DEDICATION

Tino, knowledge is power and enthusiasm

pulls the switch

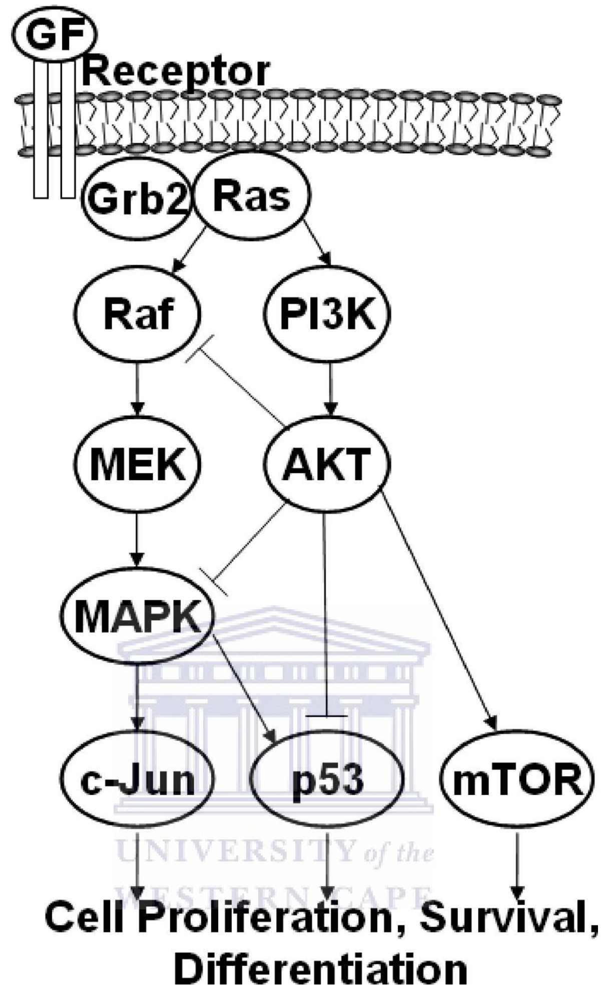

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Figure 1.1. Schematic representation of Ras-MAPK/PI3K signalling pathways.

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.

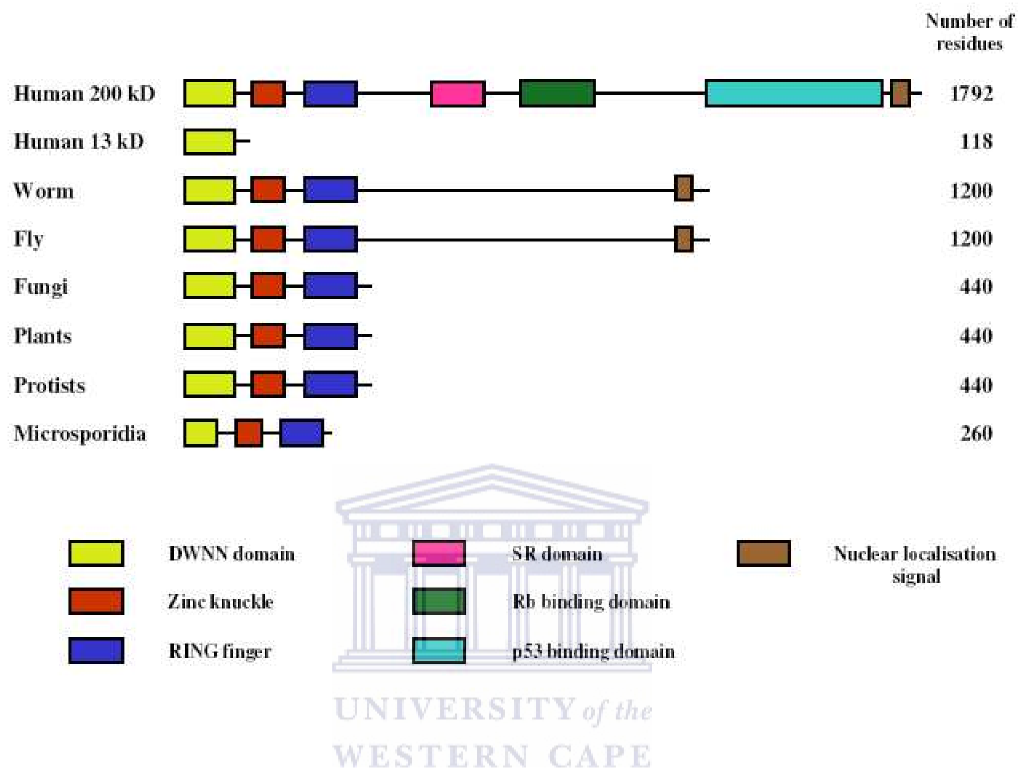

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Figure 1.2. The domain structure of the RBBP6 family of proteins

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.

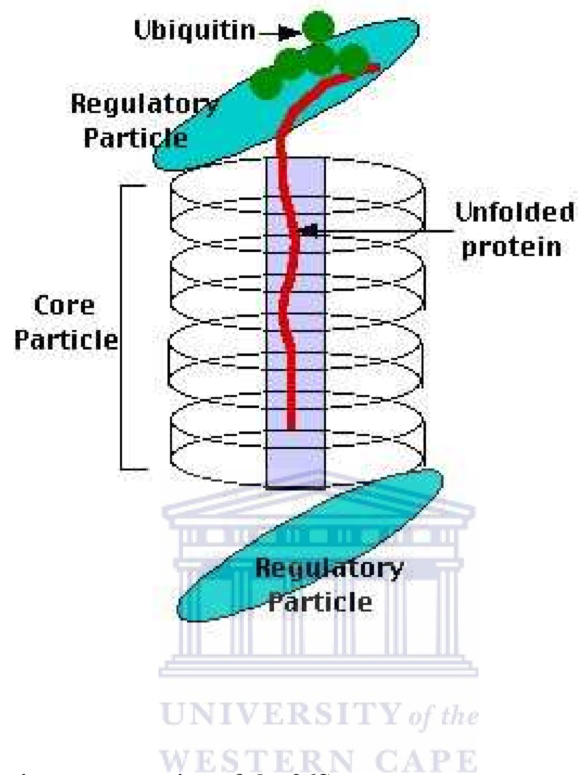

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Figure 1.3. A schematic representation of the 26S proteasome

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).

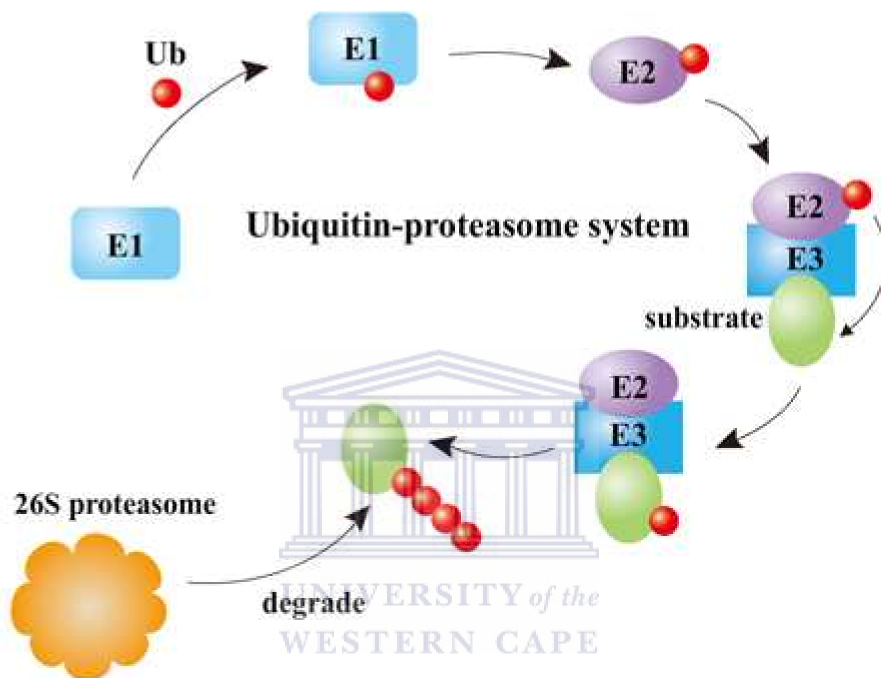

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Figure 1.4. Schematic representation of ubiquitin-proteasome system

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.

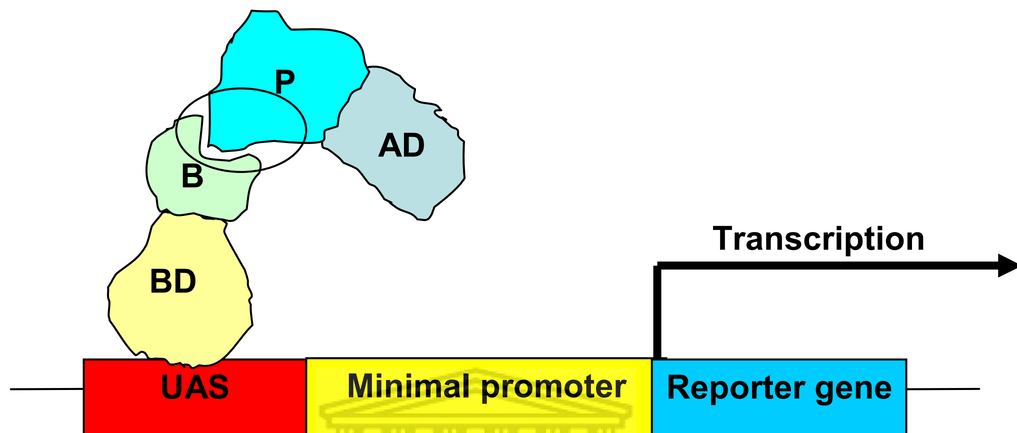

Figure 1.5. **The principle of Y2H system**

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.

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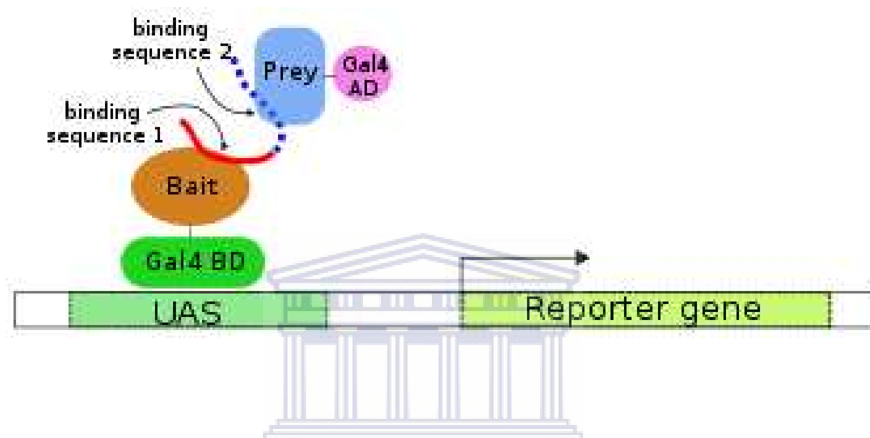

Figure 1.6. The principle of the yeast 3-hybrid system

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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.

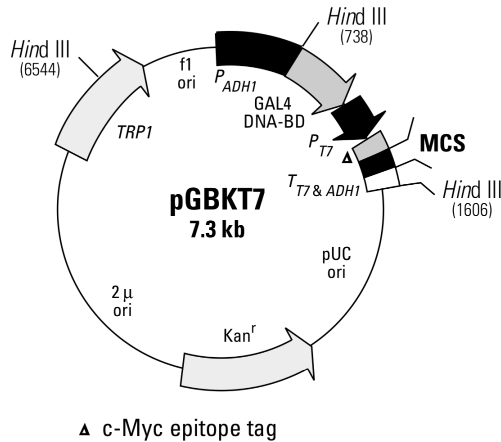
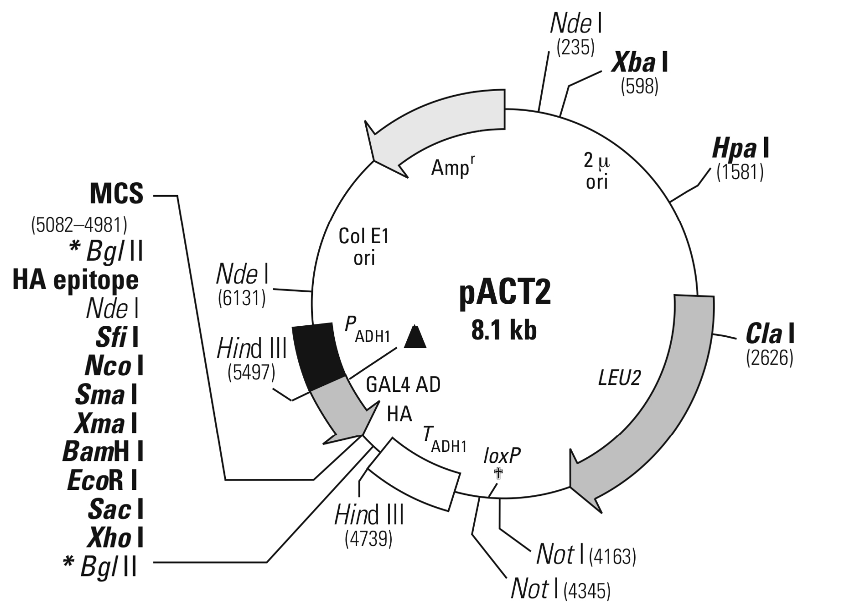


Figure 2.1. Restriction map and multiple cloning site (MCS) of pGBKT7

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).



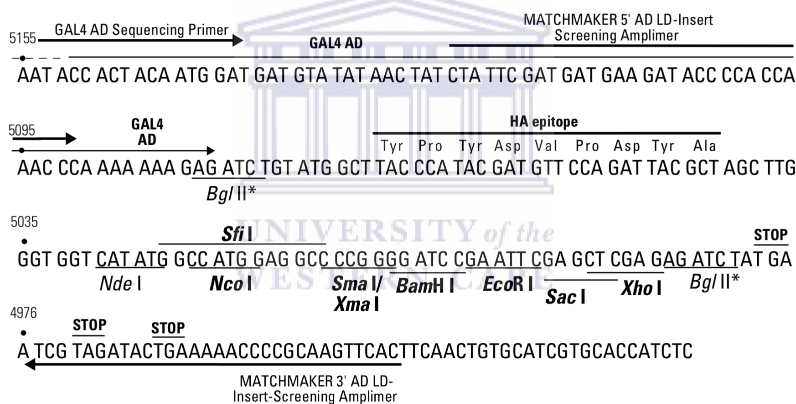


Figure 2.2. Restriction map and multiple cloning site (MCS) of pACT2

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.

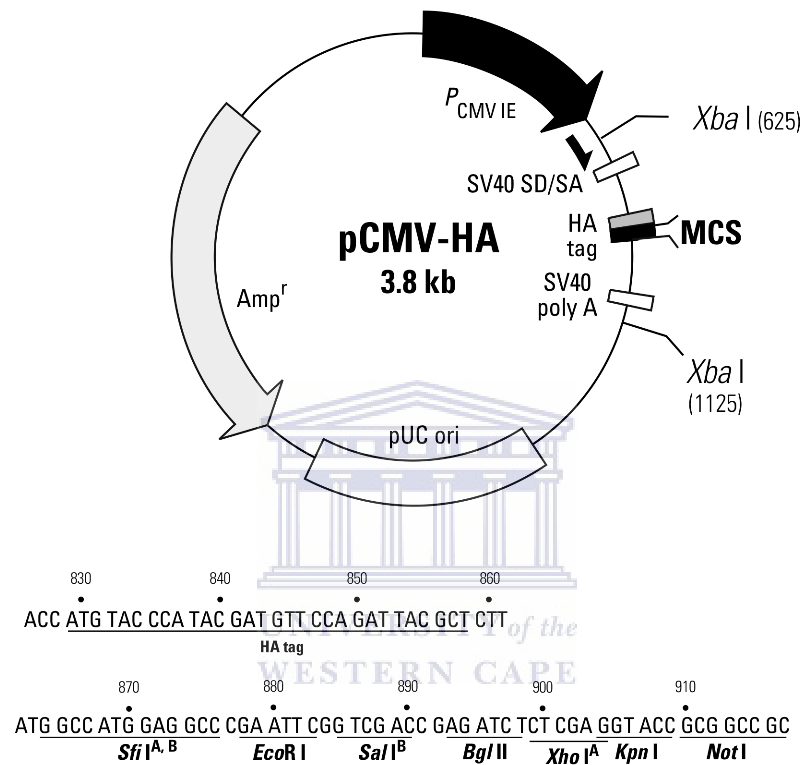


Figure 2.3. Restriction map and multiple cloning site (MCS) of pCMV-HA

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.*

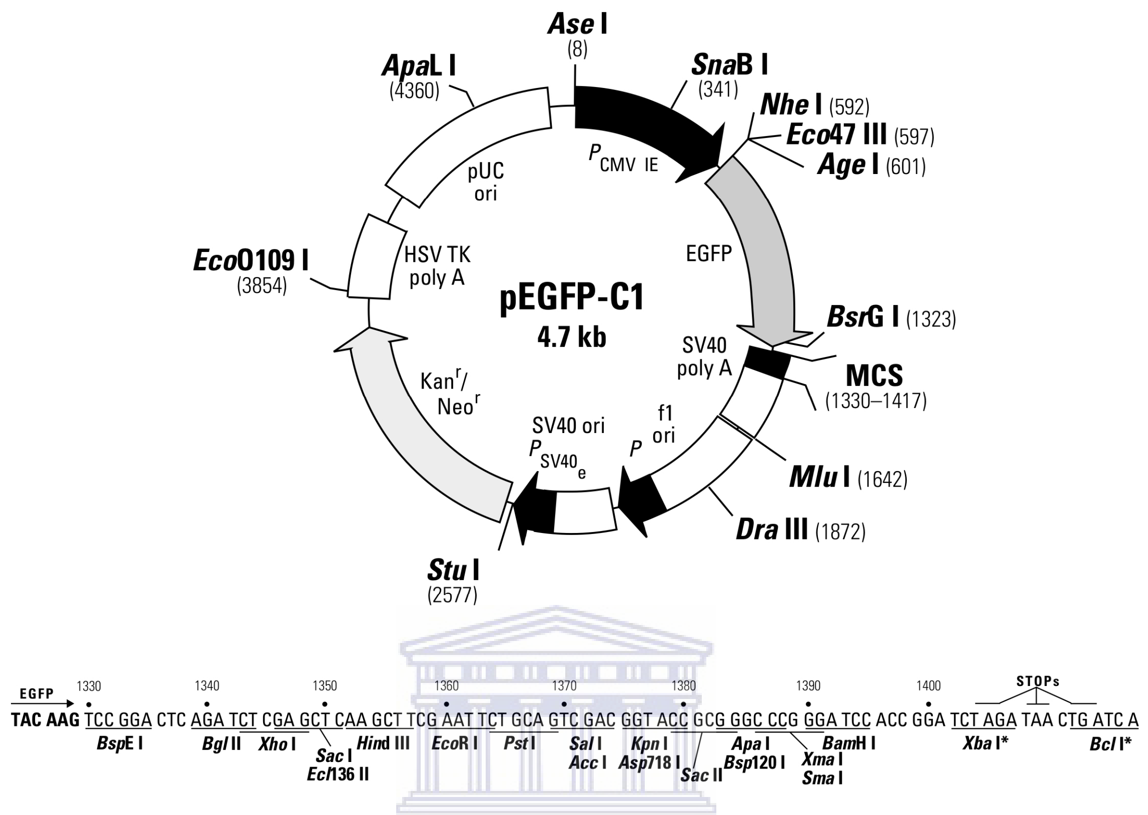


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Figure 2.5. Restriction map and multiple cloning site (MCS) of pEGFP-C1

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.

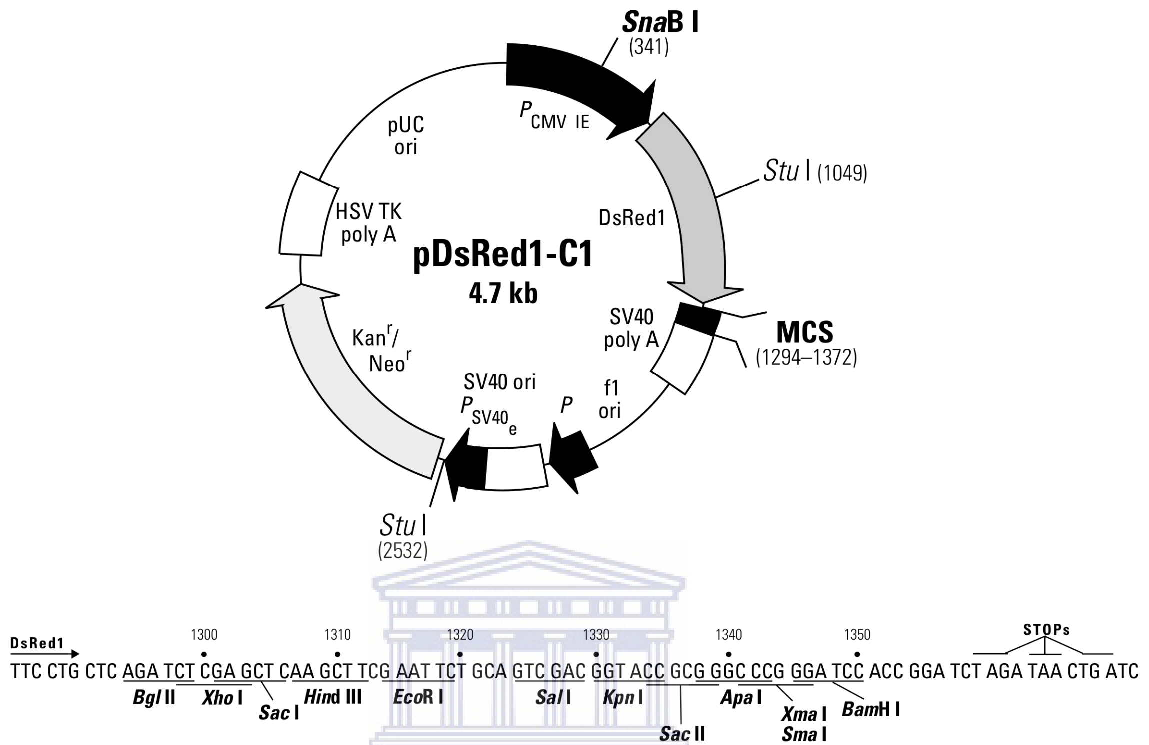

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Figure 2.6. Restriction map and multiple cloning site (MCS) of pDsRed1-C1

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.

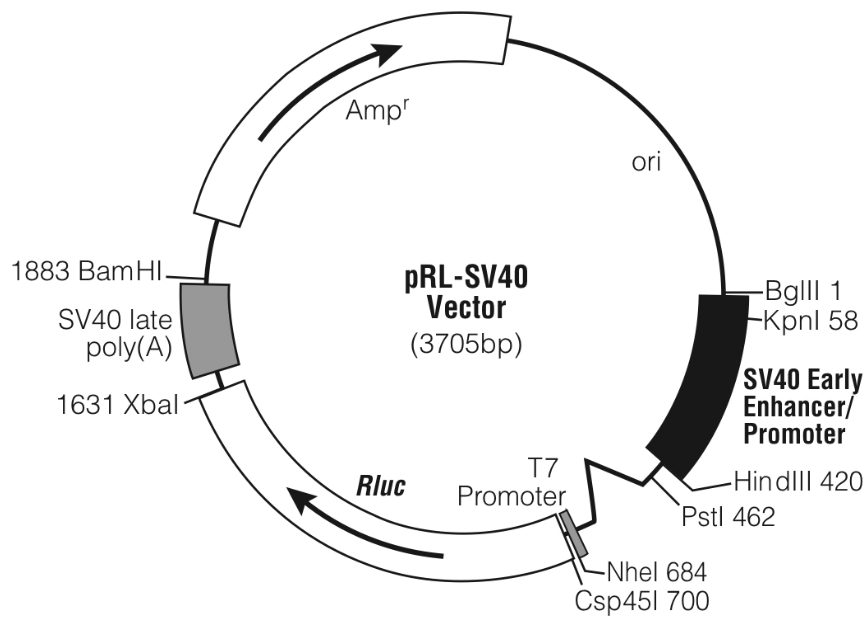

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Figure 2.7. The vector map of pRL-SV40 (Promega)

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*.

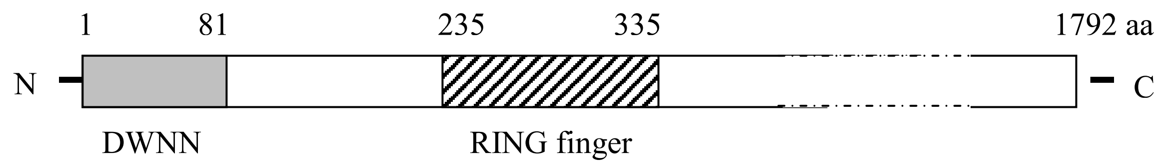

Figure 3.1. RBBP6 domains cloned into pGBKT7

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

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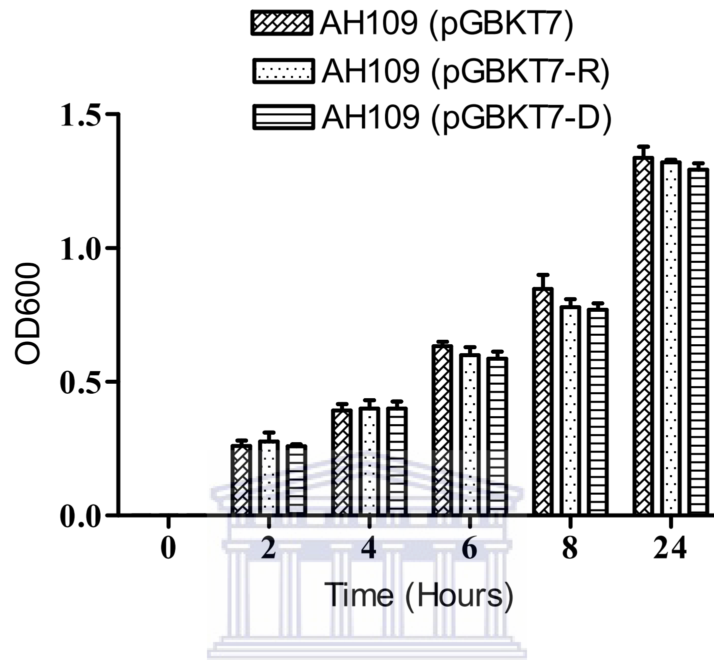

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Figure 3.2. Bait toxicity test

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

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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)	-	-	-	+	+

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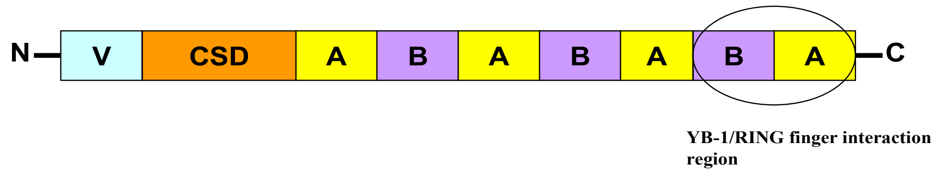
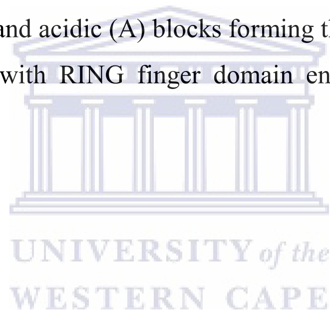


Figure 4.2. Mapping of the YB-1/RING interaction region

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).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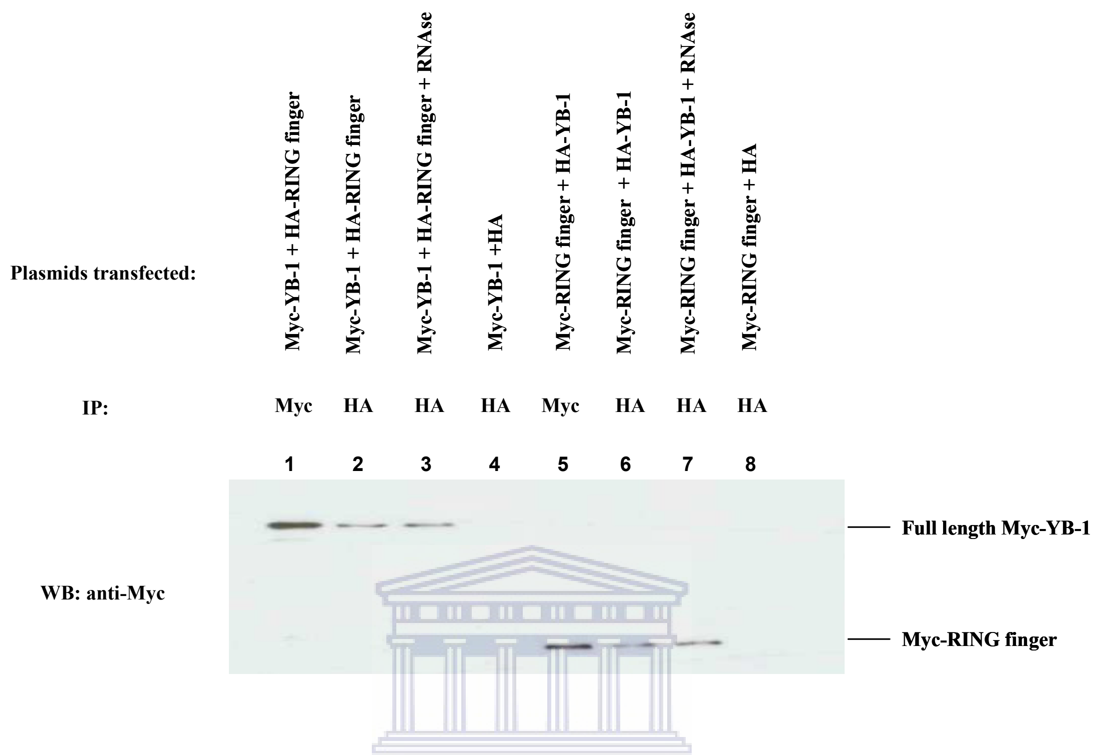

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Figure 4.4. RING finger co-immunoprecipitates full length YB-1 *in vivo* and *vice versa.*

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.

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

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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

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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.

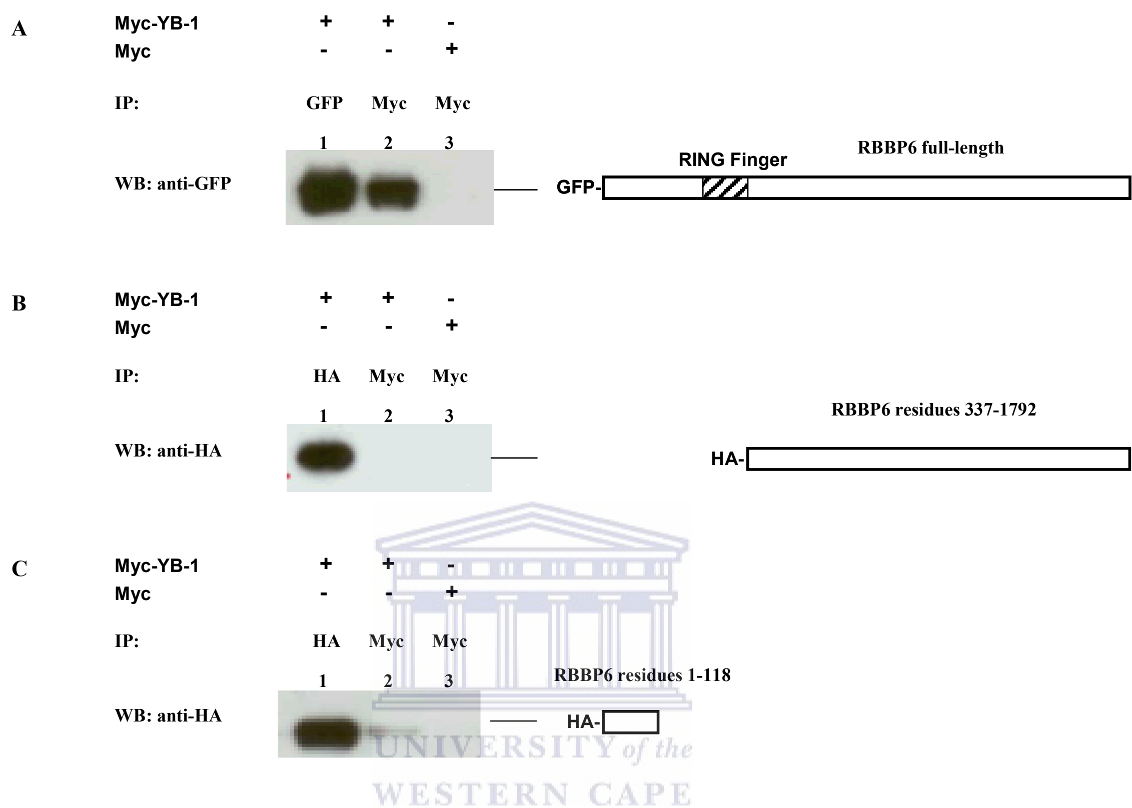

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Figure 4.6. Full-length YB-1 interacts with full-length RBBP6 *in vivo*

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.

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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

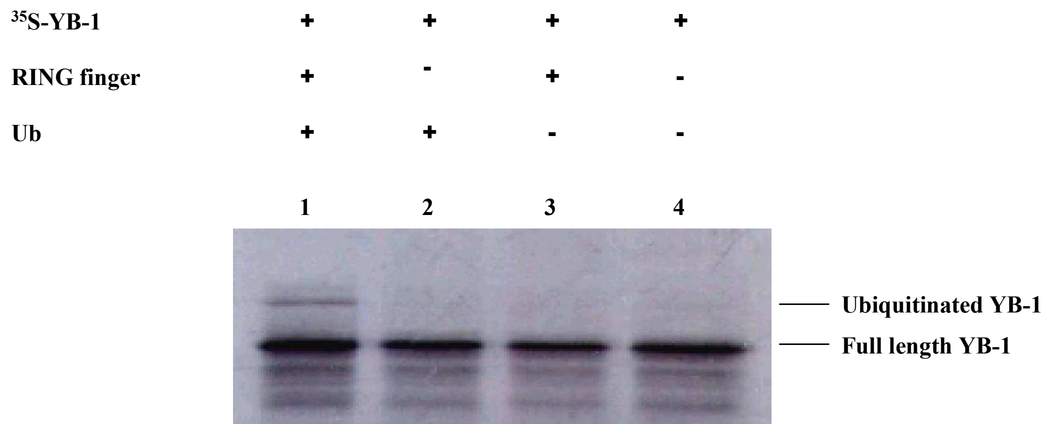

Figure 4.7. RING finger ubiquitinates YB-1 *in vitro*

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).

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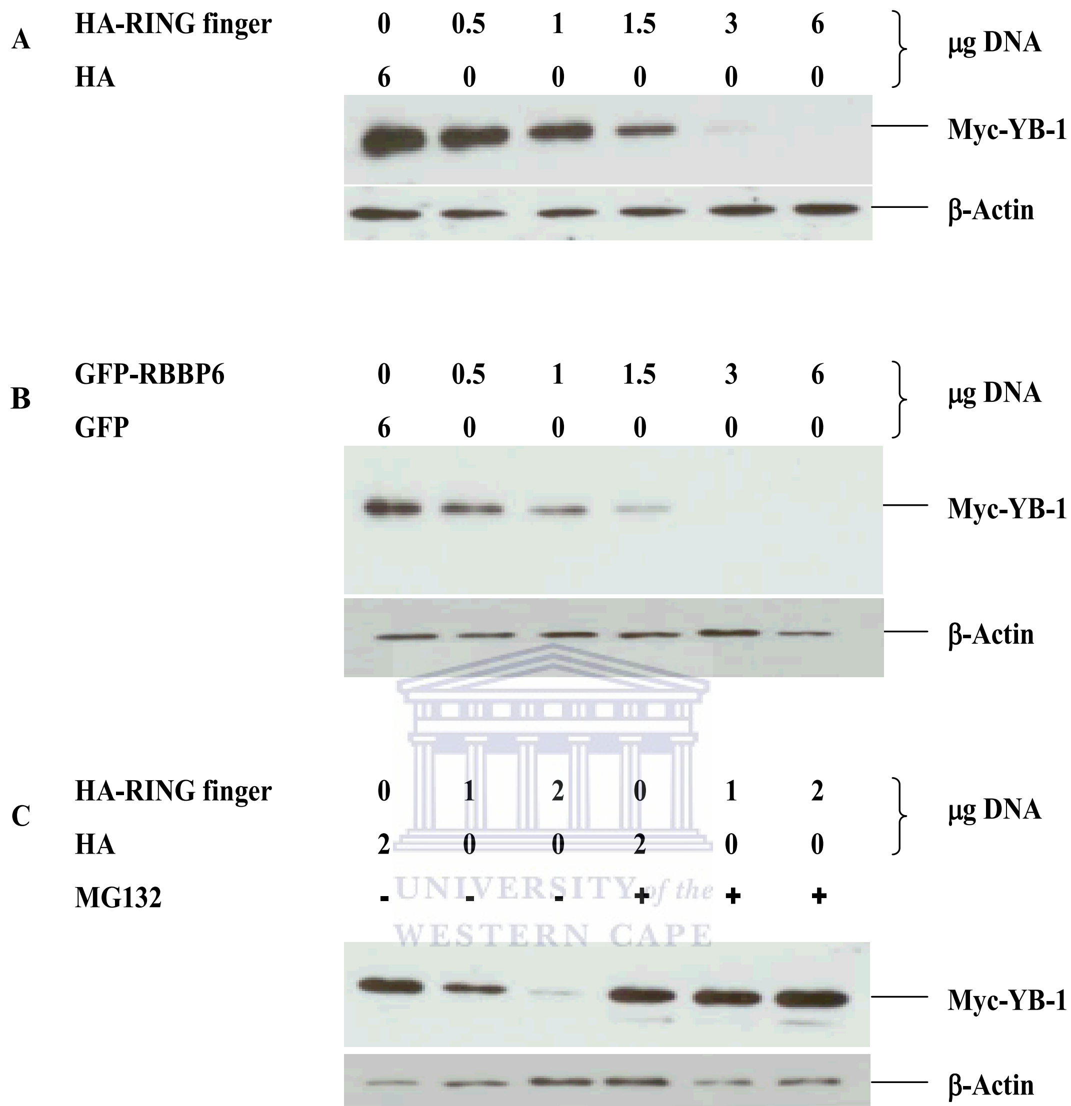

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Figure 4.8. RBBP6 suppresses YB-1 levels *in vivo.*

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.

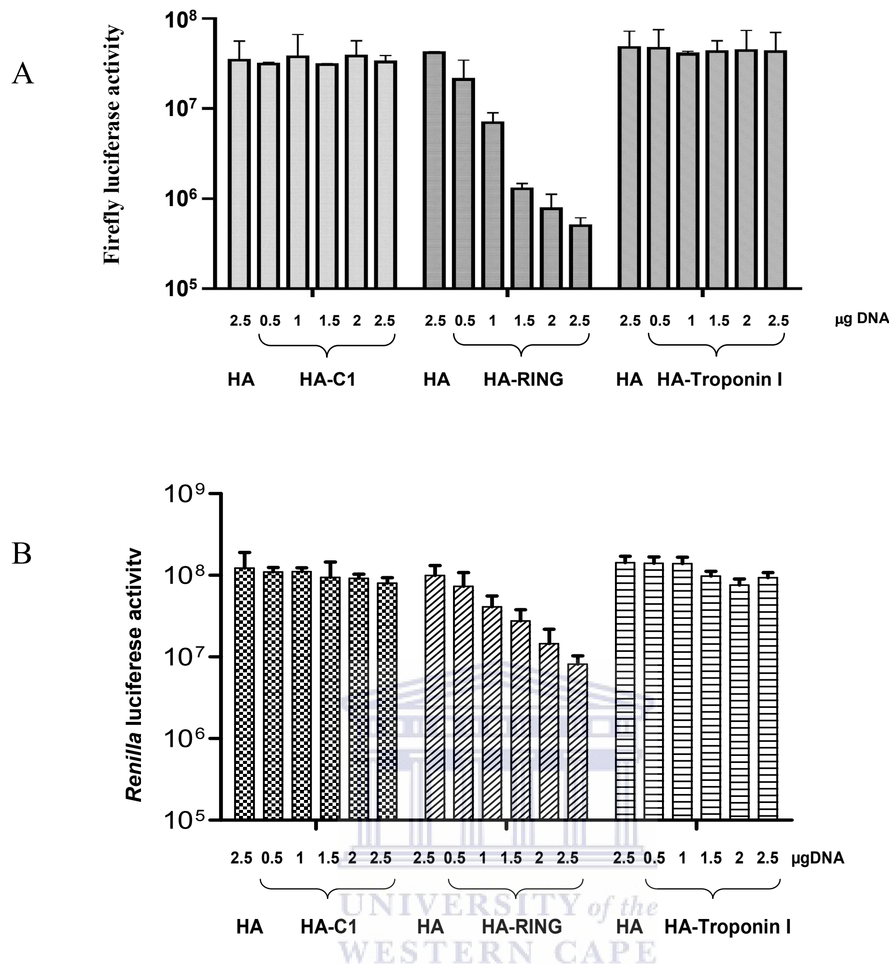

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Figure 4.9. RING finger represses YB-1 transactivational activity

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.

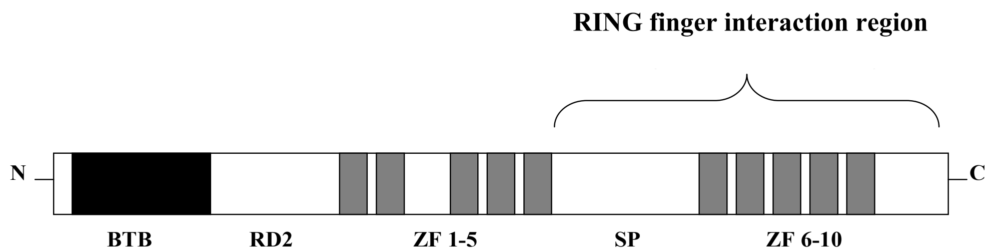
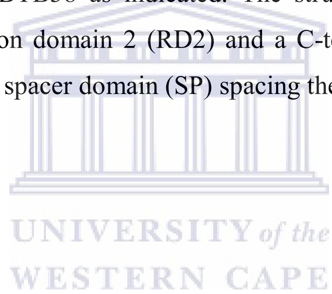


Figure 4.10. Mapping of zBTB38/RING interaction region

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.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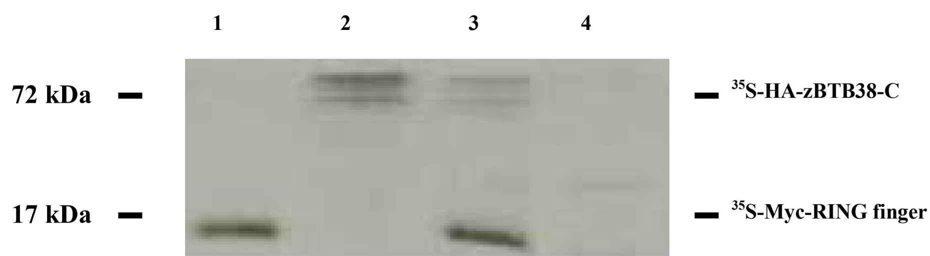

Proteins added:

^{35}S-Myc-RING	+	-	+	-
^{35}S-HA-zBTB38-C	-	+	+	+

IP:

anti-Myc	+	-	+	+
anti-HA	-	+	-	-

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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.

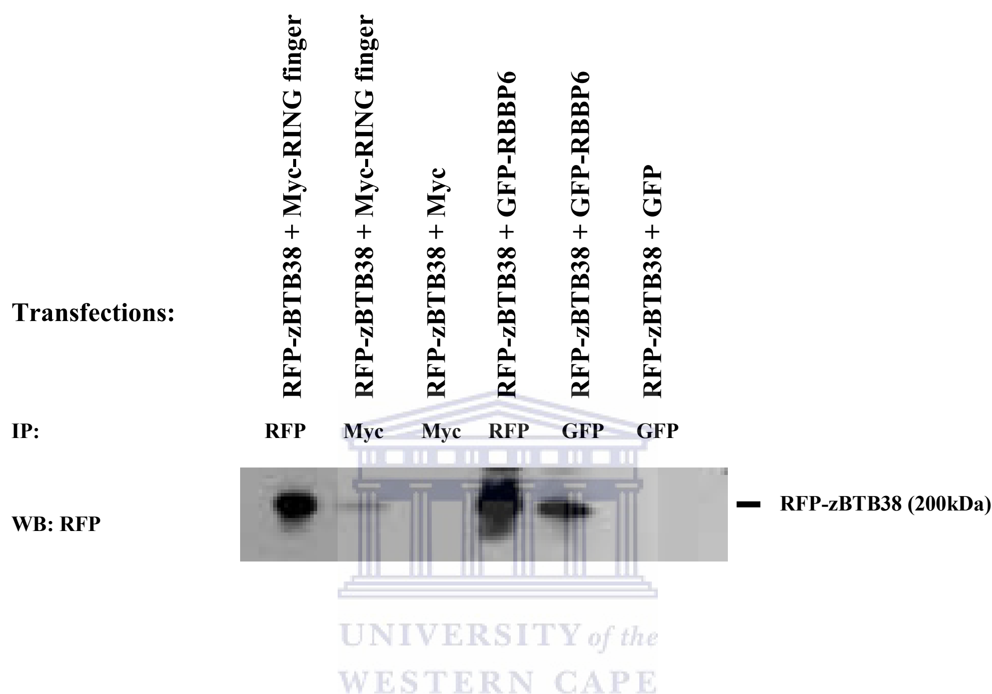

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Figure 4.12. Exogenous RING finger and full length RBBP6 co-immunoprecipitate exogenous zBTB38 *in vivo*

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.

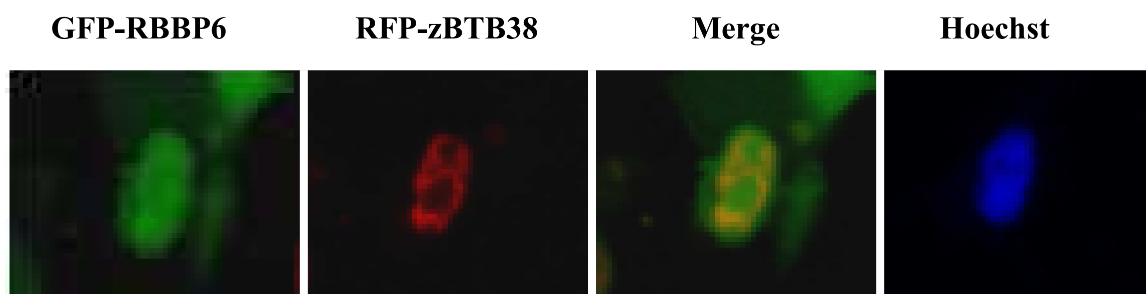

Figure 4.13. RBBP6 co-localizes with zBTB38 in transfected cells

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.

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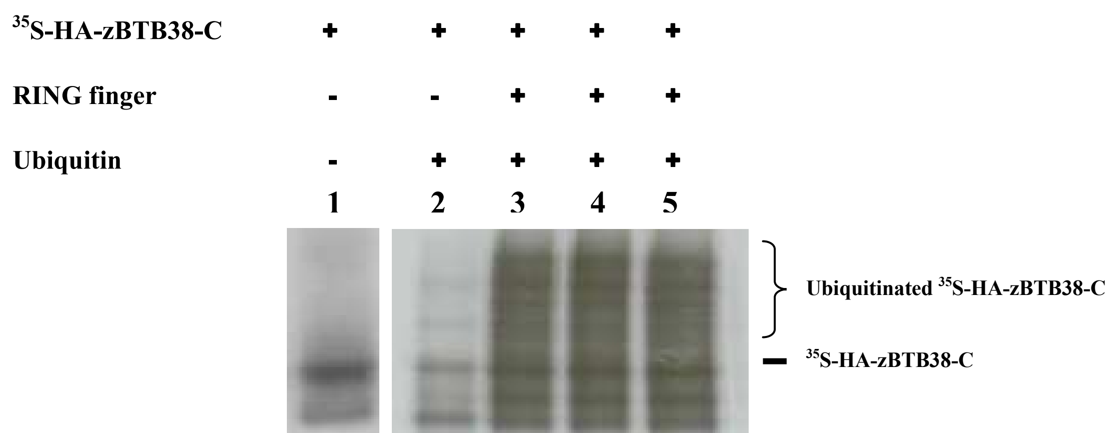

Figure 4.14. RING finger enhances ubiquitination of zBTB38 *in vitro*.

^{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).

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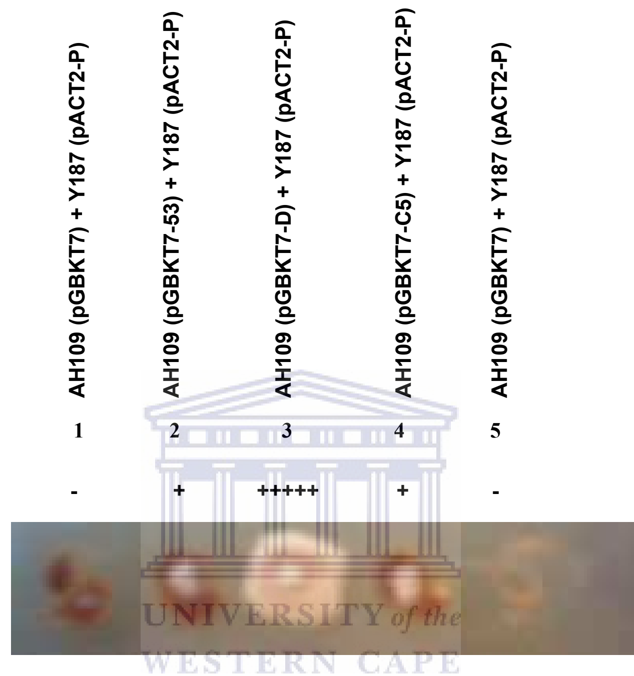

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Figure 5.1. An example of library prey (pACT2-P) that interacts DWNN only

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.

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 e snRPG were subcloned into the *SalI/XhoI* and *EcoRI/X* ly, of both pCMV-HA and pCMV-Myc plasmids using n in Table 5.3, to generate pHA-DWNN, pMyc-DWNN, -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

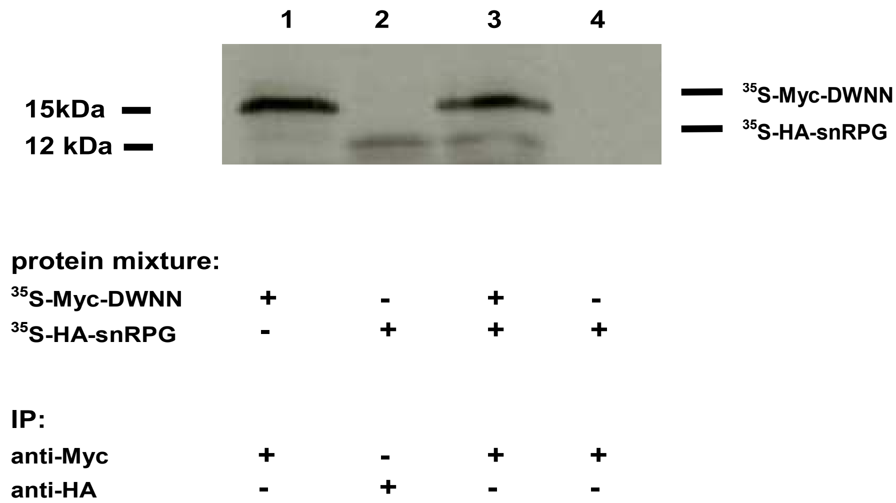

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Figure 5.2. DWNN co-immunoprecipitates snRPG proteins *in vitro*

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.

Plasmids transfected: Myc-DWNN (lysate) | Myc-DWNN + HA-snRPG | Myc-DWNN + HA-snRPG | Myc-DWNN + HA | Myc-snRPG (lysate) | HA-DWNN + Myc-snRPG | HA-DWNN + Myc-snRPG | Myc-snRPG +HA

IP: - | Myc | HA | HA | - | Myc | HA | HA

1 2 3 4 5 6 7 8

WB: anti-Myc

Myc-DWNN

Myc-snRPG

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.

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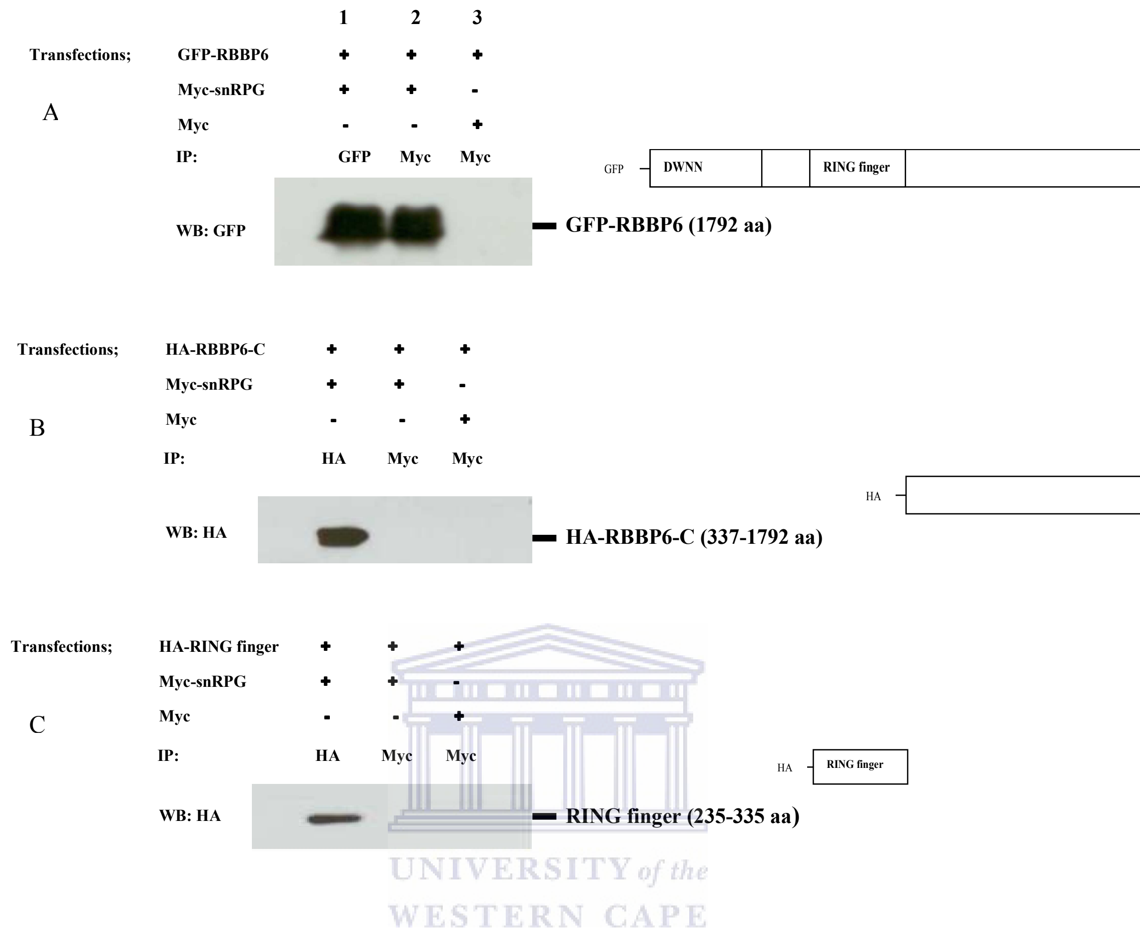

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Figure 5.4. snRPG interacts with full- length RBBP6 *in vivo*

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.

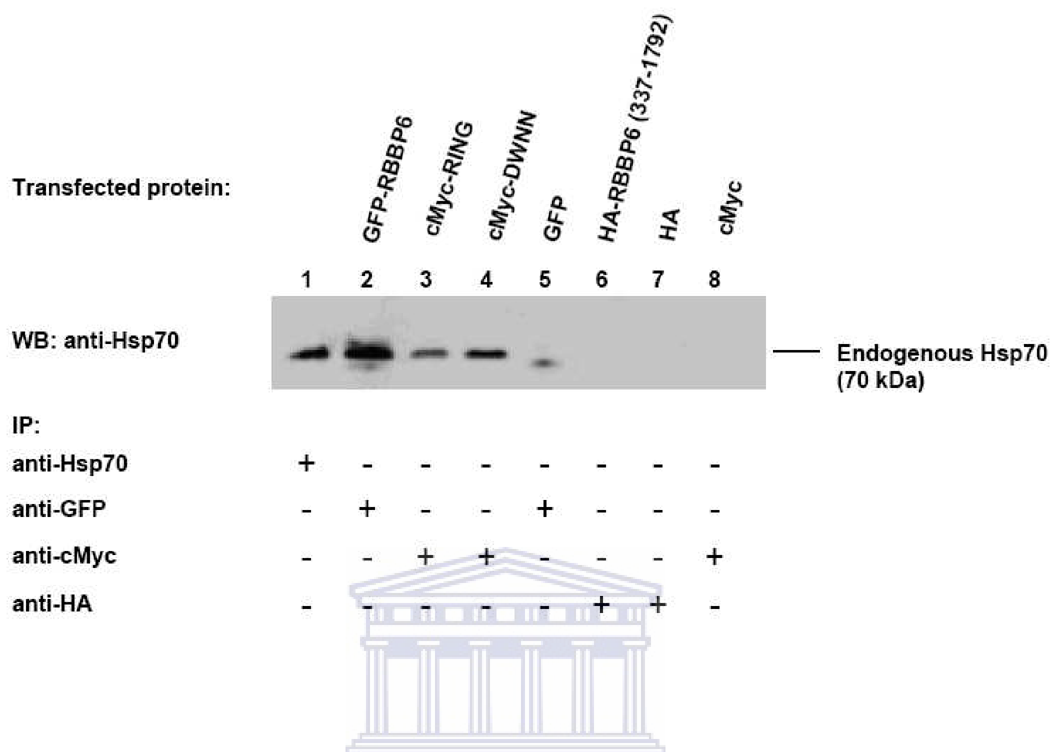
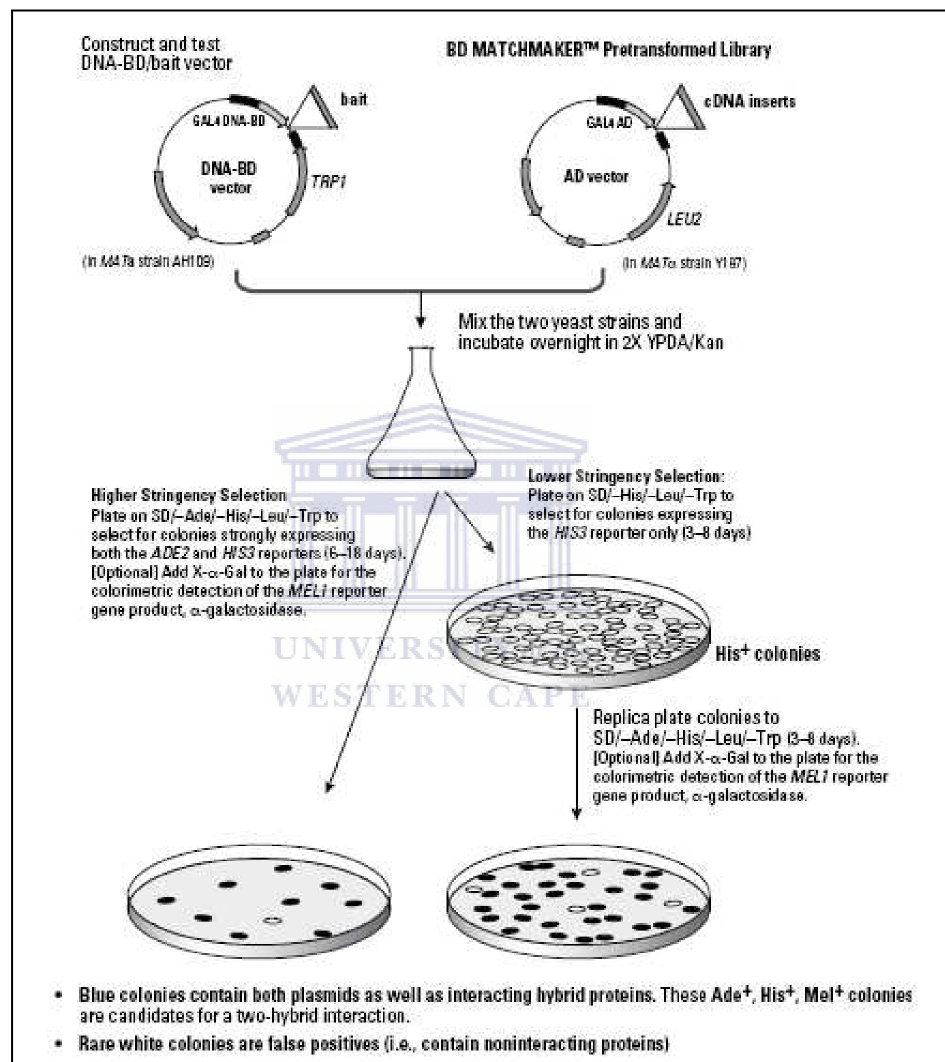


Figure 5.5. RBBP6 co-immunoprecipitates endogenous Hsp70

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.

APPENDIX I

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



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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.

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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