P membrane (Amersham Pharmacia) using a Mini Protean II™ system (Bio-Rad). Before transfer, the membrane was pre- wetted in methanol for 10 seconds and equilibrated in Transfer Buffer for 10 minutes. Likewise, the SDS PAGE gels were also equilibrated in Transfer Buffer for 10

min. To avoid air being trapped between different layers of the blotting sandwich, assembling of the sandwich was done in a container filled with transfer buffer and pre-wet each part. The blotting sandwich was mounted using the following components in their order; a porus pad, 2 sheets of Whatman 3MM paper, the equilibrated polyacrylamide gel, the PVDF membrane, 2 sheets of Whatman paper and a porous pad. The mounting was done making sure no air was trapped. The blotting sandwich was mounted into the gel holder unit and inserted into the transfer cell (Bio-rad, Richmond, CA, USA), filled with transfer buffer. The blotting sandwich cassette was inserted into the transfer cell making sure that the polyacrylamide gel is on the anode side and blotting membrane on the cathode side. Electrotransfer was performed at 4 °C, 100 V (constant voltage) for 1 hr in pre-cooled Transfer Buffer. After transfer the membranes were stained with Ponceau S (Sigma) to check for protein transfer.

The membranes were blocked by incubating in TBST containing 5 % fetal bovine serum (FBS) for 1 hour at 4 °C with shaking on a flat Labcon shaker (Advanced African Technology, South Africa). The blocked membrane was further incubated in the appropriate primary antibody diluted in TBST for another hour at 4 °C with shaking on a Labcon shaker (Advanced African Technology, South Africa). If the primary antibody was not HRP conjugated then the membrane was washed three times for 10 min in TBST and further incubated at room temperature for I hour on a Labcon shaker (Advanced African Technology, South Africa) in appropriate secondary antibody, conjugated to horseradish peroxidase, diluted in TBST. The antibody dilution

factor depended on the particular primary or secondary antibody used. The membrane was washed three times for 10 min in TBST. Detection was performed using the ECL plus™ Western Blotting Detection System (Amersham Pharmacia), which was added to the membrane according to manufacturers' instructions. The membrane was exposed to the film at 1 minute and 5 minutes intervals and then developed.

2.17. *In vitro* ubiquitination assay

The *in vitro* ubiquitination experiments were set up using an S-100 *HeLa* Conjugation Kit (Boston Biochem Inc, USA) according to the manufacturer's instructions. Briefly, an ubiquitination reaction mixture containing 26µl of S-100 HeLa, 2µl ubiquitin aldehyde and 1.25µl protease inhibitor MG-132 were initially mixed and incubated for 15 minutes at room temperature. Following incubation, 10 µl of appropriate ^{35}S-labelled full length prey protein and 10 µl of unlabelled bait protein were added to the mixture together with 5µl ubiquitin solution as well as 5µl of ERS (Energy Regeneration Solution). This final mixture was vortexed and incubated at 37 °C for 4 hours using a rotating Labnet rotor (Labnet Inc, USA) at 10 xg, after which the resulting products were resolved by SDS-PAGE and detected by autoradiography.

2.18. Luciferase Assays

Luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The Dual-Luciferase® Reporter (DLR.) Assay System provided an efficient means of performing dual-reporter assays. In the DLR Assay, the activities of firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*, also known as sea pansy) luciferases were measured

sequentially from a single sample. The pALUC was used as an experimental plasmid that expresses the firefly luciferase while pRL-SV40 was used as a control plasmid that expressed the *Renilla* luciferase. Both pALUC and pRL-SV40 were co-transfected in HEK293 cells to produce their respective luciferases. The cells were lysed using Passive Lysis Buffer as part of the kit. The firefly luciferase reporter was measured first by adding 100 µl Luciferase Assay Reagent II (LAR II) to 20 µl lysate to generate a stabilized luminescent signal. After quantifying the firefly luminescence using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, Inc, Winooski, USA), the reaction was quenched, and the *Renilla* luciferase reaction was simultaneously initiated by adding 100 µl Stop & Glo® Reagent.

2.19. Fluorescence microscopy

HEK293 cells were co-transfected with appropriate plasmids encoding the appropriate tagged proteins with either GFP or RFP. The transfected cells were cultured on Lab-Tek™ Chambered Coverglass (Thermo Fisher Scientific, Denmark) for 48 hours in a 5 % CO_2 humidified cell culture incubator (Farma International, Miami, Florida, USA). Just before image acquisition, the culture media was removed and replaced with culture media containing a 1:200 dilution of the nucleic acid stain, Hoechst H-33342 (Sigma). Live cell images were acquired on a Zeiss LSM510 Confocal Microscope (Carl Zeiss MicroImaging Inc.)

CHAPTER 3: PREPARATION OF YEAST BAIT CONSTRUCTS

3.1. Introduction

3.2. Cloning of DWNN and RING finger encoding cDNA into pGBKT7

3.3. Toxicity tests

3.4. Determination of mating efficiency

3.5 Test for bait auto-activation of the reporter genes

CHAPTER 3: PREPARATION OF YEAST BAIT CONSTRUCTS

3.1. Introduction

The preliminary steps involved in accomplishing a Y2H screen using a gene of interest as a bait are as follows: first, cloning of the bait cDNA construct into a plasmid such as pGBKT7 in order to express the bait protein as an in-frame fusion to the GAL4 binding domain. Second, transformation of the cloned bait plasmid into appropriate yeast *Saccharomyces cerevisiae* strain such as AH109. Third, checking that the expressed bait protein is not able to activate yeast reporter genes in the absence of prey, which is known as auto-activation. Since initiation of transcription due to auto-activation is present in approximately 5 % of all cases[157], elimination of this effect is crucial. Finally, the toxicity of some bait constructs to yeast cells may compromise the screen. The level of toxicity is estimated by comparing the growth of yeast transformed with pGBKT7 with and without the insert bait sequence.

This chapter describes cloning of RING finger and DWNN domains for use as baits in separate Y2H screens. Because the structures of DWNN[5] and RING finger (Pugh and colleagues, unpublished) had been previously determined and the full NMR chemical shifts were available for both domains in our laboratory, was the basis for using the same fragments in a Y2H to facilitate structural interpretation of potential interactions, including NMR-based *in vitro* binding interactions.

3.2. Cloning of DWNN and RING finger encoding cDNA into pGBKT7

Sequences encoding the DWNN domain (residues 1-81) and the RING finger (residues 235-335), as shown in Figure 3.1, were PCR amplified from a full length cDNA of RBBP6 assembled by Dr Amanda Skepu (PhD thesis, University of the Western Cape, 2005) and cloned into the NdeI and BamHI sites of pGBKT7 to produce pGBKT7-D and pGBKT7-R, respectively.

NdeI and *BamHI* restriction sites were incorporated into the forward and reverse primers respectively, as shown in Table 3.1, and TAA stop codons were incorporated into both reverse primers.

Table 3.1. Primers used for cloning DWNN and RING finger domain constructs

Primer name	Primer sequences	Ta (°C)
DWNN Forward	5′-GAGGCGCATATGTCCTGTGTGCATTATAAATTT-3′	55
DWNN Reverse	5′-GAGGCGGGATCCTTATTTAACACCTCCAATAGGAATTC-3′	55
RING Finger Forward	5′-GAGGCGCATATGCCTCCCTTCTTACCAGAGGA-3′	55
RING Finger Reverse	5′-GAGGCGGGATCCTTACTGTTTTCGTAGTCTTTTTGTATA-3′	55

The nucleotide sequence in black font represents the sequence of the primer that first anneals to the DNA in the PCR reaction. An 'overhang' tag to facilitate restriction enzyme digestion is represented in green font, while the red and blue fonts represent the restriction enzyme site and incorporated stop codon respectively. Ta is the primer annealing temperature in degrees Celsius

Following verification of the expected sequences using fluorescent dye-terminator cycle sequencing method (3130xl Genetic Analyzers, Applied Biosystems Inc CA, USA), pGBKT7-R and pGBKT7-D were transformed into yeast *Saccharomyces cerevisiae* strain AH109 as described in Section 2.13.2.

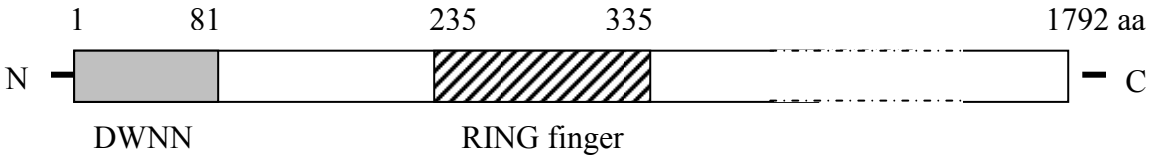


Figure 3.1. RBBP6 domains cloned into pGBKT7

A schematic representation of RBBP6 showing the DWNN and RING finger cloned into yeast bait plasmid pGBKT7.

3.3. Toxicity tests

To assess any toxic effects of DWNN and RING finger baits, pGBKT7-D, pGBKT7-R and the parental plasmid pGBKT7, were separately transformed into yeast strain AH109 and inoculated into SD/-W liquid medium and grown to stationary phase at 30 °C with shaking at 200 g (24-36 hours) in a TIH DER model LM-510R shaking incubator (SCILAB instrument Co Ltd., Taipei, Taiwan). The overgrown culture was diluted 10x with SD/-W medium and grown for an additional 24 hours under the same conditions, during which 1 ml aliquots of the medium were taken every 2 hours for the first 8 hours and then at 24 hours. The optical density at 600 nm (OD_{600}) was measured and plotted as a function of time as shown in Figure 3.2. A statistical analysis was carried out using GraphPad Prism version 4 for Windows (GraphPad Software, San Diego CA, USA).

Figure 3.2 shows that the pGBKT7-R and pGBKT7-D did not significantly reduce the growth rate in comparison to pGBKT7. It was therefore concluded that neither the RING finger nor the DWNN construct were toxic to the yeast strain AH109.

3.4. Determination of mating efficiency

To assess the mating efficiency of yeast cells transformed with DWNN and RING finger domains, a small scale yeast mating was carried out between yeast AH109 transformed with either pGBKT7-D, pGBKT7-R or the parental vector pGBKT7 and prey host strain Y187, transformed with non-recombinant parental vector pACT2.

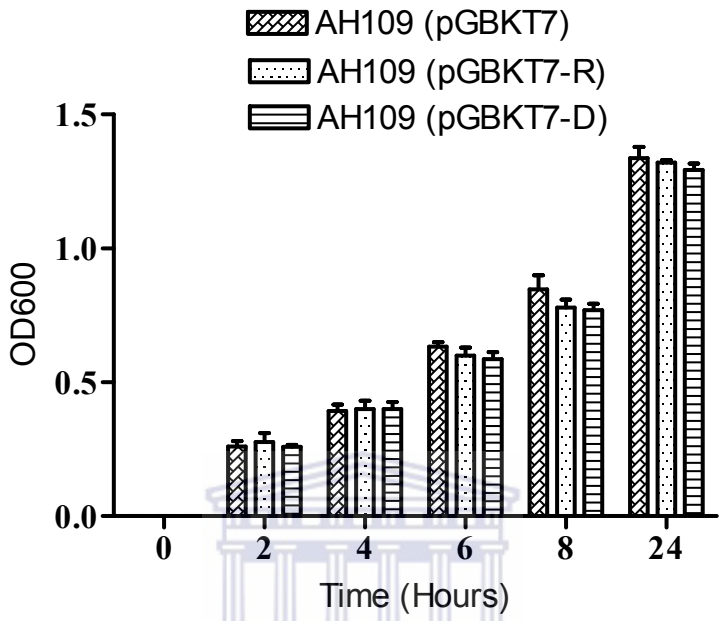


Figure 3.2. Bait toxicity test

A bar graph showing the growth of yeast strain AH109 transformed with pGBKT7, pGBKT7-R and pGBKT7-D respectively at a number of different time points. The results shown are means ± S.D. of three independent experiments. The Student's t-test compared to the empty vector at each time interval showed no significant difference, $P>0.05$. The bar graph plot was generated using GraphPad Prism version 4 for Windows (GraphPad Software, San Diego CA, USA).

Matings were carried out as described Section 2.13.4.3 and the results presented in Table 3.2. The calculated mating efficiency (Appendix 1) of the pGBKT7-D and pGBKT7-R yeast transformants were comparable to the control and, most importantly, were above the minimum of 2 % recommended by the manufacturer of the MATCHMAKER Y2H system (Clontech, USA) and would therefore result in screening of the recommended 10^6 individual clones when mated with a commercial pretransformed MATCHMAKER library.

Table 3.2. Testing the effect of baits on yeast mating efficiency

Yeast mating	Mating efficiency (%)
AH109 (pGBKT7) and Y187 (PACT2)	10.5
AH109 (pGBKT7-R) and Y187 (PACT2)	9.5
AH109 (pGBKT7-D) and Y187 (PACT2)	8.7

3.5 Test for bait auto-activation of the reporter genes

To establish whether the DWNN and RING finger baits were able to autonomously activate transcription of reporter genes *HIS3* and *ADE2*, AH109 transformed with pGBKT7-D and pGBKT7-R respectively were plated onto SD/-L, SD/-W, SD/-H, SD/-A and SD/-U agar media lacking leucine, trptophan, histidine, adenine and uracil respectively. The plates were incubated at 30 °C for 3-5 days in a Sanyo MIR262 ventilated incubator (Sanyo MIR262, Sanyo Electronic Co, Japan) after which the growth was observed and scored.

As shown in Table 3.3, neither DWNN nor RING finger resulted in auto-activation of *HIS3* or *ADE2* as evidenced by lack of growth on SD/-H and SD/-A plates respectively. These two reporter genes are only activated in the presence of interacting proteins.

Table 3.3. Testing baits for auto-activation of reporter genes in yeast

Yeast strain	SD/-A	SD/-H	SD/-L	SD/-W	SD/-U
AH109	-	-	-	-	+
AH109 (pGBKT7-R)	-	-	-	+	+
AH109 (pGBKT7-D)	-	-	-	+	+

Chapter 4: Y2H screen using RING finger domain as yeast bait

CHAPTER 4: Y2H screen using RING finger domain as yeast bait

4.1. Introduction

Along with other members of the RING finger family, the RING finger domain from RBBP6 was suspected of having E3 ligase activity prior to this study. RING finger domains typically interact directly with both the E2 enzyme and the substrate, and the respective E2's and substrates of a number of RING finger domains had previously been identified using Y2H screening.

This chapter describes a Y2H screen of a human testis cDNA library (Clontech, USA) using the RING finger from RBBP6 as bait. A number of putative interactors were identified and 2 of these were confirmed using co-immunoprecipitation assays, both *in vitro* and *in vivo*. It was furthermore shown that interaction with the RING finger leads to ubiquitination of both interactors.

4.2. Yeast library matings

The pGBKT7-R bait plasmid transformed into *Saccharomyces cerevisae* strain AH109 was used to screen a commercial human testis cDNA library cloned into the pACT2 vector and transformed into *Saccharomyces cerevisae* strain Y187 (Clontech, USA). The choice of library was based on the fact that RBBP6 had been found to be highly expressed in testis[1]. The screening of an estimated $6.5x10^6$ library clones yielded 2220 clones in which transcription of the *HIS3* reporter gene was activated as evidenced by their ability to grow on TDO selection medium (lacking leucine, tryptophan and histidine). The clones from TDO medium plates were transferred to QDO selection medium

(lacking leucine, tryptophan, histidine and adenine) on which 550 clones survived, indicating additional activation of transcription of the *ADE2* reporter gene. These clones were then transferred to QDO selection medium supplemented with X-α-gal (a chromogen substrate for α-galactosidase enzyme) to select yeast clones that activate transcription of *MEL1* as evidenced by blue colouration of the colonies. 101 yeast clones concurrently exhibited activation of all three interaction-associated reporter genes (*HIS3*, *ADE2* and *MEL1*), and these clones were further subjected to interaction specificity mating tests.

4.3. Interaction specificity tests

The yeast clones expressing all three reporter genes (*HIS3*, *ADE2* and *MEL1*) were analyzed further. Prey plasmids were separated from bait plasmids by extracting the plasmids from yeast colonies as described section 2.13.4.6 and transforming them into *E. coli* DH5α, using ampicilin to select for prey plasmid-transformed bacteria. The bait plasmid-transformed bacteria, because of lack of ampicilin resistance marker, would not grow on amplicilin supplemented media. The prey plasmids were then extracted from the bacteria as described in Section 2.5.4 and re-transformed into yeast strain Y187. These transformed Y187 strains were then used in interaction specificity tests.

The aim of interaction specificity test is to identify preys that activate reporter genes in the presence of the RING finger bait, but not in the absence of RING finger bait nor in the presence of any of the following heterologous baits: pGBKT7-C5, encoding the C5 immunoglobulin-like domain of cardiac myosin

binding protein-C, pGBKT7-53, encoding murine p53 and the parental plasmid pGBKT7.

The interaction specificity test was set up as follows: yeast strain Y187, transformed with the prey plasmid was separately mated with yeast AH109, transformed with either pGBKT7-R or heterologous bait plasmids, after which the resulting diploid clones were cultured on SD/-L-W plates for 3-4 days at 30 °C in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan). The resulting yeast colonies were transferred onto QDO selection plates and incubated for another 3-4 days at 30 °C, after which the growth was observed and scored.

Figure 4.1 shows a typical result of yeast clones scored as 'positive' depending on the robustness of growth otherwise they were scored as 'negative' for no growth. Following the scoring trend, Table 4.1 shows that of the 101 putative preys, 16 showed noticeable specific interactions with RING finger bait and not in the presence of heterologous baits.

4.4. Identification of putative prey interactors

The Y2H screen identified 16 putative interactors that consistently activated the yeast reporter genes (*HIS3*, *ADE2* and *MEL1*) in a RING finger-specific manner. The prey plasmids were sequenced using the fluorescent dye-terminator cycle sequencing method (3130xl Genetic Analyzers, Applied Biosystems Inc CA, USA) and the portions of the sequences in the Human Genome database and the encoded proteins were determined using BLAST searches (www.ncbi.nlm.nih.gov/blast) as shown in Table 4.1.

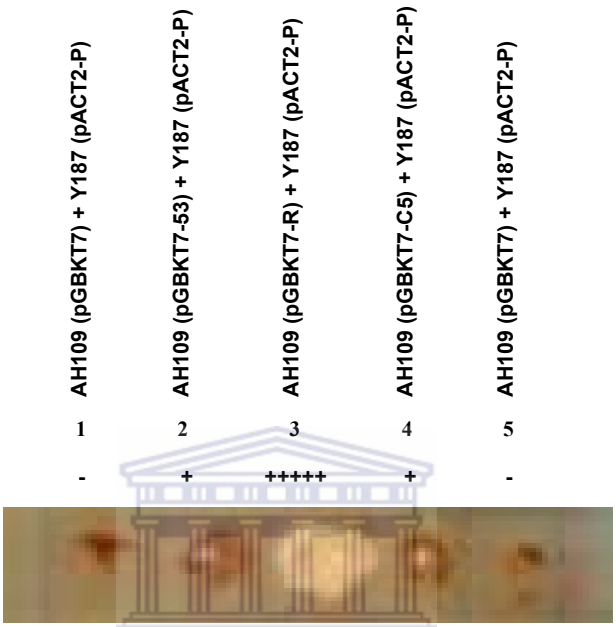


Figure 4.1. An example of a library prey (pACT2-P) which interacts only with RING finger

Diploid colonies containing the prey plasmid (pACT2-P) grew on QDO medium only in the presence of RING finger-containing bait plasmid pGBKT7-R (lane 3), but not in the presence of p53 (lane 2), C5 (lane 4) or parental plasmids (lanes 1 and 5). The complete set of results is shown in Table 4.1.

Table 4.1. Scoring for yeast growth after heterologous bait matings

Clone number	AH109 (pGBKT7) + Y187 (pACT2-P)	AH109 (pGBKT7-53) + Y187 (pACT2-P)	AH109 (pGBKT7-R) + Y187 (pACT2-P)	AH109 (pGBKT7-C5) + Y187 (pACT2-P)	AH109 (pGBKT7) + Y187 (pACT2-P)
92	-	-	-	-	-
95	-	-	-	-	-
101	-	+	-	+	-
111	+	+	+	+	+
135	+	-	-	-	+
144	-	-	-	-	-
158	-	-	-	-	-
232	-	-	+++++	-	-
237	-	-	-	-	-
246	++	-	-	-	++
249	-	-	-	-	-
253	-	-	-	-	-
260	-	-	-	-	-
268	+++++	+++++	+++++	+++++	+++++
268	-	-	-	-	-
291	-	-	-	-	-
294	++	++	++	++	++
296	-	-	-	-	-
304	-	-	-	-	-
343	-	-	-	-	-
344	++	++	++	++	++
344	-	-	-	-	-
350	-	-	-	-	-
358	-	-	-	-	-
359	-	-	-	-	-
394	-	+	+++++	+	-
480	-	-	-	-	-
487	-	-	-	-	-
489	-	-	-	-	-
490	-	-	-	-	-
501	++	-	++	++	++

Clone number	AH109 (pGBKT7) + Y187 (pACT2-P)	AH109 (pGBKT7-53) + Y187 (pACT2-P)	AH109 (pGBKT7-R) + Y187 (pACT2-P)	AH109 (pGBKT7-C5) + Y187 (pACT2-P)	AH109 (pGBKT7) + Y187 (pACT2-P)
512	++	+	++	+	++
699	-	-	-	-	-
797	++	++	++	+	++
798	++	+++	++	+++	++
807	-	-	-	-	-
843	++	++	++	++	++
844	++	++	++	+++	++
907	-	-	-	-	-
908	++	++	++	+	++
914	++	++	++	+++	++
1019	++	+++	++	+++	++
1020	+++	+++	+++	+++	+++
1021	-	-	-	-	-
1022	++	+++	++	+++	++
1039	-	-	+++++	-	-
1043	-	-	-	-	-
1044	-	-	-	-	-
1054	++	++	++	++	++
1058	-	-	-	-	-
1068	-	-	-	-	-
1080	++++	++++	++++	++++	++++
1081	-	-	-	-	-
1091	++	+++	++	+++	++
1093	-	-	+++++	-	-
1119	++	+++	++	+++	++
1189	-	-	-	-	-
1191	-	+++	+	+++	-
1192	-	-	-	-	-
1193	++	+++	++	+++	++
1194	-	-	-	-	-
1195	-	-	+++++	-	-
1200	-	-	-	-	-
1201	++	++	++	++	++
1202	-	-	+++++	-	-
1208	-	-	-	-	-

Clone number	AH109 (pGBKT7) + Y187 (pACT2-P)	AH109 (pGBKT7-53) + Y187 (pACT2-P)	AH109 (pGBKT7-R) + Y187 (pACT2-P)	AH109 (pGBKT7-C5) + Y187 (pACT2-P)	AH109 (pGBKT7) + Y187 (pACT2-P)
1209	-	-	-	-	-
1210	-	++	+++++	-	-
1211	+	-	-	-	+
1212	-	-	+++++	-	-
1213	++	+	++	+	++
1216	++	++	++	+++	++
1217	-	-	+++++	-	-
1218	-	-	-	-	-
1223	++	++	++	++	++
1224	++	+++	+++	+++	++
1227	-	-	+++++	-	-
1228	++	++	++	+++	++
1229	-	-	+++++	-	-
1231	-	-	-	-	-
1234	++++	++++	++++	++++	++++
1242	-	-	-	-	-
1262	++	++	++	+++	++
1400	-	+	+++++	+	-
1411	-	-	+++++	-	-
1447	++	+++	+++	+++	++
1448	-	-	-	-	-
1804	-	+	+++++	-	-
1805	++	+++	+++	+++	++
1824	-	-	-	-	-
2001	++	+	++	+	++
2003	-	-	-	-	-
2004	-	-	+++++	-	-
2005	-	-	+++++	+	-
2006	-	-	-	-	-
2007	++	+++	+++	+++	++
2034	-	-	-	-	-
2204	-	-	-	-	-
2223	+	+	+	+	+
2226	-	-	-	-	-
2227	++	+++	+++	+++	++

On analyzing the DNA sequences encoding these putative interactors, only 3 sequences gave rise to meaningful biological products, of which 2 sequences encoded the same fragment of Y-Box binding protein 1 (YB-1) and the other sequence encoded part of a protein called zinc finger and BTB domain containing 38 (zBTB38). These plausible preys were subjected to additional verification assays. The other 13 prey constructs were discarded since their sequences did not correspond to known proteins in either NCBI Entrez (www.ncbi.nlm.nih.gov) or Ensemble (www.ensembl.org) protein databases. This, however, is expected as only one sixth of the clones represented in Matchmaker™ pre-transformed oligo-dT primed cDNA libraries represent known protein products (Clontech MATCHMAKER Two-Hybrid Assay Kit User Manual).

4.5. YB-1 binds to RING finger through its C-terminal region

Mammalian YB-1 consists of 3 highly conserved domains: an Ala/Pro-rich N-terminal domain, a cold-shock domain (CSD) that mediates RNA and DNA interactions, and an Arg/Lys-rich C-terminal domain[158]. In the latter domain, clusters of about 30 basic or acidic residues alternate, creating a B/A repeat; this region is thought to be involved in protein-protein and RNA-protein interactions[158,159]. From the Y2H screen, the 2 prey plasmids that were identified to be interacting with RING finger encoded the last 62 amino acid residues of YB-1; which correspond approximately to the last B/A repeat as shown in Fig 3.4. This region will be referred to in the rest of this thesis as YB-1-C.

Table 4.2. Identities of putative RING finger interactors

Clone Number	*Nucleotide Blast identity*	*Nucleotide Accession Number (E-value)*	*In-frame Protein Hit*	*Protein Accession Number (E-value)*
232, 1039	Homo sapiens Y box binding protein 1 (YBX1)	NM_004559.3 (0.0)	Homo sapiens Y box binding protein 1 (YBX1)	AAH18393.1 (8e-40)
1195	Homo sapiens zinc finger and BTB domain containing 38 (ZBTB38)	XM_001133510.1 (0.0)	Homo sapiens zinc finger and BTB domain containing 38 (ZBTB38)	NP_001073881 (1e-163)
394	Homo sapiens CD81 molecule (CD81)	NM_ 004356.3 (0.0)	unnamed protein product [Tetraodon nigroviridis]	CAG04182.1 (7e-06)
1217	Homo sapiens mitochondrion, complete genome	NC_001807.4 (0.0)	NADH dehydrogenase subunit 4	ABC60558.1 (6e-55)
1093	Homo sapiens chromosome 7 genomic contig, alternate assembly (based on CRA_TCAGchr7v2)	NT_ 079592.2 (0.0)	No significant similarity found	-
1202	Homo sapiens ribosomal protein S14 (RPS14), transcript variant 3	NM_005617.3 (0.0)	No significant similarity found	-
1210	Homo sapiens leucine-rich repeats and calponin homology (CH) domain containing 4 (LRCH4).	NM_002319.3 (1e-136)	No significant similarity found	-
1212	Homo sapiens mitochondrion, complete genome	NC_001807.4 (0.0)	No significant similarity found	-
1227	Homo sapiens v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1)	NM_005238.2 (0.0)	No significant similarity found	-
1229	Homo sapiens chromosome 21 open reading frame 63 (C21orf63)	NM_058187.3 (0.0)	No significant similarity found	-
1400	Homo sapiens UPF3 regulator of nonsense transcripts homolog B (yeast)	NM_023010.2 (5e-46)	No significant similarity found	-
1411	Homo sapiens chromosome 18 open reading frame 20 (C18orf20)	NM_152728.1 (0.0)	No significant similarity found	-
1804	Homo sapiens chromosome 7 genomic contig, reference assembly	NT_007933.14 (0.0)	No significant similarity found	-
2004	Homo sapiens mitochondrion, complete genome	NC_001807.4 (0.0)	No significant similarity found	-
2005	Homo sapiens chromosome 10 open reading frame 104 (C10orf104),	NM_173473.2 (0.0)	No significant similarity found	-

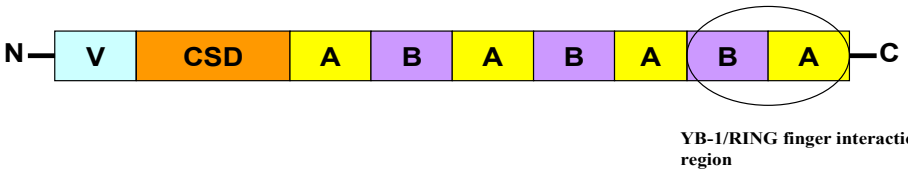


Figure 4.2. Mapping of the YB-1/RING interaction region

Schematic diagram of YB-1 showing the variable N-terminal region (V), the cold shock domain (CSD) and the alternating base (B) and acidic (A) blocks forming the C-terminal domain. Both YB-1 clones identified as interacting with RING finger domain encoded the last acid/base repeat (encircled).

4.6. RING finger co-immunoprecipitates YB-1-C *in vitro*.

To confirm the above interaction in the absence of GAL4 domains, the RING finger and the C-terminal fragment of YB-1 (YB-1-C) were PCR amplified from their respective bait and prey plasmids using the primers set out in Table 2.1. These PCR-generated fragments were used to express ^{35}S-labelled proteins in an *in vitro* transcription/ translation system as described in Section 2.13.5 and 2.13.6., incorporating Myc and HA tags, respectively. When subjected to SDS-PAGE, RING finger and YB-1-C migrated with the expected sizes of 17 and 14 kDa, respectively (Figure 4.3, lanes 1 and 3). Immunoprecipitation with anti-Myc antibody in the presence of Myc-RING and HA-YB-1-C resulted in the detection of HA-YB-1-C (lane 2), whereas no HA-YB-1-C was precipitated in the absence of Myc-RING (lane 4), indicative of an interaction between them.

4.7. RING finger co-immunoprecipitates full length YB-1 *in vivo*

Following confirmation of the interaction using *in vitro* co-immunoprecipitation assays, the interaction was followed up *in vivo* to confirm whether the RING finger can interact with full length YB-1 in the context of the cell. A construct encoding full length YB-1 (pEGFP-YB-1) which was a kind gift from Prof. Lev P. Ovchinnikov (Institute of Protein Research of the RAS, Russia), was amplified and cloned into the *EcoRI* and *XhoI* sites of the pCMV-HA and pCMV-Myc mammalian expression plasmids using the primers in Table 4.3, yielding pHA-YB-1 and pMyc-YB-1 respectively. Similar constructs were made for the RING finger domain, yielding pHA-RING finger and pMyc-RING finger respectively.

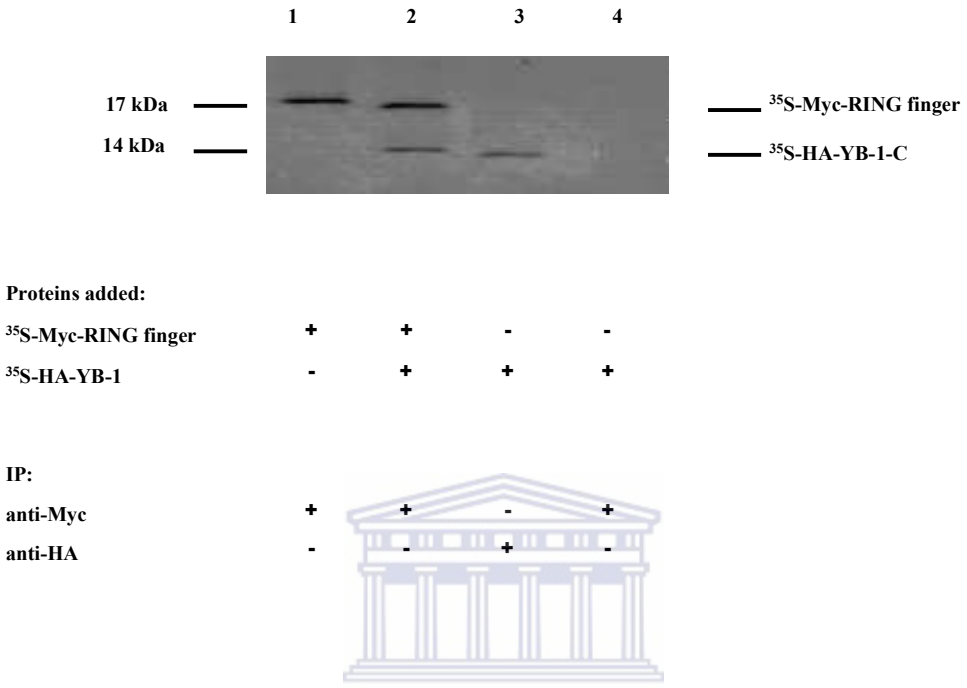


Figure 4.3. RING finger co-immunoprecipitates YB-1 protein *in vitro*

Autoradiograph of immunoprecipitation of ^{35}S-labelled *in vitro* generated proteins; antibodies used in the immunoprecipitation reactions are as indicated. Immunoprecipitation with anti-Myc antibody resulted in co-immunoprecipitation of the 14-kDa HA-YB-1-C fragment in the presence (lane 2), but not in the absence (lane 4), of the 17 kDa Myc-RING. Lanes 1 and 3 correspond to individual immunoprecipitates of HA-YB-1-C and Myc-RING finger, respectively.

Appropriate combinations of HA- and Myc-tagged constructs were transfected into HEK293 cells and immunoprecipitations were performed using anti-HA and anti-cMyc agarose conjugated antibodies as shown in Figure 4.4. Exogenously expressed full-length YB-1 and RING finger were able to precipitate each other (Fig. 4.4 lanes 2 and 4 respectively), while the exogenously expressed RING finger was also able to precipitate endogenous full-length YB-1 (Fig. 4.5). Since RBBP6 and YB-1 are both known to interact with mRNA, a possibility that needed to be investigated was that the interaction was not direct but was mediated by mRNA. The co-immunoprecipitation of RING finger and YB-1 was not decreased as a result of pre-treatment with RNAse A (Fig 4.4, lanes 3 and 7), indicating that the interaction is direct and is not meditated by mRNA. Endogenous YB-1 was also co-immunoprecipitated by RING finger even after RNAse A treatment (Figure 4.5 lane 3), further indicating direct interaction of RING finger and YB-1. The high molecular weight smears were evident in Figure 4.5 are discussed in detail in Section 4.9. Also, constructs for mammalian expression of the C-terminal fragment of YB-1 (YB-1-C) were made and transfected into HEK293 cells, but the protein could not be visualized on Western blot and so was abandoned in favour of the full length YB-1.

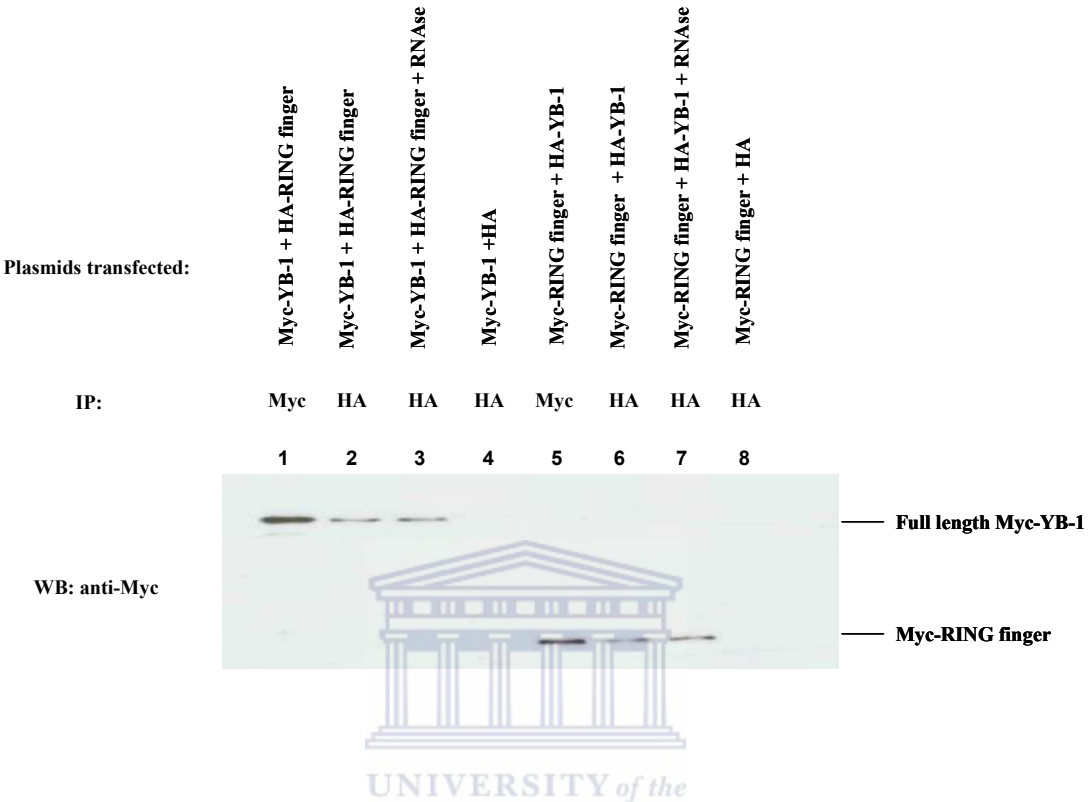


Figure 4.4. RING finger co-immunoprecipitates full length YB-1 *in vivo* and *vice versa.*

HEK293 cells were transfected with combinations of HA- and Myc-tagged constructs as indicated and immunoprecipitated as indicated. RNase A was added to the lysates as described in section 2.14.2.3. Anti-HA antibodies were able to precipitate Myc-YB-1 in the presence (lane 2) but not in the absence (lane 4) of HA-RING. The same result was obtained in the presence of RNase (lane 3), indicating that the interaction between Myc-YB-1 and HA-RING is direct and not mediated by RNA. Conversely, anti-HA antibodies were able to precipitate Myc-RING in the presence (lane 6) but not in the absence (lane 8) of HA-YB-1.

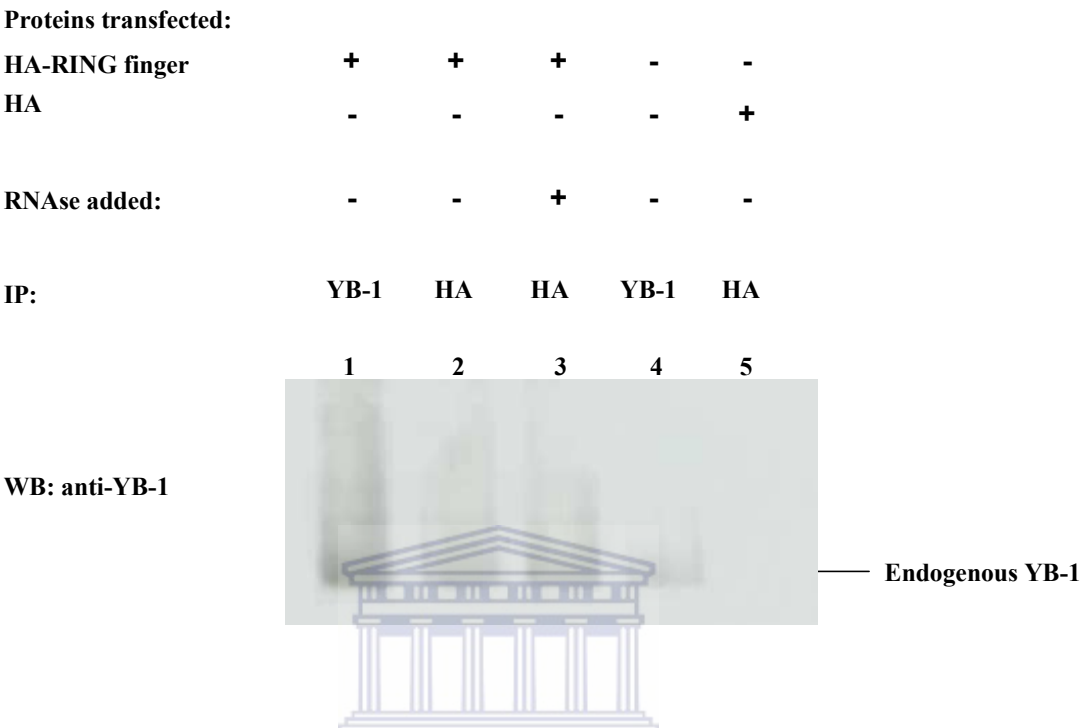


Figure 4.5. RING finger co-immunoprecipitates endogenous YB-1 *in vivo.*

HEK293 cells were transfected with HA-RING or HA alone as indicated and RNase A was added to the lysates as described in Section 2.14.2.3. Exogenous HA-RING was able to precipitate endogenous YB-1 (lane 2), whereas HA alone was not (lane 5). Addition of RNase had no effect on the amount of YB-1 precipitated (lane 3), indicating that the interaction does not involve RNA. Endogenous YB-1 (immunoprecipitated with anti-YB-1 to increase the signal) shows evidence of ubiquitin-like modification in the presence of transfected HA-RING (lane 1), but much less in the absence of HA-RING (lane 4). These modifications are also visible when YB-1 is co-immunoprecipitated with HA-RING using anti-HA antibodies (lanes 2 and 3).

Table 4.3. Primers for cloning YB-1/ RING finger into pCMV-HA and pCMV-Myc

Primer name	Primer sequences	Ta (°C)
Full length YB-1 Forward	5′-GAGGGCCGAATTCAAAGCAGCGAGGCCGAGACC-3′	55
Full length YB-1 Reverse	5′-GAGGGCACCTCGAGATTATACACAAAGACAATTATTTAAGACCT-3′	55
RING Finger Forward	5′-GAGGCGCGAATTCCTCCCTTCTTACCAGAGGA-3′	55
RING Finger Reverse	5′-GAGGCGCGCTCGAGATTACTGTTTTCGTAGTCTTTTTGTATA-3′	55

The nucleotide sequence in black font represents the sequence of the primer that first anneals to the DNA in the PCR reaction. An 'overhang' tag to facilitate restriction enzyme digestion is represented in green font, while the red and blue fonts represent the restriction enzyme site and incorporated stop codon respectively. Ta is the primer annealing temperature in degrees Celsius

4.8. YB-1 interacts with full length RBBP6 *in vivo*

Following confirmation that YB-1 interacts *in vivo* with the RING finger from RBBP6, the next task was to investigate whether YB-1 was able to interact with full length RBBP6. A mammalian construct expressing GFP-tagged full length RBBP6 (described in Section 3.2) together with constructs encoding the C-terminus (residues 337 to1792) and N-terminus (residues 1 to 118) of RBBP6 as shown in Fig 4.6, were used to investigate the interaction further. The N and C-terminal fragments were cloned into the *SalI/XhoI* and *XhoI/NotI* sites respectively of pCMV-HA mammalian expression vector, using primers given in Table 4.4.

Fig 4.6 shows that full length YB-1 was able to precipitate full length RBBP6 (panel A, lane 2), but not the C-terminus fragment (panel B, lane 2). YB-1 was also able to precipitate the N-terminal DWNN domain (panel C, lane 2), although from the weak intensity of the band it may be inferred that the

interaction is weaker than with the RING finger. Nevertheless, Fig 4.6 suggests that both the DWNN and the RING finger interact with YB-1. However the region of YB-1 interacting with the DWNN domain may not be the same as that interacting with the RING finger.

Table 4.4. Primers for cloning N- and C-terminal of RBBP6 into pCMV-HA

Primer name	Primer sequence	Ta (°C)
N-terminal RBBP6 Forward	5′-GAGGCGCGGTCGACCTCCTGTGTGCATTATAAATTTTC-3′	55
N-terminal RBBP6 Reverse	5′-GAGGCGCTCGAGTTAGGCAGTCTTTGTAAGCTGGG-3′	55
C-terminal RBBP6 Forward	5′-GAGGCGCTCGAGGTCCTCCTCCACCACCCCCA-3′	55
C-terminal RBBP6 Reverse	5′-GAGGCGGCGGCCGCTTACACAGTGACAGATTTCACTT-3′	55

The nucleotide sequence in black font represents the sequence of the primer that first anneals to the DNA in the PCR reaction. An 'overhang' tag to facilitate restriction enzyme digestion is represented in green font, while the red and blue fonts represent the restriction enzyme site and incorporated stop codon respectively. Ta is the primer annealing temperature in degrees Celsius

4.9. RING finger ubiquitinates YB-1 *in vitro* and *in vivo*

Although it had been previously suggested that RBBP6 was an E3 ubiquitin ligase due to the presence of the RING finger domain[5], no substrate had previously been identified. In order to investigate whether YB-1 is ubiquitinated by the RING finger domain, ^{35}S-labelled full-length YB-1 and unlabelled RING finger protein were expressed in an *in vitro* transcription/translation system (Section 2.13.5 and 2.13.6) and used in an *in vitro* ubiquitination assay as described in Section 2.18.

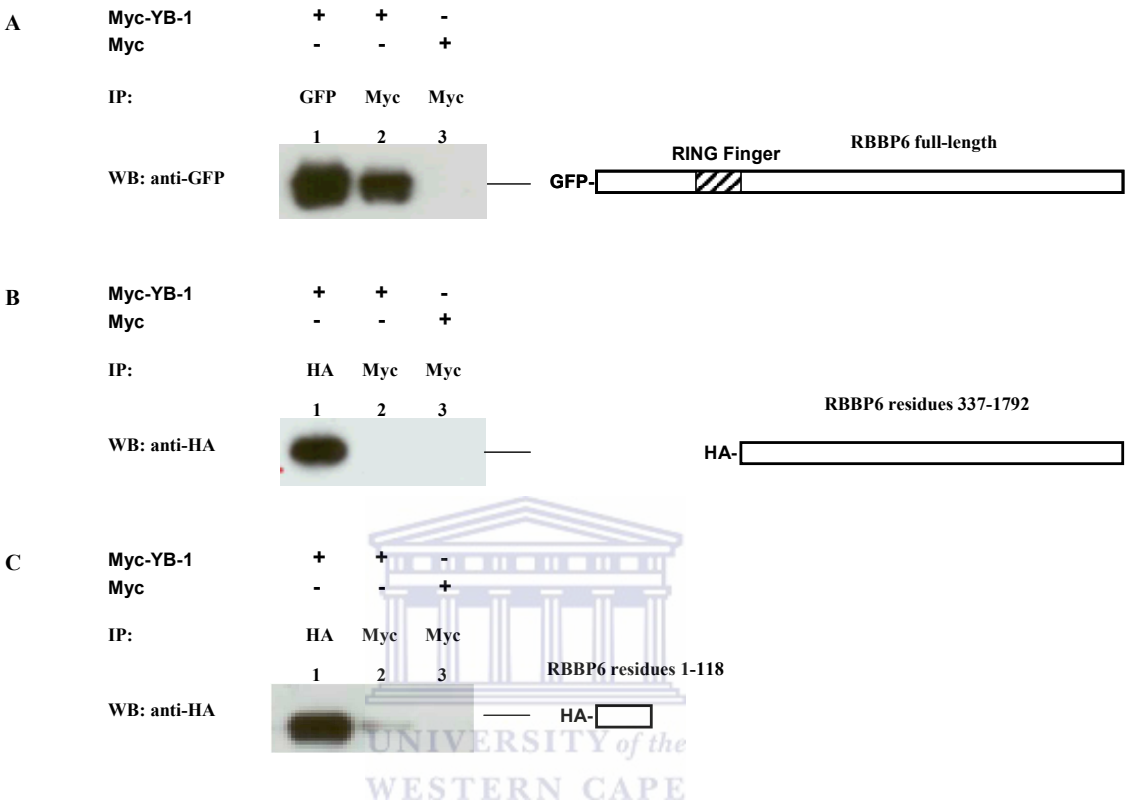


Figure 4.6. Full-length YB-1 interacts with full-length RBBP6 *in vivo*

HEK293 cells were co-transfected with Myc-YB-1 and either GFP-RBBP6 or HA-tagged fragments of RBBP6, as shown schematically on the right of the figure. (A) Myc-YB-1 was able to precipitate full-length GFP-RBBP6 (lane 2), but Myc alone was not (lane 3). However, Myc-YB-1 was not able to precipitate the C-terminal part of RBBP6 (B), which lacks the RING finger domain, although the N-terminal part, which contains the ubiquitin-like DWNN domain, interacted weakly with YB-1 (C, lane 2).

Higher molecular weight bands consistent with polyubiquitinated YB-1 were visible when YB-1 was incubated with RING finger and ubiquitin (see Fig 4.7) but not when either the RING finger or ubiquitin was omitted, indicating not only that the RING finger is capable of catalyzing the ubiquitination of YB-1 *in vitro* but also that it is ubiquitin that is being attached to YB-1 and not some other ubiquitin like-modifiers such as SUMO or NEDD8[39,160].

Full-length YB-1 for use in *in vitro* was first amplified from pEGFP-YB-1 (described in Section 4.7) using the primers given in Table 4.5 and then cloned into the *EcoRI* and *XhoI* sites of pACT2 vector. It was re-amplified using the pACT2 primers given in Table 2.1, to produce a fragment incorporating the T7 promoter which was then used to produce ^{35}S-labelled protein using the TNT *in vitro* transcription/translation system, as described in Section 2.13.5 and 2.13.6. Unlabelled RING finger was produced in the same manner described in Section 4.6, with substitution of ^{35}S-methionine for unlabelled methionine.

Table 4.5. Primers for cloning YB-1 full length into pACT2

Primer name	Primer sequence	Ta (°C)
YB-1 full length Forward	5′-GAGGGCCGAATTCAAAGCAGCGAGGCCGAGACC-3′	55
YB-1 full length Reverse	5′-GAGGGCACCTCGAGATTATACACAAAGACAATTATTTAAGACCT-3′	55

The nucleotide sequence in black font represents the sequence of the primer that first anneals to the DNA in the PCR reaction. An 'overhang' tag to facilitate restriction enzyme digestion is represented in green font, while the red and blue fonts represent the restriction enzyme site and incorporated stop codon respectively. Ta is the primer annealing temperature in degrees Celsius

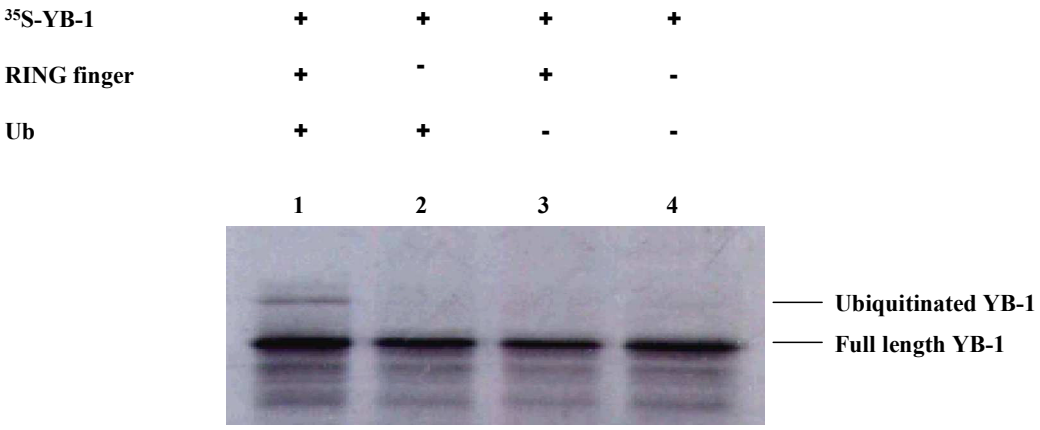


Figure 4.7. RING finger ubiquitinates YB-1 *in vitro*

Autoradiograph showing *in vitro* ubiquitination of YB-1 by RING finger protein. ^{35}S-labelled YB-1 and unlabelled RING finger were produced in an *in vitro* transcription/ translation system (Promega), and used in an *in vitro* S-100 *HeLa* ubiquitination assay system (Boston-Biochem). YB-1, RING finger and ubiquitin substrates were added as indicated. All reactions were precipitated with anti-YB-1 antibodies prior to SDS-PAGE analysis to amplify the signal. YB-1 was ubiquitinated in the presence of RING finger and ubiquitin (lane 1) but not when either or both of them were omitted (lanes 2–4).

4.10. YB-1 ubiquitination leads to proteasomal degradation

Modification of proteins by ubiquitin can have many consequences, including proteasomal degradation. In order to investigate whether YB-1 is degraded in the proteasome as a consequence of ubiquitination by RBBP6 or RING finger, HEK293 cells were co-transfected with Myc-YB-1 and increasing amounts of HA-RING or GFP-RBBP6; the amount of YB-1 present in the cells was visualised on Western blot using anti-Myc antibodies as shown in Figure 4.8. YB-1 levels decreased in a dose-dependent manner with increasing amounts of RING finger or full-length RBBP6. However, the effect was abolished when MG132 was added to block the proteasome, indicating that degradation by the proteasome is responsible for the decrease in YB-1 levels.

4.11. RING finger represses YB-1 transactivation

Since expression of RBBP6 leads to a decrease in intracellular levels of YB-1, a similar decrease in the transactivational activity of YB-1 is to be expected. To confirm this, HEK293 cells were transfected with pALUC, a plasmid encoding a firefly luciferase reporter gene under the control of the YB-1 inducible promoter. Transfection of HA-RING resulted in a dose-dependent decrease in the ability of endogenous YB-1 to activate a luciferase reporter as indicated by decrease in luciferase activity as shown in Figure 4.9. No such effect was observed when the RING finger was replaced with two control constructs (donated by Professor JC Moolman-Smook, Stellenbosch University, South Africa), encoding the C1 domain from human cardiac myosin binding protein C and cardiac troponin I, respectively.

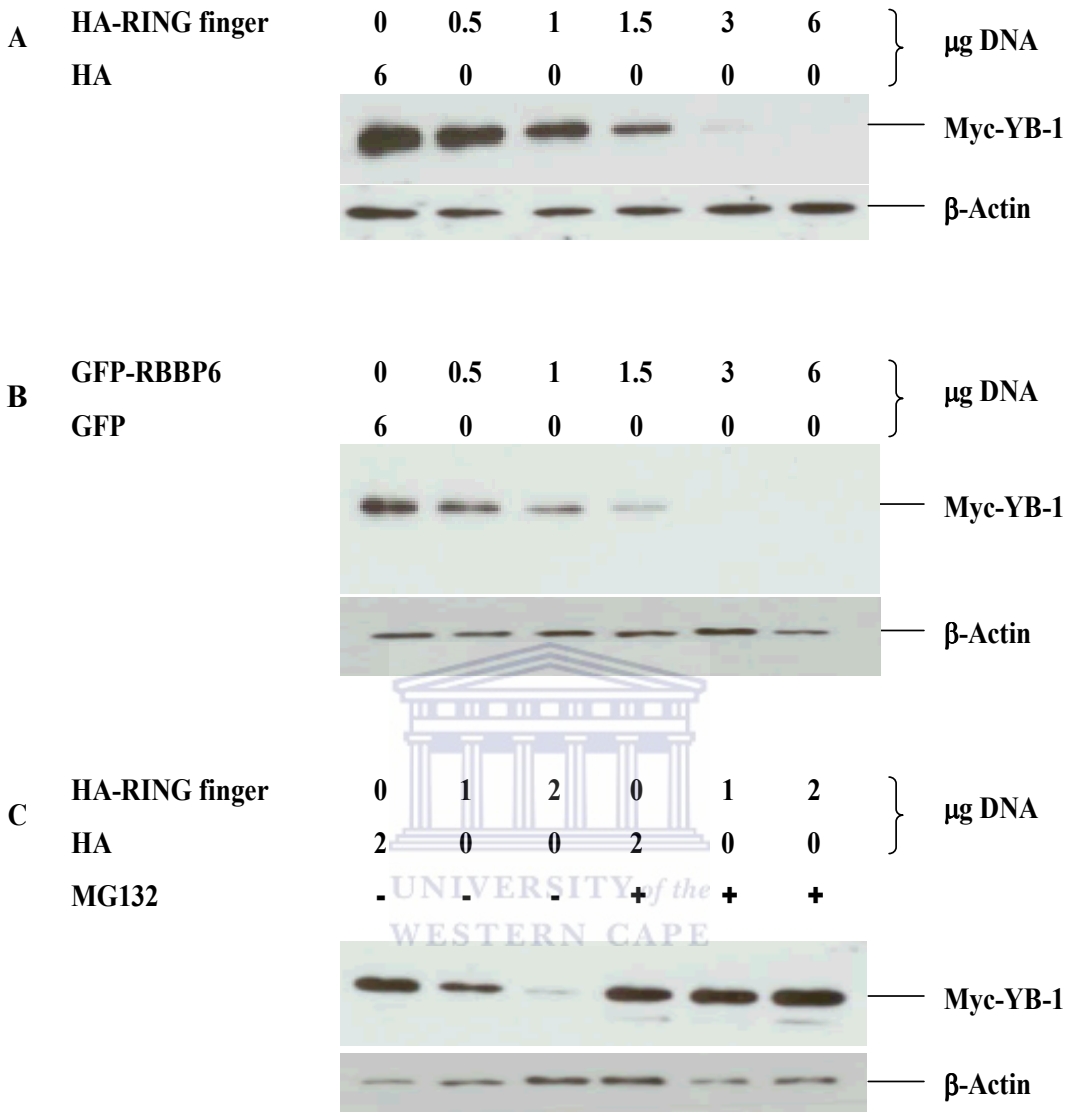


Figure 4.8. RBBP6 suppresses YB-1 levels *in vivo*.

HEK293 cells were co-transfected with Myc-YB-1 and with increasing amounts of HA-RING or GFP-RBBP6 as indicated. Six micrograms each of HA and GFP respectively served as controls. (A) Exogenously expressed HA-RING suppressed levels of exogenously expressed Myc-YB-1 in HEK293 cells in a dose-dependent manner. (B) A similar effect was observed using full-length GFP-RBBP6. (C) However, the effect was abolished following treatment of the cells with the proteasomal blocker MG132, indicating that the suppression of YB-1 is due to its degradation in the proteasome.

The Dual-luciferase Assay system (Promega) utilizes a second form of luciferase (*Renilla*) under the control of a constitutive SV-40 promoter to serve as an internal control. Typically this would be expected to show only small variations as was observed for the two control constructs (Figure 4.8 (B)). In the case of RING finger, however *Renilla* luciferase activity also decreased in a dose dependent manner, although much less than for the Firefly luciferase. Since a decrease of this magnitude is not likely to be due to variations in loading levels, it was concluded that *Renilla* was not suitable as a control. The Firefly luciferase activity values were therefore quoted in absolute terms, rather than normalized by the *Renilla* levels. A similar decrease was observed when the SV-40 driven *Renilla* reporter was replaced by a CMV-driven *Renilla* reporter (data not shown). The reason for the repression from these constitutive promoters is being investigated, but is outside the scope of this thesis.

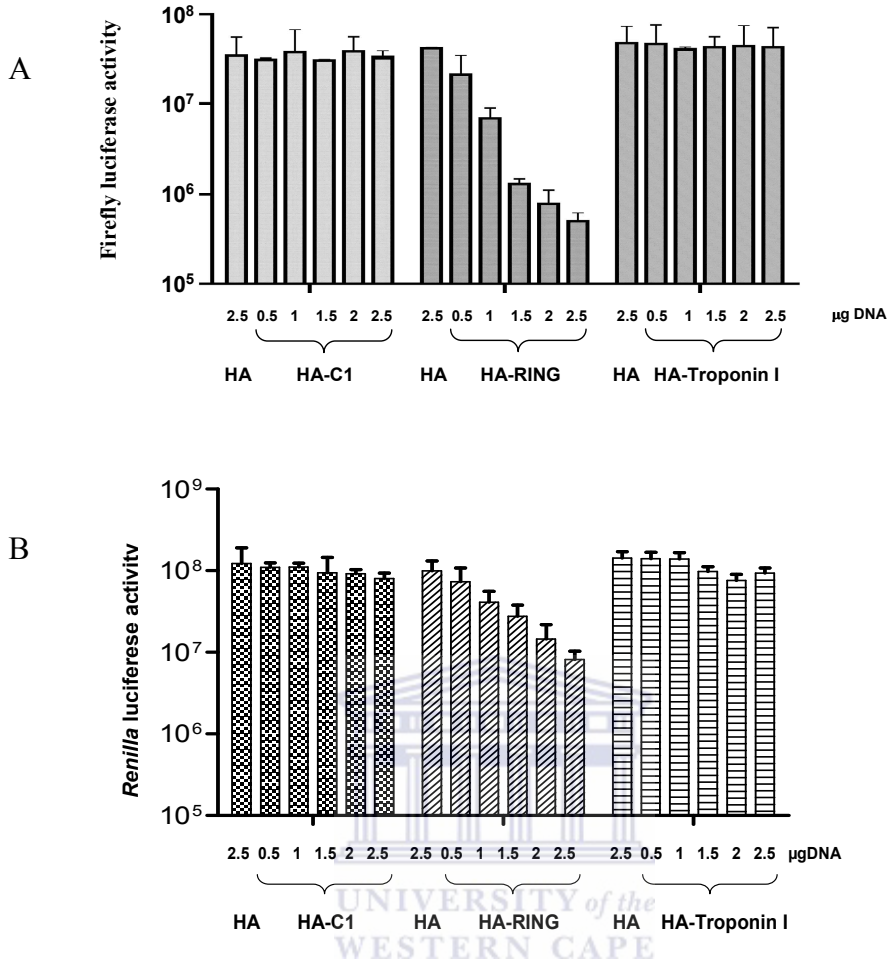


Figure 4.9. RING finger represses YB-1 transactivational activity

HEK293 cells were co-transfected with the YB-1-transducible reporter construct pALUC, pRL-SV40 (internal control) and increasing amounts of HA-RING as indicated. Luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega). (A) Exogenously expressed RING finger repressed expression of luciferase reporter driven from the YB-1-inducible promoter, consistent with the observed decrease in YB-1 levels (see Figure 4.8). No such effect was observed using two external control constructs, viz. the C1 domain from cardiac myosin binding protein C and cardiac troponin I. As the *Renilla* reporter was also suppressed by the RING in a dose-dependent fashion (panel B), luciferase values were not normalised with respect to *Renilla* values but are quoted in absolute terms. Bars indicate standard deviations calculated on the basis of three independent measurements.

4.12. zBTB38 binds to RING finger through its C-terminal region

zBTB38 is a second protein identified as interacting with the RING finger domain from RBBP6. zBTB38 contains an N-terminal BTB domain and a C-terminal region containing 10 clusters of zinc finger residues[161]. The region encoded by the Y2H prey clone corresponded to the last 631 amino acid residues on the C-terminal region of zBTB38 protein thereafter denoted as zBTB38-C as shown schematically in Figure 4.10.

4.13. RING finger co-immunoprecipitates zBTB38-C *in vitro*

To confirm the interaction in the absence of GAL4 domains, the RING finger and the C-terminal fragment of zBTB38 (zBTB38-C) were PCR amplified from their respective bait and prey plasmids to produce fragments that were used to express ^{35}S-labelled proteins in an *in vitro* transcription/translation system as described in section 2.13.5 and 2.13.6., incorporating Myc and HA tags, respectively. When subjected to SDS-PAGE the RING finger and zBTB38-C migrated with the expected sizes of 17 and 72 kDa, respectively (Figure 4.11, lanes 1 and 2). Immunoprecipitation with anti-Myc antibody in the presence of Myc-RING and HA-zBTB38-C resulted in the detection of HA-zBTB38-C (lane 3), whereas no HA-zBTB38-C was precipitated in the absence of Myc-RING (lane 4), indicating the presence of an interaction between them.

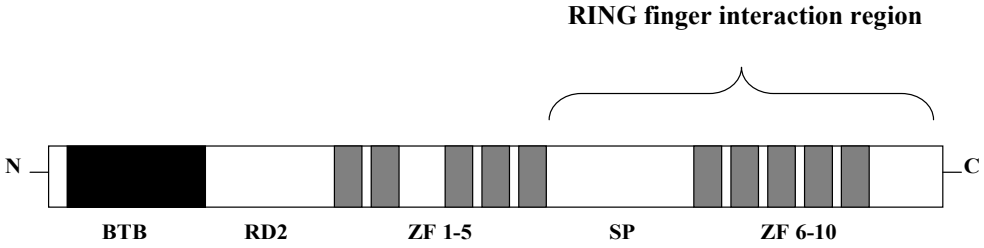


Figure 4.10. Mapping of zBTB38/RING interaction region

The prey plasmid containing the zBTB38 construct that was identified in Y2H screen encoded the last 631 amino acid residues of zBTB38 as indicated. The structural organization of zBTB38 comprises a BTB domain, repression domain 2 (RD2) and a C-terminal region consisting of 10 clusters of Zinc fingers containing a spacer domain (SP) spacing the Zinc fingers apart.

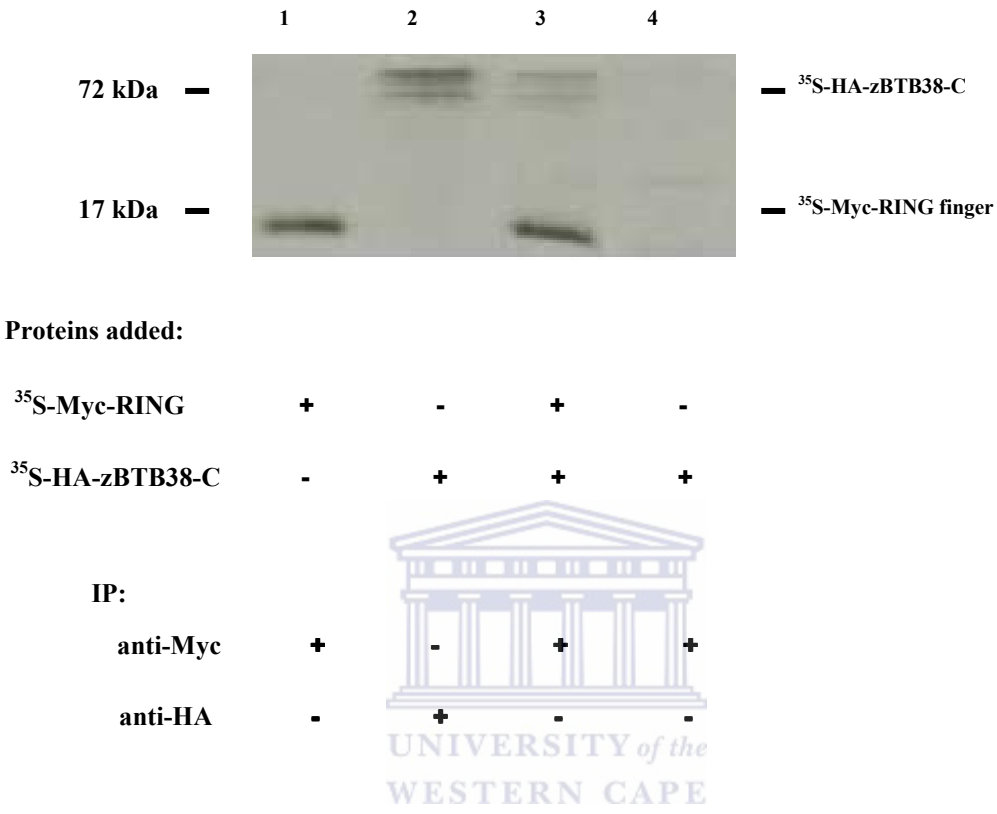


Figure 4.11. RING finger co-immunoprecipitates zBTB38-C *in vitro*

Autoradiograph of the immunoprecipitation assays of Met-^{35}S-labelled *in vitro*-transcribed/translated proteins; antibodies used in the immunoprecipitation reactions are as indicated. Immunoprecipitation with anti-Myc antibody resulted in co-immunoprecipitation of the 72-kDa HA-zBTB38-C fragment in the presence (lane 3), but not in the absence (lane 4), of the 17 kDa Myc-RING. Lanes 1 and 2 are individual immunoprecipitates of Myc-RING finger and HA-zBTB38-C respectively to serve as markers for the expected sizes of the proteins.

4.14. zBTB38 interacts with RING finger and RBBP6 *in vivo*

Following confirmation of the interactions using *in vitro* co-immunoprecipitation assays, the interaction was further verified in transfected mammalian cells. A mammalian construct encoding full length RFP-zBTB38 was a kind donation from Dr Pierre-Antoine Defossez, Paris University, France[162]. RFP-zBTB38, Myc-RING finger and GFP-RBBP6 were co-transfected into HEK293 cells as shown in Figure 4.12 and co-immunoprecipitation assays performed after 48 hours. Western blotting was carried out using anti-RFP antibodies. Figure 4.12 shows that both full-length RBBP6 and the RING finger were able to precipitate full-length zBTB38 *in vivo*.

4.15. RBBP6 co-localizes with zBTB38

A preliminary fluorescence microscopy study was conducted to determine whether RBBP6 and zBTB38 can be observed occupying the same intracellular compartment. HEK293 cells were transfected with GFP-RBBP6 and RFP-zBTB38 after which live cell images were acquired on a Zeiss LSM510 Confocal Microscope (Carl Zeiss MicroImaging Inc.). GFP-RBBP6 appears as green, RFP-zBTB38 as red and areas of co-localization appear as yellow. The cell nucleic region is shown in blue due to Hoechst stain. Both proteins are localized within the nucleus. Areas of yellow can be seen within the nucleus, indicating co-localization of the proteins within those regions. Intriguingly, the overlapping of the proteins is observed in defined subnuclear structures that may correspond to nuclear speckles. This observation is consistent with the expected localization of zBTB38, which is known to be a nuclear associated protein that is recruited to the chromocenters[163]

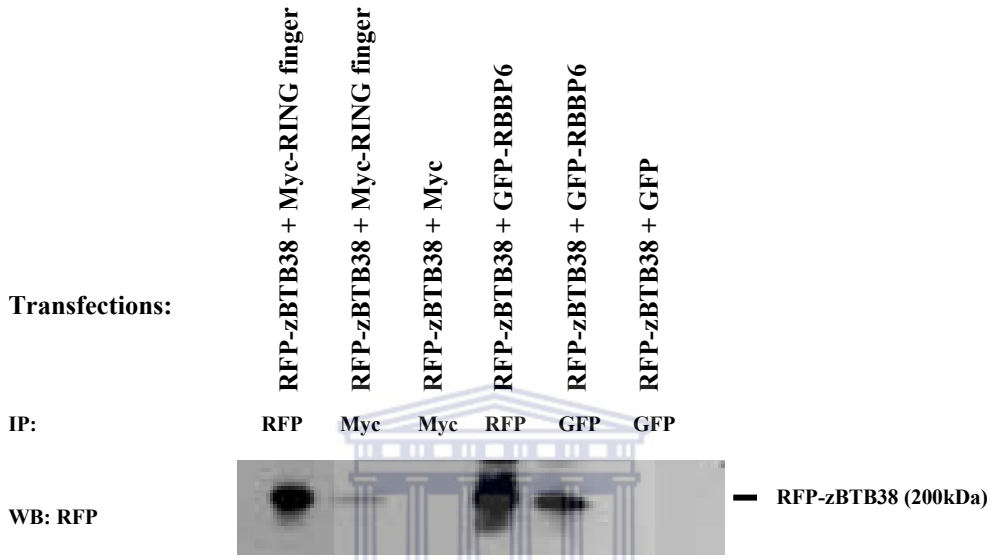


Figure 4.12. Exogenous RING finger and full length RBBP6 co-immunoprecipitate exogenous zBTB38 *in vivo*

Exogenous full length RFP-zBTB38 was imminoprecipitated by anti-Myc antibodies in the presence of (lane 2), but not in the absence (lane 3) of exogenous RING finger. Similarly, RFP-zBTB38 was co-immunoprecipitated in the presence (lane 5) but not in the absence (lane 6), of full length RBBP6. RFP-zBTB38 was detected using anti-RFP antibodies.

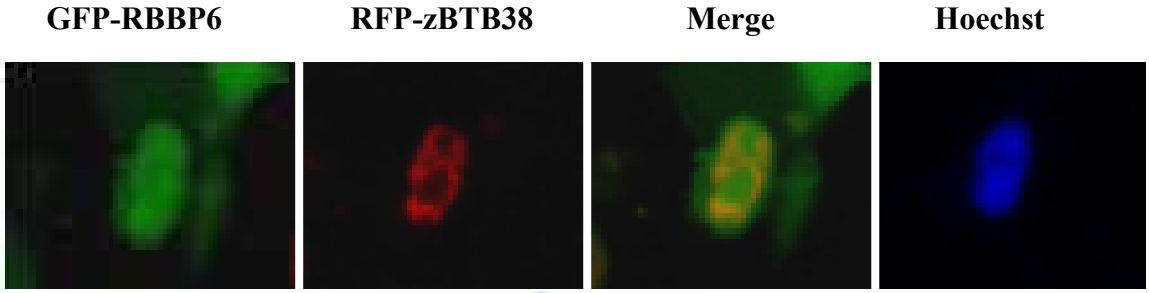


Figure 4.13. RBBP6 co-localizes with zBTB38 in transfected cells

The co-localization image that was taken on live cells, showing an overlap of exogenous RBBP6 and exogenous zBTB38 localisation in live cells. The co-localisation was observed in defined structures within the nuclear region, which may correspond to nuclear speckles.

4.16. RING finger polyubiquitinates zBTB38 *in vitro*

In section 4.9 it was shown that the interaction of RING finger with YB-1 leads to ubiquitination of YB-1. To investigate whether zBTB38 is ubiquitinated by the RING finger domain, ^{35}S-labelled HA-zBTB38-C and unlabelled Myc-RING finger protein were expressed in an *in vitro* transcription/translation system the same way as in Section 4.9 and used in an *in vitro* ubiquitination assay again as described in Section 4.9. All reactions were precipitated with anti-HA antibodies, subjected to SDS-PAGE and visualized by autoradiograph as shown in Figure 4.14.

Higher-molecular-weight bands consistent with polyubiquitinated zBTB38-C were visibly enhanced when zBTB38 was incubated with RING finger (lanes 3-5) compared to the ubiquitination that occurred in the absence of RING finger (lane 2), thereby confirming that the RING finger enhances the ubiquitination of zBTB38-C. Full length zBTB38 could not be used as an ubiquitination substrate because the efficiency of *in vitro* translation was too low.

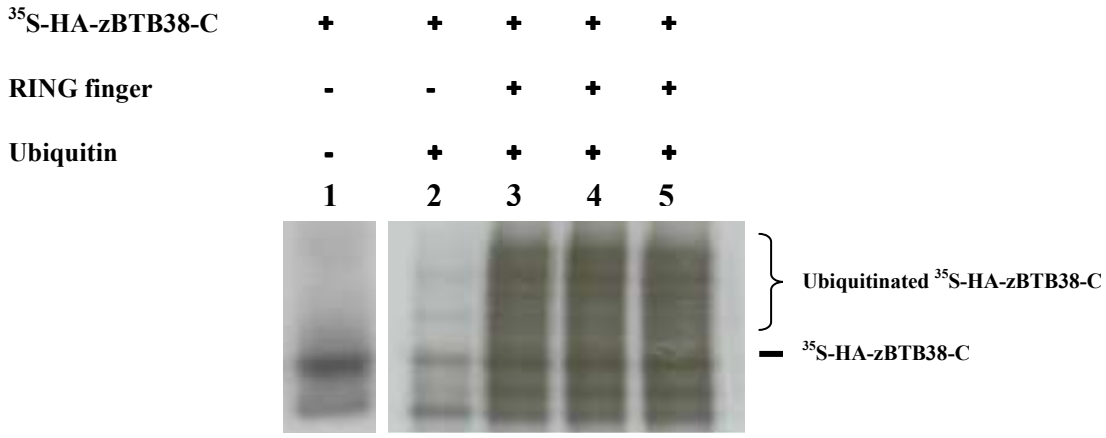


Figure 4.14. RING finger enhances ubiquitination of zBTB38 *in vitro*.

^{35}S-labelled zBTB38-C and unlabelled RING finger were produced in an *in vitro* transcription/translation system and added to an *in vitro* S-100 *HeLa* ubiquitination kit as indicated. All reactions were precipitated with anti-HA antibodies prior to SDS-PAGE analysis to amplify the signal. RING finger enhances *in vitro* ubiquitination of zBTB38-C as shown in lanes 3-5, compared to levels of ubiquitination in the absence of RING finger (lane 2).

Chapter 5: Y2H screen using DWNN domain as yeast bait

5.1. Introduction

5.2. Yeast library matings

5.3. Interaction specificity tests

5.4. The identification of putative prey interactors

5.5. DWNN co-immunoprecipitates snRPG *in vitro*

5.6. DWNN co-immunoprecipitates snRPG *in vivo*

5.7. snRPG interacts with full length RBBP6 *in vivo*

5.8. RBBP6 interacts with heat shock protein 70kDa *in vivo*

CHAPTER 5: Y2H screen using DWNN domain as yeast bait

5.1. Introduction

The N-terminus of all members of the RBBP6 family comprises of an ubiquitin-like domain known as the DWNN domain that is also expressed as an independent domain in higher eukaryotes[5]. Due to the function of RBBP6 as an E3 ubiquitin ligase, the DWNN domain is likely to play some role in ubiquitination, although the exact nature of the role is not yet known. Pugh and colleagues speculated that the independently expressed domain may act as a novel ubiquitin-like modifier similar to SUMO or NEDD8[5]. Another possibility is that the domain acts as a protein-protein interaction motif, recruiting E2 enzymes or substrates to the RING finger domain.

This chapter describes a Y2H screen of a human testis cDNA library using the DWNN domain from RBBP6 as bait. A number of putative interactors were identified and 2 of these were confirmed using co-immunoprecipitation assays, both *in vitro* and *in vivo*.

5.2. Yeast library matings

pGBKT7-D was transformed into *Saccharomyces cerevisae* strain AH109 and used to screen a commercial human testis cDNA library cloned into the pACT2 vector that was received pre-transformed into *Saccharomyces cerevisae* strain Y187 (Clontech, USA). The screening of an estimated 5.4 x10^6 library clones yielded 520 clones in which transcription of the interaction-reporter gene *HIS3* was activated as evidenced by their ability to grow on TDO selection medium (medium lacking leucine, tryptophan and histidine).

The yeast clones from TDO medium plates were transferred to QDO selection medium (medium lacking leucine, tryptophan, histidine and adenine) on which 102 clones survived, indicating additional activation of transcription of the interaction-reporter gene *ADE2*. These clones were then transferred to QDO selection medium supplemented with X-α-gal (a chromogen substrate for α-galactosidase enzyme) to select yeast clones that activate transcription of another interaction-reporter gene *MEL1* by show of blue colony coloration. There were 55 yeast clones that exhibited consistency in activating three interaction-reporter genes (*HIS3*, *ADE2* and *MEL1*), and these clones were further subjected to interaction specificity bait mating tests.

5.3. Interaction specificity tests

Prey plasmids from the 55 clones expressing all 3 reporter genes were extracted and re-transfromed into yeast strain Y187 in a similar fashion as described in Section 4.3 and subsequently used to test the specificity of the interaction as again described in Section 4.3. An example of a prey that interacted specifically with the DWNN domain is shown in Fig 5.1. Table 5.1 shows that of the 55 putative preys, 21 showed strong interaction with DWNN and little or no interaction with other heterologous baits (indicated in red).

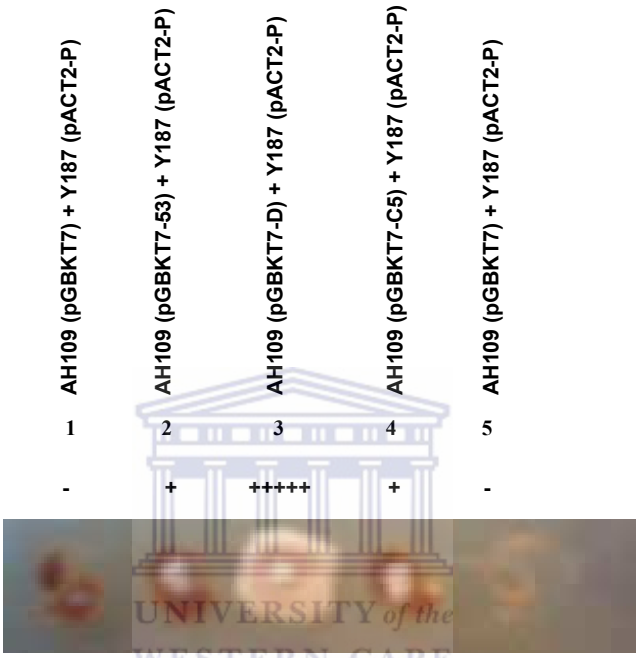


Figure 5.1. An example of library prey (pACT2-P) that interacts DWNN only

Diploid colonies containing the prey plasmid (pACT2-P) grew on QDO medium only in the presence of DWNN-containing bait plasmid pGBKT7-D (lane 3), but not in the presence of p53 (lane 2), C5 (lane 4) or parental plasmids (lanes 1 and 5). The complete set of results is shown in Table 5.1.

Table 5.1. Scoring for yeast growth after heterologous bait matings

Clone number	AH109 (pGBKT7) + Y187 (pACT2-P)	AH109 (pGBKT7-53) + Y187 (pACT2-P)	AH109 (pGBKT7-D) + Y187 (pACT2-P)	AH109 (pGBKT7-C5) + Y187 (pACT2-P)	AH109 (pGBKT7) + Y187 (pACT2-P)
11	-	-	-	-	-
19	+++++	++++	+++++	++++	+++++
20	-	-	+++++	-	-
24	-	+	+++++	-	-
26	-	-	+++++	+	-
29	-	+	+++++	+	-
31	+++++	++++	+++++	++++	+++++
38	+++++	+++++	++++	++++	+++++
44	+++++	+++++	+++++	+++++	+++++
47	-	+	+++++	+	-
56	+++++	+++++	+++++	+++++	+++++
62	-	-	+++++	-	-
63	+++++	+++++	+++++	+++++	+++++
64	+++	+++	+++	+++	+++
66	+++++	+++++	++++	++++	+++++
67	+++	+++	+++	+++	+++
68	+++++	++++	+++++	++++	+++++
74	+++++	+++++	+++++	+++++	+++++
76	+++++	+++++	+++++	+++++	+++++
81	+++	+++	+++	+++	+++
82	+++++	++++	+++++	+++++	+++++
83	+++++	+++++	+++++	++++	+++++
85	+++	+++	+++	+++	+++
88	+++++	+++++	+++++	+++++	+++++
93	-	-	+++++	-	-
95	+++++	+++++	+++++	+++++	+++++
97	+++++	+++++	+++++	+++++	+++++
99	-	+	+++++	-	-
104	-	-	+++++	+	-

Clone number	AH109 (pGBKT7) + Y187 (pACT2-P)	AH109 (pGBKT7-53) + Y187 (pACT2-P)	AH109 (pGBKT7-D) + Y187 (pACT2-P)	AH109 (pGBKT7-C5) + Y187 (pACT2-P)	AH109 (pGBKT7) + Y187 (pACT2-P)
108	+++	+++	+++	+++	+++
127	+++++	++++	++++	++++	+++++
131	-	-	+++++	-	-
139	+++++	+++++	+++++	+++++	+++++
140	+++++	+++++	+++++	+++++	+++++
150	-	-	+++++	+	-
151	+++++	+++++	++++	+++++	+++++
160	-	+	+++++	-	-
167	+++++	+++++	+++++	+++++	+++++
176	+++	+++	+++	+++	+++
176	-	-	+++++	-	-
177	+++++	+++++	+++++	+++++	+++++
178	+++++	+++++	+++++	+++++	+++++
179	-	-	+++++	-	-
181	+++	+++	+++	+++	+++
190	+++++	+++++	+++++	+++++	+++++
210	-	+	+++++	-	-
211	-	-	+++++	-	-
212	-	-	+++++	-	-
225	+++++	+++++	+++++	+++++	+++++
231	-	+	+++++	-	-
232	+++++	+++++	+++++	+++++	+++++
232	+++	+++	+++	+++	+++
234	+++++	+++++	+++++	++++	+++++
235	-	-	+++++	-	-
242	-	-	+++++	-	-

5.4. The identification of putative prey interactors

The Y2H screen identified 21 putative interactors that consistently activated the yeast interaction-specific reporter genes *HIS3*, *ADE2* and *MEL1* as well as showing interaction-specificity with the DWNN bait only. The identified putative preys were sequenced using the fluorescent dye-terminator cycle sequencing method (3130xl Genetic Analyzers, Applied Biosystems Inc CA, USA) and their identity determined by BLAST searches of Human Genome database sequences as well as encoded proteins as shown in Table 5.1

On analyzing the DNA sequences encoding these putative interactors, 8 prey sequences gave rise to 6 preys with known identity from which 2 DNA sequences repeatedly identified the same prey. The other 13 prey constructs were discarded since their sequences did not correspond to known proteins in either NCBI Entrez (www.ncbi.nlm.nih.gov) or Ensemble (www.ensembl.org) protein databases. This, however, is expected as only one sixth of the clones represented in Matchmaker™ pre-transformed oligo-dT primed cDNA libraries represent known protein products (Clontech MATCHMAKER Two-Hybrid Assay Kit User Manual).

Among the 6 identified preys, 2 preys encoded small nuclear ribonucleoprotein polypeptide G and heat shock 70kDa protein 14 (see Table 5.2) and were prioritized for further verification assays. The reasons they were initially chosen were as follows: (i) both preys were identified more than once thereby increasing the likelihood of a genuine interaction, (ii) previous studies about the prey proteins gave an indication that their interactions with RBBP6

may provide physiological relevant information. For instance, RBBP6 has been previously linked to splicing role[1] so, its interaction with snRPG, a splicing factor, may be physiologically relevant. Also proteins containing both ubiquitin like domain and RING finger domain such as BAG-1/CHIP protein complex have been found to associate with heat shock protein 70[35,36] to facilitate chaperone dependent ubiquitination and similarly, association of RBBP6 with Hsp70 may unveil another novel role of RBBP6.

Table 5.2. Putative DWNN ligands identified from Y2H library screen

Clone Number	*Nucleotide Blast identity*	*Accession Number (E-values)*	*In-frame Protein Hit*	*Accession Number (E-values)*
93	Homo sapiens heat shock 70kDa protein 14 (HSPA14), transcript variant 1	NM_016299.2 (0.0)	Heat shock 70kDa protein 14 [Homo sapiens]	NP_057383 (2e-41)
131	Homo sapiens heat shock 70kDa protein 14 (HSPA14), transcript variant 1	NM_016299.2 (8e-86)	Heat shock 70kDa protein 14 [Homo sapiens]	NP_057383 (2e-23)
211	Homo sapiens small nuclear ribonucleoprotein polypeptide G (SNRPG)	NM_003096 (0.0)	Small nuclear ribonucleoprotein polypeptide G [Homo sapiens].	NP_003087 (2e-35)
62	Homo sapiens small nuclear ribonucleoprotein polypeptide G (SNRPG)	NM_003096.2 (0.0)	Small nuclear ribonucleoprotein polypeptide G [Homo sapiens]	NP_003087 (4e-36)
20	Homo sapiens DnaJ (Hsp40) homolog, subfamily B, member 1 (DNAJB1)	NM_006145 (0.0)	DnaJ (Hsp40) homolog, subfamily B, member 1 [Homo sapiens]	NP_006136 (3e-35)
104	Homo sapiens RAN binding protein 9 (RANBP9)	NM_005493.2 (0.0)	RANBP9 protein [Homo sapiens].	AAH52781 (6e-101)
150	Homo sapiens gametogenetin (GGN)	NM_152657.3 (0.0)	Gametogenetin protein 1a [Homo sapiens].	AAP31500 (1e-52)
176	Homo sapiens Niemann-Pick disease, type C2 (NPC2)	NM_006432 (0.0)	Niemann-Pick disease, type C2 [Homo sapiens]	EAW81178 (5e-39)
47	Homo sapiens chromosome 20 genomic contig, reference assembly	NT_028392.5 (0.0)	No significant similarity found	-

24	Homo sapiens misc_RNA (MGC88374), miscRNA	XR_041747 (5e-97)	No significant similarity found	-
179	Homo sapiens misc_RNA (MGC88374), miscRNA	XR_041747.1 (4e-85)	No significant similarity found.	-
212	Homo sapiens nuclear receptor interacting protein 1 (NRIP1)	NM_003489.2 (0.0)	No significant similarity found	-
231	Homo sapiens misc_RNA (LOC653602), miscRNA	XR_041664.1 (0.0)	No significant similarity found	-
26	Homo sapiens CDC28 protein kinase regulatory subunit 2 (CKS2)	NM_001827.1 (8e-112)	No significant similarity found	-
242	Homo sapiens chromosome 9 genomic contig, reference assembly	NT_008413.17 (0.0)	No significant similarity found	-
210	Homo sapiens chromosome 15 genomic contig, reference assembly	NT_010194.16 (0.0)	No significant similarity found	-
160	Homo sapiens chromosome 8 genomic contig, reference assembly	NT_008183.18 (2e-168)	No significant similarity found	-
99	Homo sapiens chromosome 1 genomic contig, reference assembly	NT_021877.18 (1e-177)	No significant similarity found	-
29	Homo sapiens prion protein 2 (dublet) (PRND)	NM_012409.2 (0.0)	No significant similarity found	-
235	Unnamed protein product [Homo sapiens]	BAC03955 (2e-10)	No significant similarity found	-
95	Homo sapiens chromosome 12 genomic contig, reference assembly	NT_009714.16 (0.0)	No significant similarity found	-

5.5. DWNN co-immunoprecipitates snRPG *in vitro*

To confirm the above interaction in the absence of GAL4 domains, the DWNN and the Y2H screen-identified small nuclear ribonucleoprotein polypeptide G (snRPG) were PCR amplified from their respective bait and prey plasmids to produce PCR fragments that were used to express as ^{35}S-labelled proteins in an *in vitro* transcription/translation system, incorporating Myc and HA tags,

respectively. When subjected to an SDS-PAGE the DWNN and snRPG proteins migrated with the expected sizes of 15 and 12 kDa, respectively (Figured 5.2, lanes 1 and 2). Immunoprecipitation with anti-Myc antibody in the presence of Myc-DWNN and HA-snRPG resulted in the detection of HA-snRPG (lane 3), whereas no HA-snRPG was immunoprecipitated in the absence of Myc-DWNN (lane 4), indicative of an interaction between them.

5.6. DWNN co-immunoprecipitates snRPG *in vivo*

Following confirmation of the interactions using *in vitro* co-immunoprecipitation assays, the interaction was further followed up *in vivo* to confirm whether the interaction between DWNN and snRPG takes place within the cell. Constructs encoding DWNN and snRPG were subcloned into the *SalI/XhoI* and *EcoRI/XhoI* sites, respectively, of both pCMV-HA and pCMV-Myc plasmids using the primers as shown in Table 5.3, to generate pHA-DWNN, pMyc-DWNN, pHA-snRPG and pMyc-snRPG constructs.

Table 5.3. Primers for cloning DWNN/ snRPG into pCMV-HA and pCMV-Myc

Primer name	Primer sequences	Ta (°C)
DWNN Forward	5′-GAGGCGCGGTCGACCTCCTGTGTGCATTATAAATTTTC-3′	55
DWNN Reverse	5′-GAGGCGCTCGAGTTAGGCAGTCTTTGTAAGCTGGG-3′	55
snRPG Forward	5′-GAGGCGCGAATTCGGAGCAAAGCTCACCCTCCCG-3′	55
snRPG Reverse	5′-GAGGCGCTCGAGTTATACTCGTTCCAAGGCTTC-3′	55

The nucleotide sequence in black font represents the sequence of the primer that first anneals to the DNA in the PCR reaction. An 'overhang' tag to facilitate restriction enzyme digestion is represented in green font, while the red and blue fonts represent the restriction enzyme site and incorporated stop codon respectively. Ta is the primer annealing temperature in degrees Celsius

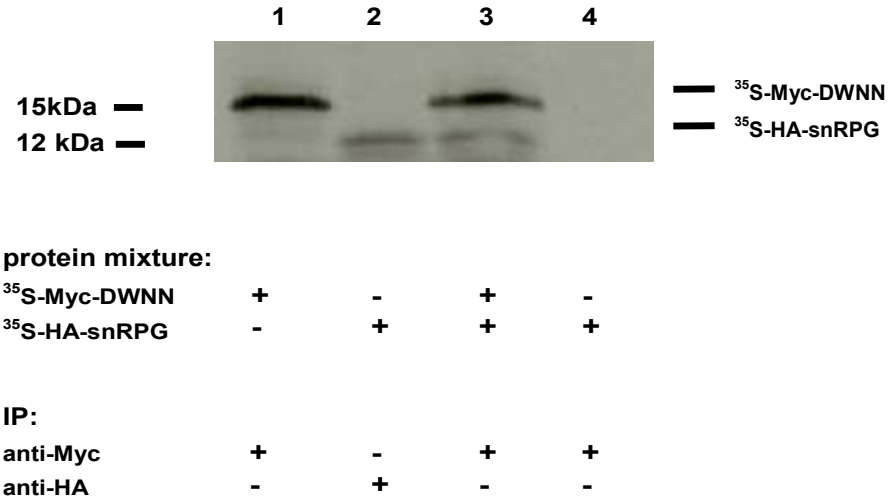


Figure 5.2. DWNN co-immunoprecipitates snRPG proteins *in vitro*

Autoradiograph of immunoprecipitation of ^{35}S-labelled *in vitro* transcribed /translated proteins; antibodies used in the immunoprecipitation reactions are as indicated. Immunoprecipitation with anti-Myc antibody resulted in co-immunoprecipitation of the 12-kDa HA-snRPG fragment in the presence (lane 3), but not in the absence (lane 4), of the 15 kDa Myc-DWNN. Lanes 1 and 2 are individual immunoprecipitates of Myc-DWNN and HA-snRPG respectively.

Co-transfection of appropriate combinations of HA- and Myc-tagged constructs was carried out using HEK293 cells and immunoprecipitations were performed using anti-HA and anti-cMyc agarose conjugated antibodies. Exogenous DWNN and snRPG were able to precipitate each other as shown in Figure 5.3.

5.7. snRPG interacts with full length RBBP6 *in vivo*

It was investigated whether the interaction of snRPG with DWNN was true in the context of the full length RBBP6. Using a GFP-tagged full-length cDNA construct of RBBP6, it was shown that exogenous snRPG was able to precipitate full-length RBBP6 but not with truncated versions C-terminal RBBP6 fragment and RING finger domain lacking the DWNN domain indicating the interaction of snRPG with RBBP6 is via the DWNN domain as shown in Figure 5.4. The primers for generating HA-RING finger and C-terminal RBBP6 are the same as used in Figures 4.3 and 4.4 respectively.

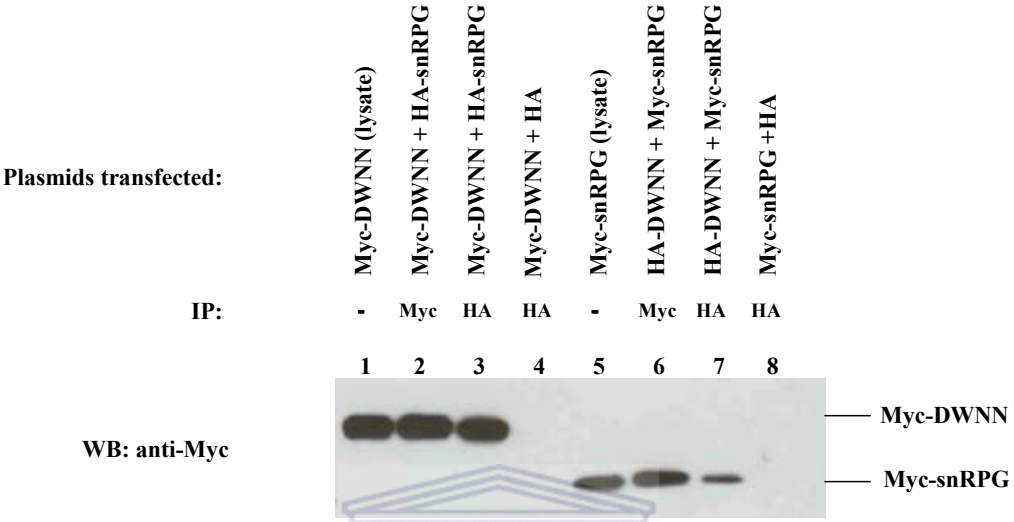


Figure 5.3. DWNN immunoprecipitates snRPG *in vivo* and *vice versa*

HEK293 cells were transfected with combinations of HA- and Myc-tagged constructs as indicated and immunoprecipitated using the indicated agarose conjugated antibodies. Anti-HA antibodies were able to precipitate Myc-DWNN in the presence (lane 3), but not in the absence (lane 4), of HA-snRPG. Conversely, anti-HA antibodies were able to precipitate Myc-snRPG in the presence (lane 7) but not in the absence (lane 8) of HA-DWNN, thereby confirming interaction of the two proteins.

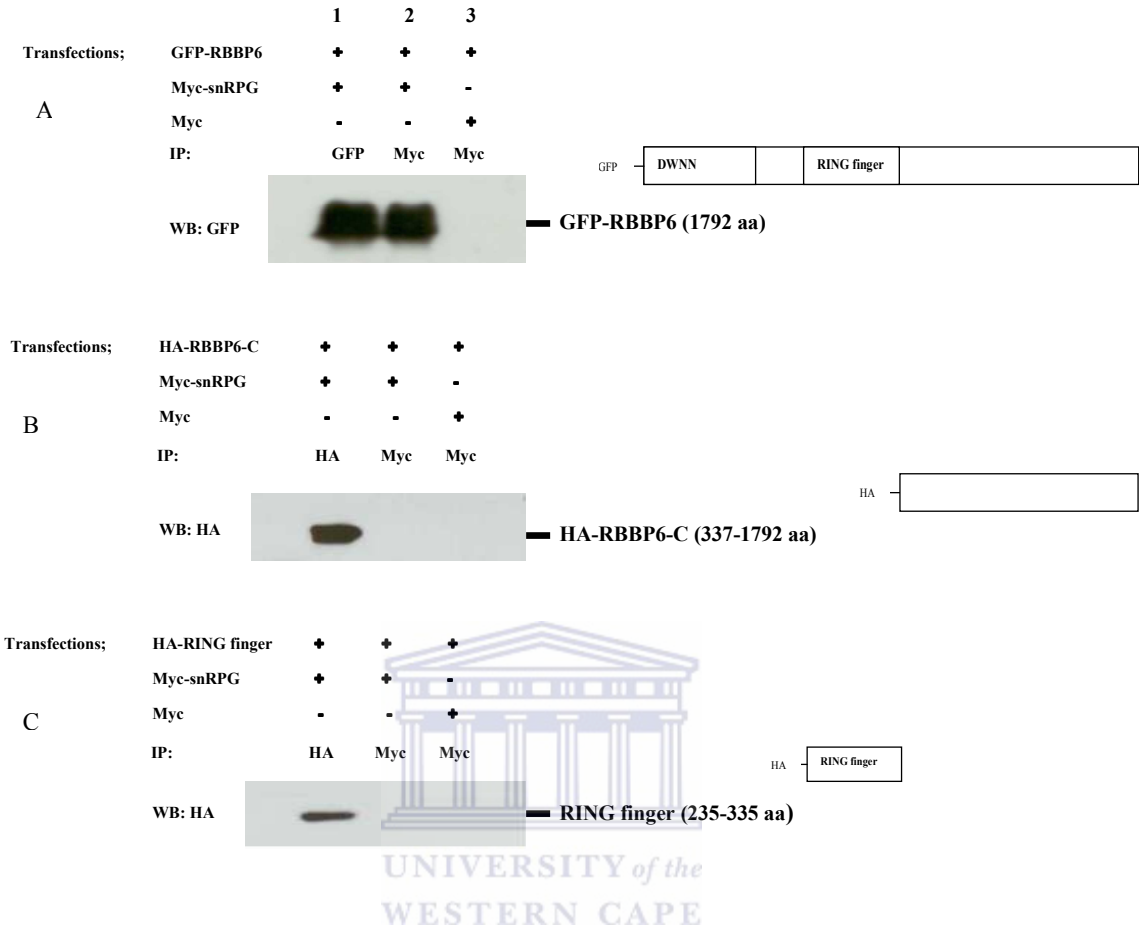


Figure 5.4. snRPG interacts with full- length RBBP6 *in vivo*

HEK293 cells were co-transfected with Myc-snRPG as indicated, and GFP-RBBP6 or HA-tagged regions of RING finger and RBBP6-C as shown. Myc-snRPG was able to precipitate full-length GFP-RBBP6 (A lane 2), but Myc alone was not (lane 3). However, Myc-snRPG was not able to precipitate either the C-terminal part of RBBP6 (panel B lane 2) or the RING finger (panel C lane 2) lacking the DWNN domain indicating snRPG interacts with RBBP6 only through the N-terminal region (residues 1-234), which contains the DWNN domain and the zinc finger domain.

5.8. RBBP6 interacts with heat shock protein 70kDa *in vivo*

Heat shock 70kDa protein 14 (HSPA14), was identified more than once in the Y2H screen as shown in Table 5.1. Both prey constructs encoded the C-terminal 86 amino acids of the protein. The gene that encodes HSPA14 is one of 21 human genes encoding proteins belonging to heat shock protein 70 (Hsp70) subfamily. HSPA14 is both structurally and functionally similar to other members of Hsp70 protein including the heat shock 70kDa protein 1 (HSPA1) that has been previously shown to interact with proteins containing ubiquitin-like domain[164,165]. HSPA14 shares common receptors on human dendritic cells (DCs) with Hsp70 and can interact with DCs, promoting DC maturation and stimulating secretion of the proinflammatory cytokines interleukin 12p70 (IL-12p70), IL-1β, tumor necrosis factor-α (TNF-α), and the chemokines IP-10, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and normal T cell expressed and secreted (RANTES)[166].

Because of the similarities that exist between HSPA14 and HSPA1 and the availability of commercial anti-HSPA1 antibodies, it was decided to confirm whether the interaction existed between the DWNN domain and HSPA1, which will be referred to in what follows simply as Hsp70.

To investigate if endogenous Hsp70 interacts with RBBP6 *in vivo* as well as mapping the region of RBBP6 interaction, single transfections in HEK293 cells were carried out with previously discussed full length GFP-RBBP6, Myc-DWNN domain (residues 1-118 aa), Myc-RING finger domain (residues 235-335 aa) and HA-RBBP6-C (residues 337-1792 aa) as shown in Figure 5.5.

Immunoprecipitation of the respective exogenous proteins via their respective tags and detection with anti-Hsp70 antibody (anti-HSPA1) (Stressgen) led to the result shown if Fig 5.5. Endogenous Hsp70 could be precipitated by full length RBBP6 (lane 2), by the DWNN domain (lane 4), by the RING finger (lane 3), but not by the C-terminus of RBBP6 (lane 6).

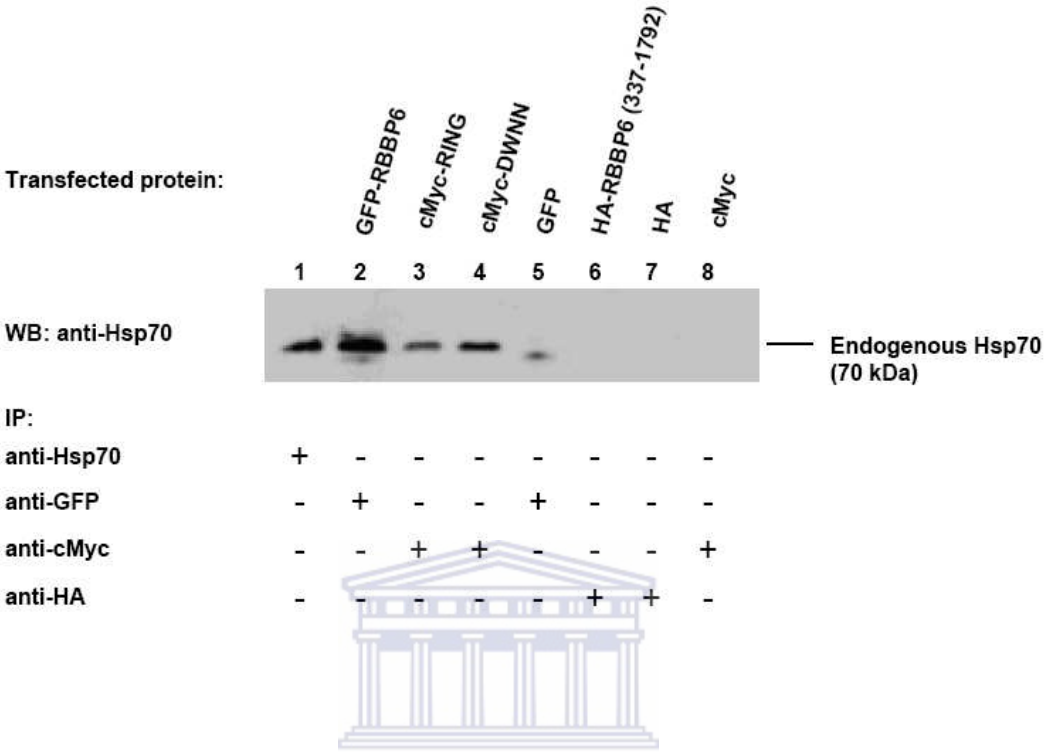


Figure 5.5. RBBP6 co-immunoprecipitates endogenous Hsp70

HEK293 cells were transfected various tagged fragments of RBBP6 as shown. Immunoprecipitation of these tagged proteins using their respective antibodies, Hsp70 was precipitated by RBBP6, DWNN and RING finger but the C-terminal RBBP6 (337-1792) did not result in Hsp70 precipitation (Lane 2, 3, 4 and 6). As negative controls, transfection of HEK293 cells with non recombinant parental vectors containing GFP, Myc or HA did not result in Hsp70 pull-down thereby indicating that Hsp70 precipitation was due to its interaction with respective proteins as indicated (Lane 5, 7 and 8). Also as positive control for the expected size of Hsp70, the Hsp70 was precipitated by the anti-Hsp70 antibodies and detected by the same antibody. Lane 1 is loaded with untransfected cell lysate as a control marker for the presence of Hsp70 endogenous protein.

CHAPTER 6: GENERAL DISCUSSION

CHAPTER 6: GENERAL DISCUSSION

6.1. Introduction

The study focused on characterizing RBBP6 through identification of its protein interactors using Y2H system. Among other approaches that include analysis of gene expression patterns and phylogenetic profiles, protein-protein interaction data has been used to predict the functions of unknown proteins[167,168]. Using the protein-protein interaction approach, a Y2H screen was conducted using RING finger and DWNN domains of RBBP6 as baits respectively. The identified interactors, through their known functions, would provide an insight into the possible functional roles of RBBP6.

To facilitate the screening, cDNA encoding the regions of DWNN and RING finger from RBBP6 was cloned into bait plasmid pGBKT7. The cloned prey constructs were tested in yeast for background effects that included toxicity and autoactivation of interaction-specific reporter genes. According to the recommendations specified by the manufacturer of the MATCHMAKER cDNA library (Clontech), both the DWNN and RING finger baits qualified to be used in the Y2H screening exercise.

Yeast 2-hybrid system was used in 2 different screens: first, using RING finger as a bait and second, using DWNN as a bait in another screen. From the respective Y2H screens, the RING finger identified YB-1 and zBTB38 proteins as plausible interactors that were further investigated while DWNN

domain identified 6 plausible interactors from which snRPG and Hsp70 were prioritized for further investigations.

6.2. RBBP6 and YB-1 interaction

A novel interaction between YB-1 and RBBP6 was identified from the study. YB-1, also known as DNA binding protein B (dbpB) and nuclease sensitive protein 1 (NSEP1), is a transcription factor that binds to the inverted CCAAT box and is a member of the cold shock family of proteins[169]. YB-1 performs a wide variety of cellular functions, including transcriptional and translational regulation[170,171], DNA repair [172], drug resistance[173] and stress responses to extracellular signals[174]. Mammalian YB-1 consists of three domains: an N-terminal domain which is involved in transactivation, a central cold-shock domain (CSD) and a C-terminal domain that is thought to be involved in nucleic acid as well as protein-protein interactions[158]. The B/A repeats of the C-terminal region may adopt a charged zipper structure; our study maps the region of interaction to the last B/A repeat of YB-1 (Figure 4.2).

In humans, YB-1 interacts directly with eIF4E, which initiates transcription by binding to the 5'-cap of mRNA transcripts[175]. The yeast homologue of eIF4E, Cdc33p[176], associates closely with the CPF complex, which includes Mpe1p, the yeast homologue of RBBP6. YB-1 is also one of two main protein components of the cytoplasmic messenger ribonucleoproteins particles (mRNP's), in which mature mRNA transcripts are exported from the nucleus prior to translation[177]. The other major protein found in mRNP's is PABP[178], the yeast homologue of which, Pab1p, also associates closely

with the CPF complex[175]. As well as strengthening the link between YB-1 and RBBP6, these findings suggest that RBBP6 may interact directly with eIF4E as well as with PABP in humans.

In response to various stresses, YB-1 is cleaved by the 20S proteasome in the vicinity of residue 220, with the N-terminal part translocating to the nucleus in a process which requires the presence of transcriptionally active p53[179,180]. Once in the nucleus, YB-1 transduces the expression of a number of growth-promoting or anti-apoptotic genes[181,182] and blocks expression of pro-apoptotic genes such as p53. YB-1 binds directly to the C-terminus of p53, impairing its ability to transactivate pro-apoptotic genes such as BAX, NOXA and APAF-1[179,180]. In separate studies, YB-1 was shown to associate with transcriptional activation of the cyclin A and cyclin B1 genes, thereby setting itself as a cell cycle stage-specific transcription factor important for cell proliferation that can be used as a marker for tumor biology, as increased levels of cyclin A in breast cancer are associated with poor clinical outcome[183]. Furthermore, YB-1 is responsible for up-regulation of the *mdr1* gene, resulting in a multidrug resistant phenotype[184] and was also identified as a regulator for the expression of matrix metalloproteinase gelatinase A, which is involved in angiogenesis, tumor invasion/metastasis, and chronic inflammation[185]. These findings further implicate YB-1 as a cell cycle-regulating transcription factor that affects a multitude of biological features of cancerous cells, which might cause a highly aggressive tumor phenotype.

Within the nucleus YB-1, like RBBP6, localises to nuclear speckles, which are sites enriched in splicing factors. Unlike RBBP6, YB-1 is not itself an SR protein, yet has been shown to play a similar role to SR proteins in regulating the alternative splicing of CD44 in breast and ovarian cancer[186-188]. This suggests that YB-1 may play a role in coupling splicing to tumourigenesis.

The preceding discussion reveals significant functional overlap of RBBP6 and YB-1, with both playing essential roles in the otherwise poorly related areas of tumourigenesis and mRNA processing/splicing. Consistent with these findings, the study has established that RBBP6 regulates YB-1 through ubiquitination. Furthermore, it was shown that the YB-1 ubiquitination has a downstream effect on its transactivational activity. Although YB-1 is already known to be ubiquitinated by the SCF (Skp-1/Cul 1/Fbox) ubiquitin-ligase complex[148], that probably suggests that YB-1 ubiquitination is context dependent since YB-1 is a multifunctional protein. The other possibility could be that the RBBP6 may act as a scaffold to YB-1 ubiquitination in a similar way to the role of PACT in p53 ubiquitination via Hdm2 protein[2], hence suggesting RBBP6 to be acting as an E4 ligase.

In conclusion, the common involvement of YB-1 and RBBP6 in the up to now largely separate areas of mRNA processing and tumourigenesis suggests not only that they are functionally related, but also that the interaction between them may hold the key to understanding the relationship between these two major areas of modern biology.

6.3. RBBP6 and zBTB38 interaction

A zinc finger protein zBTB38 was also identified and confirmed to interact with RBBP6 protein. Although much is still to be unveiled about the cellular functions of zBTB38 protein, it has been implicated in transcriptional regulation of a number of genes through binding to methylated DNA[189] via methyl-CpG recognition through its zinc fingers[190]. Kaiso and ZBTB4 are 2 other proteins known to recognize methyl-CpG via zinc fingers.

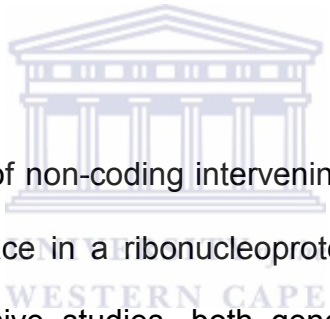
Kaiso was originally identified by Y2H screening in a search for proteins that interact with the p120 catenin[191] and later independently identified as a component of a protein complex that binds to a region of the mouse *S100A4* gene in a methylation-dependent manner[192]. Kaiso contains three tandem zinc fingers at the C terminus and a BTB domain at the N terminus[193]. Kaiso was also biochemically identified from HeLa cell nuclear extracts as a component of the NCoR corepressor complex including HDAC3, GPS2 and TBL1/TBLR1[194]. Kaiso directly binds to NCoR via its BTB domain; this type of interaction with corepressors involving the BTB is a common feature of BTB-zinc finger transcription factors[195]. Kaiso recruits the NCoR complex to the *MTA2* promoter in a DNA methylation-dependent manner, resulting in hypoacetylation and methylation at K9 of H3 at the promoter region[194]. Therefore, this establishes that Kaiso is a DNA methylation-dependent transcriptional repressor of the *MTA2* gene. However, as the cultured cells do not reflect the normal patterns of DNA methylation[196], it remains unknown whether *MTA2* is an actual target of Kaiso in normal cells and at present there is no information about methylated target genes of Kaiso in normal cells.

ZBTB38 is a Kaiso-like Zinc finger that contain a BTB domain that bind methylated DNA through recognition of single methylated CpG sequence. It was also shown that ZBTB38 represses transcription of methylated promoters in a reporter assay[197]. Moreover, zBTB38 localizes to densely methylated pericentromeric heterochromatin regions in mouse cells in a DNA methylation-dependent manner[198] and recruits several co-repressors that act on histones, thus contributing to the establishment of pericentric heterochromatin[199,200]. A mouse zBTB38 homologue, CIBZ, recruits a C-terminal binding protein (CtBP) to pericentromeric foci from a diffuse nuclear localization in interphase cells. CIBZ was found physically associating with CtBP via a conserved CtBP binding motif, PLDLR[201]. CtBP1 was originally identified as a cellular protein that binds to the C-terminal region of adenovirus E1A oncoprotein and negatively regulates oncogenic transformation[202].

Based on what is known about zBTB38 protein, it is rational to suggest that RBBP6 may be recruited to pericentromeric heterochromatin through zBTB38 binding and regulates zBTB38 functions through mechanisms that involves ubiquitination. Consistent with this speculation, preliminary co-localisation results showed co-localisation of zBTB38 and RBBP6 in the nuclear region of HEK293 cells. Furthermore, it was found that RING finger ubiquitinates zBTB38 *in vitro*. To further elucidate the physiological relevance of the RBBP6/zBTB38 interaction, further functional studies need to be explored, together with identification of zBTB38 target genes.

6.4. RBBP6 and snRPG interaction

A novel interaction between snRPG and the DWNN domain of RBBP6 was among the interactions identified from the Y2H screen. The protein snRPG, also known as sm-G, is a small ribonuclear protein found in small RNA/protein complexes called small ribonuclear protein particles (snRNPs) [203], involved in splicing regulation. Pre-mRNA splicing is a highly dynamic process involving transient RNA-RNA, RNA-protein and protein-protein interactions [203] and an essential cellular mechanism that generates processed mRNAs that can be translated into different protein isoforms from a single gene because of alternative splicing, thus increasing the coding repertoire of the genome [203,204].



Splicing involves removal of non-coding intervening sequences called introns and the process takes place in a ribonucleoprotein complex known as the spliceosome [203]. Extensive studies, both genetic and biochemical, in a variety of systems have revealed that essential components of the spliceosome include five small RNAs U1, U2, U4, U5 and U6, each of which functions as a RNA, protein complex called an snRNP[205]. In addition to snRNPs, the spliceosome contains non-snRNP protein factors that are required for the splicing mechanism[206].

A recent study revealed the presence of RBBP6 as part of the molecular architecture of the purified human pre-mRNA 3' processing complex[207], thereby suggesting RBBP6 as an essential non-snRNP protein factor required during splicing. Furthermore, the RBBP6 yeast homologue, Mpe1p, was

shown be part of the Yeast Cleavage and Polyadenylation Factor that is essential for the specific cleavage and polyadenylation of pre-mRNA[208].

Interaction of RBBP6 with snRNPs was first speculated to play a role in splicing when RBBP6 co-immunoprecipitated snRNPs from HeLa cell nuclear extract[1] and RBBP6's translocation to nuclear speckles (splicing sites) during mitosis[1]. Our study confirms this interaction, particularly showing specific interaction of snRPG with the ubiquitin-like DWNN domain of RPPB6. Intriguingly, ubiquitin and ubiquitin-like proteins have also been implicated in splicing regulation [209-211] with recent evidence that ubiquitin plays a role in the maintenance of U4/U6-U5 triple snRNP levels through regulation of U4/U6 duplex unwinding [212]. Similarly, DWNN, as an ubiquitin-like domain, may play a key role in the assembly of the Sm proteins onto snRNA, which is an important and critical step of the biogenesis of the snRNPs[213]

Very little is known about the manner in which the Sm proteins recognize and interact with the Sm site RNA element. A direct contact between the snRPG protein and the 5' part of the Sm site element within HeLa U1 snRNP particles was demonstrated by cross-linking approaches[214]. Notably, neither the snRPG protein nor any of the other Sm proteins contain established RNA binding motifs[215], and no single Sm protein or heteromeric complex can directly interact with the U snRNA in a stable manner *in vitro* [216]. This suggests that both the RNA interaction surface and binding specificity of the Sm proteins are determined by interactions among the Sm protein complexes or modified by non-snRNP proteins such as splicing factors Prp8 and ubiquitin

like protein Hub1[217-220]. Investigation of whether RBBP6 is responsible for the pre-assembly of the snRNPs or the actual role in pre-mRNA splicing or both warrants further investigation.

6.5. RBBP6 and Hsp70 interaction

Heat shock proteins (HSP) are a family of proteins which cooperate with the Ub-proteasome system in the quality control of cellular proteomes, ensuring proper folding, intracellular transport and repair or degradation of mis-translated, mis-folded or aged proteins. These proteins are typically induced after cellular heat shock and are modulated by nutrient deprivation and oxidative stress. Since HSPs act on caspase-dependent and independent apoptosis of tumour cells they are ideal targets of therapies aimed at modulating programmed cell death[221,222].

The DWNN domain from RBBP6 identified HSPA14 as an interactor from the Y2H screen. Further confirmation of the interaction of RBBP6 with Hsp 70 protein *in vivo* provided a valuable insight on yet another possible role that RBBP6 plays. More specifically, the interaction of Hsp70 with RBBP6 was shown to be via the DWNN and the RING finger domains.

The interaction found in this study is consistent with what was previously found for parkin, a Parkinson's disease (PD) related E3 ligase that contains both a RING finger domain and an ubiquitin-like domain[223,224]. Overexpression of parkin reduces aggregation and cytotoxicity of an expanded polyglutamine ataxin-3 fragment[225]. Parkin forms a complex with the expanded polyglutamine proteins, Hsp70 and the proteasome, which may

be important for the elimination of the expanded polyglutamine protein. Hsp70 enhances parkin binding and ubiquitination of the expanded polyglutamine protein *in vitro*, suggesting that Hsp70 may help to recruit misfolded proteins as substrates for the ubiquitin E3 ligase activity of parkin. Loss of parkin function and the resulting proteasomal impairment may contribute to the accumulation of toxic aberrant proteins in neurodegenerative diseases including PD[226]

In another study, it was shown that a 35-kDa protein called CHIP (carboxyl terminus of Hsp70-interacting protein) was also a candidate for a ubiquitin ligase that plays a role in protein quality control[227]. On the other hand, another ubiquitin-like domain protein, BAG-1 (Bcl2-associated athanogene-1), has been shown to modulate the chaperone activity of Hsc70 and Hsp70 in the mammalian cytosol and nucleus. Remarkably, BAG-1 possesses a ubiquitin-like domain at its amino terminus, suggesting a link to the ubiquitin/proteasome system[228]. However, previous studies have shown that the proteasome binding activity of BAG-1 (through its ubiquitin-like domain) and its stimulation of substrate release from Hsc/Hsp70 can act in concert with CHIP's ubiquitin ligase activity to deliver substrates to the proteasome such that they will be recognized as marked for degradation[35,36].

Consistent with the parkin and CHIP/BAG-1 roles in Hsp70 chaperone activity, the RBBP6 through its ubiquitin-like DWNN and the RING finger domain takes part in Hsp70 chaperone dependent ubiquitination. In addition

the RBBP6 RING finger and CHIP U-box domain adopts the same molecular structure and they both belong to the same family of E3 ligases (Pugh *et al* unpublished data). This makes it possible for the RBBP6 to play a similar function as the CHIP/BAG-1 complex in facilitating ubiquitination of protein in an Hsp70 dependent fashion, whereby DWNN assumes the role of BAG-1.

Through its RING finger domain, RBBP6 has shown to ubiquitinate both YB-1 and zBTB38. Therefore, another functional possibility of the interaction of RBBP6 with respect to Hsp70 may lead to Hsp70 ubiquitination to regulate its cellular levels.

HSPA14 shares a number of definitive characteristics with Hsp70 subfamily members such as heat inducible, as demonstrated in heat-stressed HeLa cells and human dendritic cells (DCs). It also binds the same receptors (TLR2, TLR4, CD91) and stimulates DCs to secrete cytokines and chemokines and become mature[166]. However, it lacks strong primary sequence homology to other Hsp70 subfamily proteins and this suggests that HSPA14 could possess other novel functions. For example, it was observed that HSPA14 could induce DCs to secrete chemokine IP-10, but Hsp70 could not. These findings on the functional novelty of HSP14 are very interesting, and mechanisms of action of HSP14 with respect to interaction with RBBP6 deserve to be further explored.

6.6. Conclusion

This study was designed to provide further insight about the functional characteristics of RBBP6 protein through identification of its protein interactors. Identifying protein interactors with known functions facilitates the characterisation of RBBP6 through the 'guilty by association' concept. If two proteins interact with one another, they usually participate in the same, or related, cellular functions[229].

The following conclusions were drawn from the study:

a) RBBP6 interacts with YB-1 through the RING finger domain to regulate YB-1 dependent functions via ubiquitination and subsequent proteasomal degradation of YB-1[230].
b) RBBP6 interacts with a methyl dependent transcriptional repressor protein zBTB38 in the nucleus of mammalian cells HEK293. The RBBP6 RING finger domain ubiquitinates zBTB38 *in vitro*, suggesting a possible regulatory mechanism by RBBP6.
c) RBBP6 interacts with snRPG, a protein component of the snRNPs, via the ubiquitin-like DWNN, thereby further implicating the role of RBBP6 in mRNA processing.
d) RBBP6 has shown to interact with heat shock 70 protein thereby suggesting its involvement into Hsp70 chaperone dependent protein quality control mechanisms. Since RBBP6 has shown to be actively involved in the ubiquitination of both YB-1 and zBTB38, it is also possible that Hsp70 could be an ubiquitination substrate for RBBP6.

6.7. Future perspective

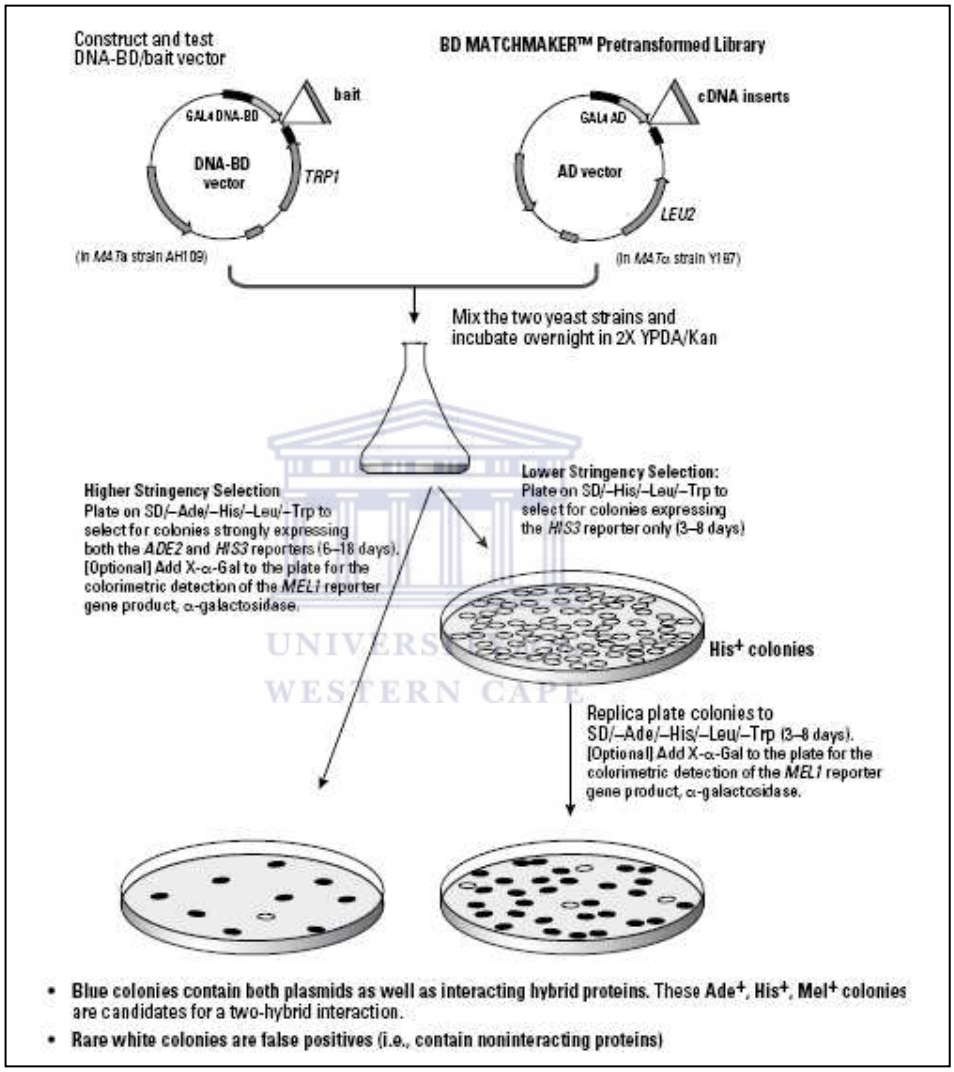
The study has created basis for interesting further studies. Firstly, interaction profiles of the protein that have been identified should be tested in RBBP6 dependent disease states or in cancer cell-lines.

Protein-protein interaction studies to further delineate the interaction interfaces should be explored further to understand the precise nature of the interactions and that information would be applied to long-term drug development attempts. Confirming such interaction using structural based methods would enable identification of the actual amino acid residues involved in the interaction and such detail is crucial on designing antagonists that could be useful in therapy.

Because RBBP6 has several functional domains on its structure, it is therefore, possible to identify other interacting proteins through other domains. Performing Y2H screen using different domain such as SR and zinc knuckle as baits would widen the interaction network of proteins associated with RBBP6 and such an exercise would result in a comprehensive understanding on the biochemical functions of RBBP6.

APPENDIX I

7.1. Schematic representation of Y2H screening using BD MATCHMAKER™ Pretransformed cDNA library from Clontech



7.2. Calculating Yeast mating efficiency

Count the number of colonies (cfu) growing on the SD/–L, SD/–W and SD/–L–W dilution plates that have 30–300 colonies after 4 days.

$$\text{Number of (cfu)/ml} = \frac{\text{cfu} \times 1000\mu\text{l/ml}}{\text{Volume plated } (\mu\text{l}) \times \text{dilution factor}}$$

1. Number of cfu/ml on SD/–L plates = viability prey partner
2. Number of cfu/ml on SD/–W plates= viability bait partner
3. Number of cfu/ml on SD/–L–W plates = viability of diploids
4. Lowest number of cfu/ml on SD/-L or SD/-W plates indicate the 'limiting partner'

$$\text{Therefore, Mating efficiency} = \frac{\text{number of cfu/ml of diploids} \times 100}{\text{number of cfu/ml of limiting partner}}$$

7.3. Calculating total number of library prey yeast clones screened

Count the number of cfu on SD/-L plates streaked with library mating culture

$$\text{Number of cfu/ml} = \frac{\text{number of colonies}}{\text{Volume plated (ml)} \times \text{dilution factor}}$$

Total number of clones screened = number of cfu/ml x final re-suspension volume

APPENDIX II

8.1. Abstract for the published article

Abstract of an article that was published in the Journal of Molecular Biology, Volume 384, Number 4, pages 908-916, 26 December 2008

RBBP6 Interacts with Multifunctional Protein YB-1 through Its RING Finger Domain, Leading to Ubiquitination and Proteosomal Degradation of YB-1

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ABSTRACT

Retinoblastoma binding protein 6 (RBBP6) is a 250kDa multi-functional protein which interacts with both p53 and pRb and has been implicated in mRNA processing. It has also been suggested to be an E3 ubiquitin ligase due to the presence of a RING finger domain, although no substrates have been identified up to now. Using the RING finger domain as bait in a yeast two-hybrid screen, we have identified Y-box binding protein 1 (YB-1) as a binding partner of BBP6, localizing the interaction to the last 62 residues of YB-1. We showed, furthermore, that both full length RBBP6 and the isolated RING finger were able to ubiquitinate YB-1, resulting in its degradation in the proteosome. As a result, RBBP6 was able to suppress the levels of YB-1 *in vivo* and to reduce its transactivational ability. In the light of the important role that YB-1 appears to play in tumourogenesis, our result suggests that RBBP6 may be a relevant target for therapeutic drugs aimed at modifying the activity of YB-1.

Keywords: RBBP6, YB-1, RING finger, ubiquitination, proteosome, p53

8.2. Abstract for the paper presented at an International Conference

Abstract of a paper presented at the International Conference "The Ubiquitin Family" held at the Cold Spring Harbor Laboratory Institute, New York, USA, on 21-25 April 2009.

RBBP6 regulates a methyl dependent transcriptional repressor protein zBTB38 through its ubiquitination

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ABSTRACT

RBBP6 (retinoblastoma binding protein 6) is a 250-kDa multifunctional protein that interacts with both p53 and pRb and has been implicated in ubiquitination pathways. Specifically, RBBP6 has been identified as a putative E3 ubiquitin ligase due to the presence of a RING finger domain. However, we have previously published involvement of RBBP6 in regulation of Y-box binding protein 1 (YB-1) through ubiquitination and subsequent proteasomal degradation of YB-1 (Chibi *et al*, 2008). In the same light, we have demonstrated that the RING finger domain as bait in a yeast two-hybrid screen interacts with the zinc finger and BTB domain containing 38 (ZBTB38) protein, leading to its poly-ubiquitination. In addition, we also observed heavy colocalization of ZBTB38 and RBBP6 in the nuclear speckles suggesting involvement in regulation of transcriptional related functions. In the light of the important role that ZBTB38 appears to play in regulating transcription of genes through binding to methylated DNA (Filion *et al*, 2006), our results suggest that RBBP6 may be a relevant target aimed at modulating ZBTB38 related transcriptional regulatory role.

DNA methylation is essential in mammals and its loss has been shown to result in apoptosis in normal cells as well as in cancer lines. The presence of DNA methylation is also required for embryonic development in mice. The key role of DNA methylation is to control gene expression, and methylated sequences undergo transcriptional repression. ZBTB38 has been observed to bind sequences containing a single methylated CpG and is a methyl-dependent transcriptional repressor, and through interaction with RBBP6 its transcription repression activity may be turned off via ubiquitination. Although the function of zBTB38 is still poorly understood, results from similar proteins suggest it plays an important role in a number of diseases ranging from developmental abnormalities to cancer. Future work will involve analysis of the effects of RBBP6-mediated ubiquitination on some of these diseases.

Keywords: RING finger domain, RBBP6, zBTB38, ubiquitination, transcriptional regulation, DNA methylation

8.3. Abstracts for articles under review for publications

Retinoblastoma binding protein 6 interacts with Small Nuclear Ribonucleoprotein G, further implicating a role in splicing

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Running title: RBBP6 plays a role in splicing

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ABSTRACT

The N-terminus of all members of the RBBP6 family comprise of an ubiquitin-like domain known as the DWNN domain that is also expressed as an independent domain in higher eukaryotes. Due to the function of RBBP6 as an E3 ubiquitin ligase, the DWNN domain is likely to play some role in this process, although the exact nature of the role is not yet known. It is speculated that the independently expressed DWNN domain may act as a novel ubiquitin-like modifier similar to SUMO or NEDD8. Another possibility is that the domain acts as a protein-protein interaction motif, recruiting E2 enzymes or substrates. In an attempt to unveil novel protein interaction partners of RBBP6, we carried out a Y2H screen of a human testis cDNA library using the DWNN from RBBP6 as bait. A number of putative interactors were identified and among them, a splicing factor, nuclear ribonucleoprotein polypeptide G (snRPG) was identified more than once. Because of the previous implication on RBBP6 in splicing activity, we decided to use co-immunoprecipitation assays, both *in vitro* and *in vivo,* to verify this important interaction. The outcome of the verifications assays confirmed and mapped the interaction of snRPG specifically via DWNN domain from RBBP6, hence further implicating the role of RBBP6 in splicing through DWNN domain motif.

Keywords; DWNN domain, snRPG, RBBP6, splicing, protein-protein interaction, ubiquitin-like domain

The RING finger domain from RBBP6 is a zinc-binding U-box that interacts with chaperone Hsp70

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Running title: The RING finger domain of RBBP6 is a zinc-binding U-box, suggesting a role for RBBP6 in protein quality control

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ABSTRACT

RBBP6 is a 250 kDa protein playing a role in a range of cellular processes including development, tumourogenesis and mRNA splicing. Through its RING finger domain it exhibits both E3 ubiquitin ligase activity, against the tumour-associated protein YB-1, and E4 activity against p53. On the basis of its primary sequence, the RING finger domain of RBBP6 has been classified both as a RING finger, due to the presence of conserved Cysteine residues, and as a U-box, due to the presence of a conserved pattern of hydrophobic residues.

We show here that, despite binding two zinc ions in common with other RING fingers, the solution structure of the RING finger domain from RBBP6 more closely resembles that of the U-boxes, in particular the U-box from CHIP (C-terminal of Hsp70-Interacting Protein). The domain homodimerises across the same interface as in U-boxes, and features the same hydrophobic groove that forms the binding site for E2 enzymes. Moreover, we show that, in common with U-box containing proteins, RBBP6 interacts with chaperone Hsp70. However, unlike in the case of CHIP, the interaction involves the RING/U-box domain as well as the N-terminal ubiquitin-like DWNN domain.

On the basis of our results we conclude that, like CHIP, RBBP6 is involved in protein "quality control", participating in the decision to refold unfolded proteins or to target them to the proteosome for degradation. However, given its role in mRNA polyadenylation, it is also possible that, like CHIP, it plays a role in transcriptional regulation by modulating the stability of mRNA transcripts in an Hsp70-dependent manner. The similarities between the structure of the RBBP6 RING finger domain and those of other U-boxes provides a structural framework for identifying residues involved in dimerisation, in the interaction with E2 enzymes and in the interaction with substrate proteins. Indeed, the fact that RBBP6 contains a U-box may provide the key to understanding the E4-like function of RBBP6 with respect to the ubiquitination of p53 by MDM2.

Keywords: RING finger, U-box, RBBP6, PACT, ubiquitination, mRNA splicing, zinc binding, CHIP, Hsp70, chaperone

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