UNIVERSITY OF ZULULAND

RECOMBINANT EXPRESSION, PURIFICATION AND STRUCTURAL CHARACTERIZATION OF *ASPERGILLUS NIGER* RING FINGER DOMAIN USING NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

By

Olufunmilayo Olukemi Akapo

Student Number: 201426758

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Supervisor: Prof. Abidemi Paul Kappo

Co-Supervisor: Prof. Albertus Basson

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ABSTRACT

The retinoblastoma binding protein 6 (RBBP6) is a 250 kDa nuclear protein which contains an N-terminal DWNN domain and a conserved RING finger domain. Previous studies determined the solution structure of the human RING finger domain using heteronuclear NMR spectroscopy. A bioinformatics analysis using the human RING finger from RBBP6 as searching bait revealed an unchanged zinc ion coordination pattern in all organisms except in three organisms: *Saccharomyces cerevisiae, Pichia pastoris* and *Aspergillus niger*. Therefore, structural characterization of the amino acid substitution observed in *A. niger* RING finger domain will add to our understanding of the biological role played by the RING finger domains. In this study, *in-silico* characterization of the *A. niger* RING finger using different bioinformatics tools was carried out. Subsequently, the RING finger was sub-cloned into a pGEX-6P-2 vector and expressed as a GST-RING fusion protein. GST-Agarose affinity chromatography was used to partially purify this protein to homogeneity. Thereafter, biophysical characterization of the purified protein was carried out using Fourier transform Infrared spectroscopy (FTIR) and 1D NMR. The predicted three-dimensional structure of *A. niger* was done using the SWISS-MODEL server and visualized on Chimera showed a consensus structure containing two α-helices and 3 random coils. This result is consistent with the secondary structure prediction performed using PSIPRED v3 and JPred 4. The predicted 3D model was validated by analyzing a Ramachandran Plot, which indicated 88.0 % of the residues are in the most favoured region. This is very close to the 90% widely accepted mark of 3D models indicative of excellent quality models. Added to this, eight B-cell epitopes were

predicted from the protein with four being continuous epitopes, while the remaining four are discontinuous epitopes. Thereafter, *A. niger* RING finger domain was sub-cloned into a pGEX-6P-2 protein expression vector and heterologously over-expressed as a GST fusion protein, which was later purified using GST-Agarose affinity chromatography. The biophysical analysis of the purified protein using a 1D proton NMR spectroscopy data revealed the appearance of a large chemical shift dispersions range from 1.0 ppm and -1.0 ppm in the methyl group and also 8.5 ppm downfield the amide region, thereby confirming the foldedness of the protein. Taken together, these results showed that the *A. niger* RING finger domain is very well folded and can be subjected to structural determination using heteronuclear NMR spectroscopy after collecting triple resonance coherence data.

Keywords: Cancer, GST, RBBP6, RING finger domain, NMR

DECLARATION

I declare that ***"*Recombinant expression, purification and structural characterization of *Aspergillus niger* RING finger domain using NMR spectroscopy*"*** 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 or acknowledged by complete references.

Candidate: Olufunmilayo Olukemi Akapo

Signature:

Date:

Supervisor: Professor Abidemi Paul Kappo

Signature:

Date:

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ABBREVIATIONS

Amp	Ampicillin
Å	Angstrom
Asn	N Asparagine
Asn	D Aspartic acid
APS	Ammonium per sulphate
Arg	R Arginine
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BPB	Bromophenol blue
CDNA	Complementary DNA
CHIP	C-terminus of HSP70 interacting protein
C-terminus	Carboxyl terminus
113Cd	Cd^{2+} Cadmium ion
CF1	Cleavage factor-1
CNOT4	CCR4-NOT transcription complex, subunit 4
CPF	Cleavage and polyadenylation factor
CV	column volume
Cys	C Cysteine
Da	Dalton
DNA	Deoxyribonucleic acid
D2O	Deuterium oxide
DTT	Dithiothreitol

DWNN	Domain with no name
E1	Ubiquitin-activating enzyme
E1A	Early region 1A
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin protein ligase
EDTA	Ethylene diaminetetra acetic acid
EEA1	Early endosome antigen 1
EL5	Elicitor-responsive protein
Fig	Figure
Fab1p	Formation of haploid and binucleate cells protein
FYVE	Fab1p, YOTB, Vac1p, EEA1
Gly	Glycine
GST	Glutathione S-transferase
Hdm2	Human double minute 2
HECT	Homologous to E6-AP carboxyl terminus
His	Histidine
HMQC	Heteronuclear multiple quantum coherence
HnRNP	Heterogeneous nuclear ribonucleoprotein
HPV	Human papilloma virus
Hr	Hour
HSQC	Heteronuclear single quantum coherence
IEEHV	Immediate-early equine herpes virus
IPTG	Isopropyl *β-D*-thiogalactoside
Kb	kilobase

KDa	kilodalton
Leu	L Leucine
LB	Luria Bertani broth
LIM	Lin11/Isl-1/Mec-3
Lys,	K Lysine
Mdm2	Mouse double minute 2
MEKK1	Mitogen-activated protein kinase
Met	M Methionine
MHz	Megahertz
Min	minutes
MRNA	Messenger RNA
Nm	nanometer
NMR	Nuclear magnetic resonance
N-terminus	Amino terminus
P44	Interferon-induced protein 44
Pab1p	Poly (A)-binding protein 1
P2P-R	Proliferation potential protein-related
PACT	p53-associated cellular protein-testes derived
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PDB	Protein data bank
PINCH	Particularly Interesting New Cysteine-Histidine Protein
PI (3) P	Phosphatidylinositol 3-phosphates

PHD	Plant homeodomain
Phe	F Phenyalanine
PMSF	Phenylmethylsulphonyl fluoride
Ppm	parts per million
PRb	Retinoblastoma gene product
Pro	P Proline
RBBP6	Retinoblastoma binding protein 6
RING	Really interesting new gene
RBQ-1	Retinoblastoma-binding Q-protein 1
S	Seconds
Ser	S Serine
SDS	Sodium dodecyl sulphate
SR	Serine/arginine
TEMED	*N,N,N',N'*-tetramethylethylenediamine
Thr	T Threonine
TNF	Tumour Necrosis factor
Tris	2-amino-2-hydroxymethylpropane-1,3diol
Trp	Tryptophan
Tyr	Y Tyrosine
Ubc	Ubiquitin conjugating enzymes
UFD2	Ubiquitin fusion degradation protein 2
V	Volts
Val	V Valine
UV	Ultraviolet

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CHAPTER 1:

GENERAL INTRODUCTION

1.1 GENERAL INTRODUCTION

Cancer is a global disease. It is estimated that in 2012, the number of people that died of various types of cancer excluding non-melanoma skin cancer are over 8.2 million worldwide (GLOBACAN, 2012). There were an estimated 14.1 million cancer cases around the world in 2012, of these 7.4 million cases were in men and 6.7 million in women. This number is expected to increase to 24 million by 2035 (GLOBACAN, 2012).

The retinoblastoma binding protein 6 (RBBP6) is now a reference point in biology due to the occurrence of retinoblastoma, an obvious culprit in the onset of cancerous events. It is a negative regulator of proteins found in vertebrates and also contains a p53-binding domain (Pugh *et al.*, 2006; Zhang *et al.*, 2005). The tumor suppressor gene p53 was observed in the cell response to various stresses such as DNA damage and uncontrolled cell proliferation signal. RBBP6 was first reported in invasive esophageal cancer while (Motadi *et al.*, 2011; Li *et al.*, 2007; Yoshitake *et al.*, 2004). RBBP6 plays diverse roles as a multifunctional protein in events such as cell cycle regulation, mRNA metabolism and cell development. Although the mechanism by which these proteins work is not yet fully understood, however, the role of p53 during the proliferation of cells is well documented. There are both negative and positive effects on the survival of a cell if there is an increase or decrease in protein expression (Motadi *et al.*, 2011). There are important roles that RBBP6 plays in modulating the action of p53 and pRB in cancer development. The degradation of p53 is initiated by RBBP6 and it is useful in studying the role of malignant cell survival in tumor microenvironments (Motadi

et al., 2011). Additionally, RBBP6 has been reported as an E3 ligase and contains the Really Interesting New Gene (RING) finger domain which is concerned with splicing associated proteins (Pugh *et al.*, 2006). RING finger domain of the RBBP6 mediates the interaction between the breast cancer associated protein (BRCA1) and its binding partner; BRCA1-Associated RING Domain (BARD). It has also been implicated in the assembly of protein complexes and participates in various cellular functions such as signal transduction, transcriptional regulation, immunoglobulin arrangement of genes and DNA repair (Kappo *et al.,* 2012).

Furthermore, it has been reported that the up-regulation and high levels of expression of RBBP6 in cancer cells of the esophagus, correlates or corresponds to increased rates of esophageal cancer cells and low survival rates of cancer patients (Pugh *et al.*, 2006, Yoshitake *et al.*, 2004). Its involvement in the cell cycle and transcription coupled with the integration of domains such as the DWNN and the RING finger domain confer on the RBBP6 the function of regulating pre-mRNA processing of proteins, which is achieved by covalently modifying them with an ubiquitin-like moiety (Pugh *et al.*, 2006).

Therefore, the translation of research findings in support of the fight against cancer in South Africa and the world in general cannot be over-emphasized. Hence, the search to find new drugs with curative effects at low costs with minimal side effects to relieve the burden of cancer is more of a necessity. Molecular oncology studies therefore have a potential of unlocking innovations into important aspect of biomedical science.

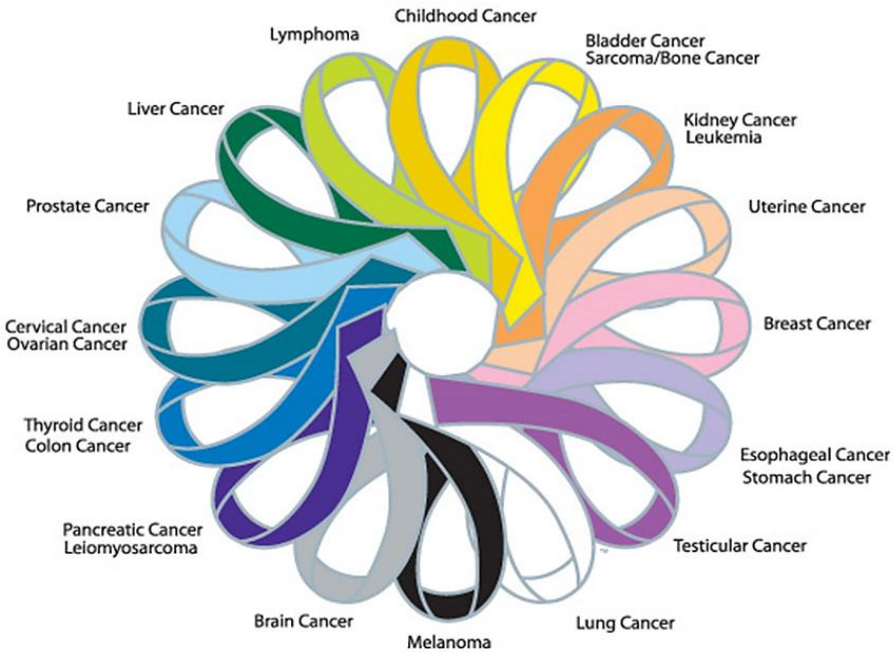


Figure 1.1: Crotchet for Cancer (Accessed on 30/04/2016 http://www.crochetforcancer.org/2012/04/03/cancerawarenessribboncolours)

CHAPTER 2:

LITERATURE REVIEW

2.1 RETINOBLASTOMA BINDING PROTEIN 6

The Retinoblastoma binding protein 6 is only found in eukaryotes and is coded for by the RBBP6 gene located on the chromosome 16p11.2-p12. It is a 250 kDa nuclear protein which contains a conserved N-terminal RING finger domain which interacts with Rb and p53 *in vivo* and *in vitro*; these domains are thought to be involved in protein degradation (Li *et al.*, 2007; Pugh *et al*., 2006; Yoshitake *et al.*, 2004). RBBP6 was first isolated from mice using p53, a known tumor suppressor as a probe. One of the most well studied regulatory molecules for cell cycle regulation, differentiation and DNA damage repair is the retinoblastoma protein (pRB) which is also a tumor suppressor (Xiong *et al.*, 2011). RBBP6 interacts with these two proteins by inhibiting the binding of p53 to DNA as well as adenovirus E1A protein to pRB.

Initially, RBBP6 was thought to be a polypeptide with an amino acid length of 948 and a predicted molecular weight of 140 kDa with multiple repeated short sequences such as SRS (12 times), YRE (4 times), VPPP (4 times), and SYSRS (3 times) (Mather *et al.*, 2005; Sakai *et al.,* 1995). The RBBP6 homologues in all eukaryotes however, appear as single copy genes, which include PACT (p53 Associated Cell Testis Derived), P2P-R, RBQ-1, Mpe 1 and SNAMA.

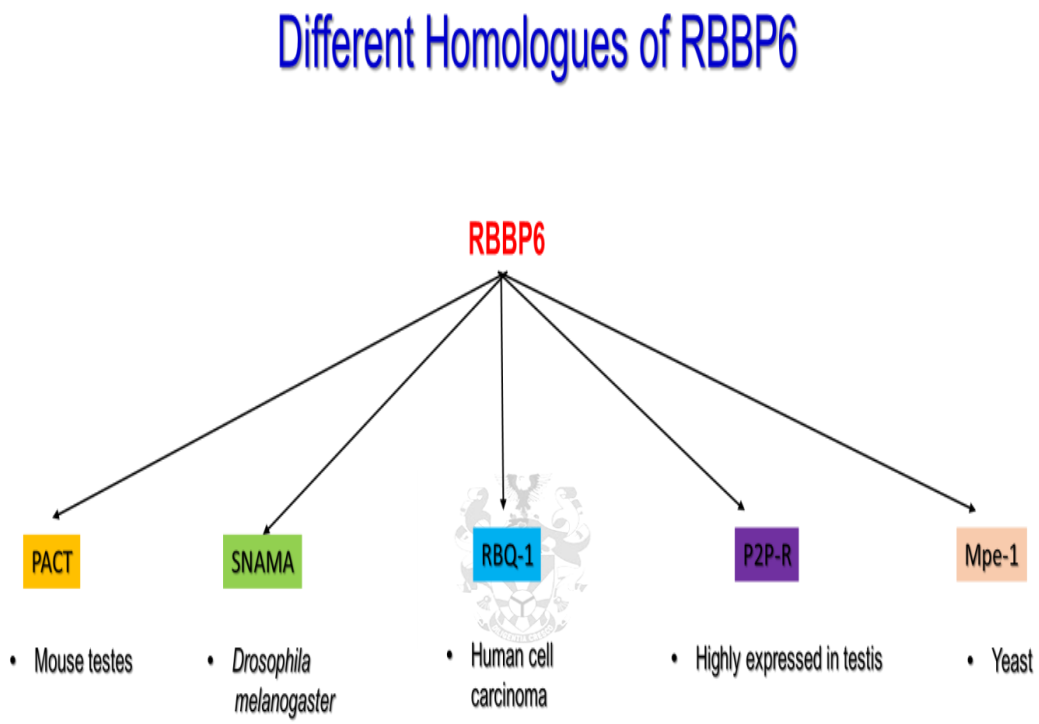


Figure 2.1: Different homologues of RBBP6 and different organisms in which they can be found. Highlighted in yellow colour is PACT and it has been reported to be found in mouse testes, green colour is SNAMA which has been reported to be found in drosophila melanogaster, in blue colour is RBQ-1 reported to be found in human cell carcinoma, highlighted in colour purple is P2P-R reported to be highly expressed in testis and the highlighted in colour pink is Mpe-1 which has been reported to be found in yeast organism.

2.1.1 PACT (p53 Associated Cell Testis Derived)

p53 was first discovered as an isoform of RBBP6 in mouse testes and later, PACT (p53 associated cellular protein-testes derived) protein was then identified by Simons and co-workers (1997) as a cellular protein that undergo protein-protein interaction with p53. It contains a serine/arginine rich N region and C terminus composed of lysine amino acids. Sakai *et al.* (1995) isolated portions of PACT first as p53 binding proteins and Rb binding proteins. The C terminal binds p53 while the N terminal binds Rb binding domain. Recombinant PACT was found to bind a wild type and not mutant p53. DNA tumor viruses produce oncoproteins which render p53 and Rb inactive. Since PACT possesses the ability to bind these two suppressor proteins, there is a possibility that it may be related to viral growth (Simons *et al.*, 1997). Previous studies have shown that there is a significant relationship between p53 and RBBP6 or PACT that plays a critical role in embryonic development and tumorigenesis (Ntwasa, 2008, Li *et al.*, 2007). Experiments done on PACT deficient mice showed that embryos end up dying early, having retarded growth and were reduced in size. Additionally, PACT interacts with RB and p53 both *in vivo* and *in vitro* (Li *et al.*, 2007).

2.1.2 P2P-R (Proliferation Potential Protein-Related)

P2P-R can be described as a nuclear protein concerned with the control and regulation of gene expression with mitosis being its main function. Since the protein lacks one exon encoding 34 amino acids, it is referred to as an alternatively spliced product arising from the P2P-R/PACT gene (Scott *et al.*, 2003). This predominant protein is expressed in several murine cell lines. Expression of P2P-R is controlled by differentiation and cell cycle events with

markedly repressed mRNA during the process of differentiation. It also interacts with scaffold attachment factor-B (SAF-B), matrix attachment regions (MARs) binding factor and with nucleolin. The protein contains an N-terminal RING type zinc finger, an SR region, a proline rich domain and a C-terminal domain rich in lysine. Phosphorylation of its binding to suppressor proteins is carried out by cdc2 and SRPK1a protein kinases. Studies have shown and supported the possibility that P2P-R can form complexes with Rb1 and/or p53 tumor suppressors which may influence gene transcription and organization of nuclear materials (Peidis *et al.*, 2010).

2.1.3 RBQ-1 (RB- Binding Q-Protein 1)

RB Binding Q-Protein-1 is one of the novel RB binding proteins. Unlike other proteins, the protein binds through a cell cycle that is dependent on an under-phosphorylated RB. Binding is disrupted by the E1A protein showing that there is more of a physiological importance of the binding to pRB (Sakai *et al.*, 1995). The protein has a molecular weight of 140kDa and is made up of 948 amino acids. The binding region is located on the C-terminus and according to PCR analysis carried out on human-hamster hybrid cells, binding takes place on the 16p11.2-p12 chromosome (Sakai *et al.*, 1995). Additionally, it contains a putative splicing region of 34 amino acids in the middle of its sequence (Sakai *et al.*, 1995).

2.1.4 MPE-1

Mpe-1 is the yeast homologue of the human RBBP6 and is a subunit of the Cleavage and Polyadenylation Factor (CPF) complex which interacts with PCF11 genetically with the novel gene Mpe-1 (YKL059c). It is an evolutionarily conserved

protein that encodes for *in vitro* pre mRNA 3'-end processing. The yeast specific cleavage and the polyadenylation of pre- mRNA in *Saccharomyces cerevisiae is formed with cleavage factor 1 (CFI) and the CPF along with the addition of Pab1p*. The protein sequences of Mpe1 include a putative RNA-binding zinc knuckle motif (Pugh *et al.*, 2006; Vo *et al.*, 2001). Vo and co-workers (2001) further revealed that Mpe1 exists in *S. pombe*, *A. thaliana* and *D. melanogaster* where the zinc knuckle and two other regions are well conserved. However, one of the cDNAs in humans which is similar to the conserved domain in Mpe1p, has been shown to code for RBBP6 protein and interact with suppressor proteins pRB1, which is involved in cell differentiation with its location at the center of mRNA processing (Vo *et al.*, 2001).

2.1.5 SNAMA (Something that sticks like glue)

SNAMA is the Drosophila *melanogaster* homologue of the human RBBP6 protein with a highly conserved structure named DWNN domain (Domain with no name). The name SNAMA is from a Xhosa word for "something that sticks like glue". SNAMA contains an open reading frame (ORF) which codes 1231 amino acids with an estimated molecular weight of 139kDa; containing 9 exons and 8 introns of SNAMA revealed by transcription. SNAMA is composed of DWNN, a zinc finger with structure CCHC which has amino acids at location between 151 & 168 and a cysteine rich RING finger-like motif (RTL) between amino acids position 217 and 250. The DWNN has a folded 3D structure similar to ubiquitin which shows that it could play a role similar to ubiquitin or could be involved in tagging other proteins. The RFL domain at the fourth RING finger of this protein has its histidine substituted for serine which shows that the protein could play an ubiquitin ligase

role. It is a highly charged basic putative protein with its C-terminal lysine-rich region containing basic residues that is more than 59% (Mather *et al.*, 2005). SNAMA has been found to be involved in apoptosis, cell survival, cell proliferation, nucleic acid metabolism and essential in development of embryo in *Drosophila melanogaster* itself but has not been shown to bind to p53 (Ntwasa, 2008; Mather *et al.*, 2005). SNAMA/Mnmp also acts in the downstream hedgehog pathway during the process of development of the compound eye of the *D. melanogaster.* Hedgehog pathway is a major pathway in human tumors development and developmental abnormalities such as brachydactylic and some limb defects. It was also found to be involved in the development of various important systems in man such as central nervous system, the circulatory and respiratory system in man (Jones *et al.*, 2006; Mather *et al.*, 2005; McMahon *et al.*, 2003; Ntwasa, 2008).

2.2 RBBP6 Domains

RBBP6 homologues found in eukaryotes have domains such as the Domain with no name (DWNN), the Zinc finger domain and the RING finger domain as single copy genes. These domains have been preserved in plants, microsporidia, protozoa, and fungi. The long C-terminal extension in mice and humans contain a proline-rich domain (residues 337–349), the Rb-binding (residues 964–1120), a SR domain (residues 679–773), and p53-binding domains (residues 1142– 1727) (Pugh *et al.*, 2006). Simons and co-workers (1995) showed that a truncated form of RBBP6, P2P-R, does not have Domain With No Name (DWNN) that binds p53 and pRB (Motadi *et al.*, 2011; Sakai *et al.*, 1995; Simons *et al.*, 1997; Witte and Scott, 1997). Additional domains have been identified in insects, mammals and

birds; a mapping carried out by Witte and Scott on the mouse isoform PACT/P2P-R revealed that such additional domains include the SR region, lysine-rich region, a region rich in arginine and features such as p53 and RB binding domains. In *Encephalitozoon cuniculi*, the domain is highly compacted and has such been said to be a minimal eukaryotic single-celled organism (Pugh *et al*., 2006).

The human eukaryotes RBBP6 homologues, additional Long C-terminal extension is included having the p53-and pRB domains of interaction. Common domains to all the above eukaryotes include ubiquitin-like DWNN domain, a zinc knuckle and a RING finger domain as the core arrangement. The protein also exists as a short form of 118 amino acids consisting of the DWNN domain alone and having a poorly conserved C-terminal extension.

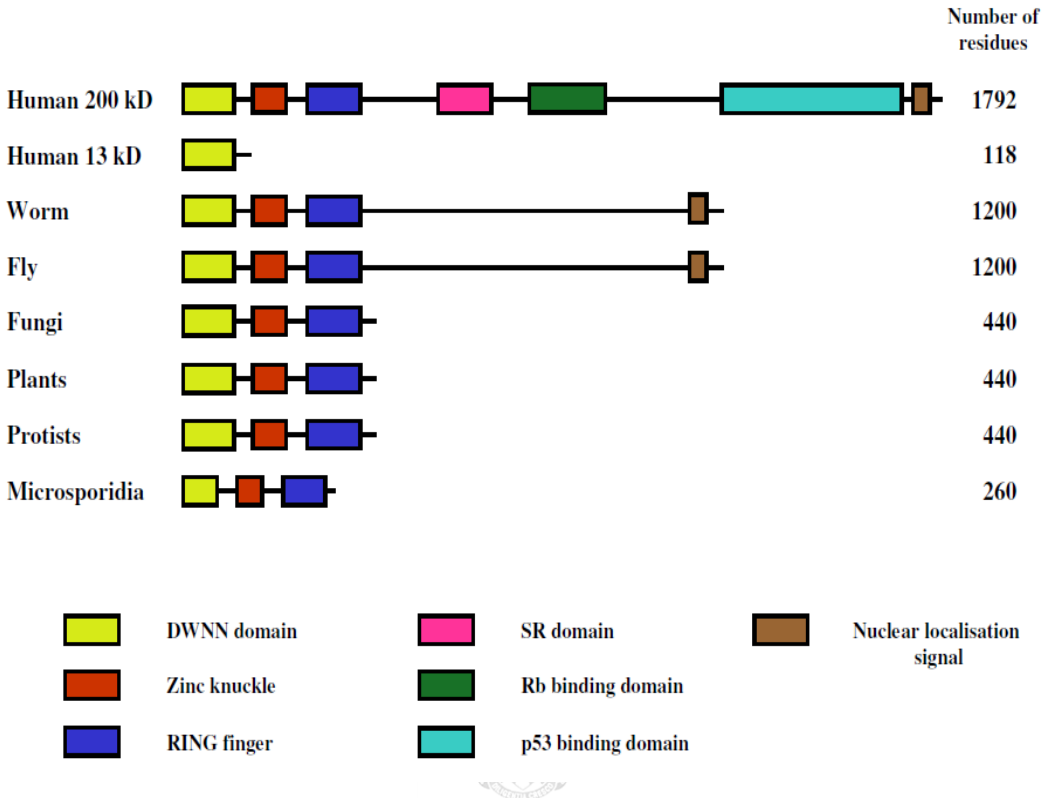


Figure 2.2: Organisation of the domain RBBP6/PACT proteins family (Adapted from Pugh *et al.*, 2006)

2.2.1 DWNN

Pugh and co-workers (2006) expressed the Domain With No Name (DWNN) in *Escherichia coli*. This is a recombinant ubiquitin-like domain found at the N-terminal domain of the RBBP6 family of splicing-associated proteins. The DWNN domain is a RBBP6 variant 3 which is expressed in vertebrates as residue 118-150 as small protein (Pugh *et al*., 2006). The DWNN corresponding to the residue 1-81 of the RBBP6 N-terminal is relatively stable as compared to the proteolytic sensitive RBBP6 protein. Using the Dali server to compare the entire Protein Data base with the DWNN structure, the findings revealed that it is close in similarity to the ubiquitin found in humans ubiquitin (PDB:1UBI). However, the similarity in the amino acid sequences between the DWNN and the ubiquitin is only 18%. According to structural determination of the DWNN structure by superimposition with the similar ubiquitin, the root mean square deviation between the two is placed as 1.88A (Pugh *et al*., 2006)

The α-helix protein structure of DWNN is arranged in an ubiquitin-like β-grasp pattern facing five stranded β-sheet namely β1, β2, β4, β5 and β7 strands respectively. The notable difference between DWNN and ubiquitin is the additional antiparallel β-sheet short section between sheets β3 and β6, residues 23–25 and 63–65 respectively leading towards the α-helix structure (Pugh *et al.*, 2006). The mRNA processing protein is modified by the ubiquitin moiety of the RBBP6 homologues containing the RING finger domain. DWNN involvement in protein splicing mode of action is yet to be fully determined (Pugh *et al.*, 2006).

2.2.2 ZINC knuckle

Zinc finger domains (ZnFs) are common DNA-binding modules that have folds of relative small proteins around one or more zinc ions. ZnFs are commonly found in clusters with small zinc litigating domains preferring different binding specifications. They facilitate interaction between nucleic acids and proteins intermolecularly. With respect to nature and arrangement of zinc binding residues, there are at least 14 different classes of Zinc fingers. There are GATA-type and Multiple GATA-types of Zinc fingers. The latter types are important in the transcriptional role they play due to their different binding selectivity and affinities. While the former type of Zinc finger bind a variety of other proteins and DNA. Classical ZnFs connect zinc ions through their cysteine and histidine residues. DNA-binding ZnFs specifically bind small molecules like protein kinase C and nuclear hormone receptor proteins. The family of ZnFs only acts exclusively in protein interactions such as ubiquitination and assembly of large protein complexes (Matthews and Sunde, 2002).

2.2.3 p53-binding domain

Regulatory region containing the p53 binding domain is at the C-terminal with residues (363-393). This domain interacts in binding p53 (oncogene) which is an important tumor suppressor protein. Pavletich and co-workers (1993) isolated a protease resistant fragment comprising of ~190 residues (residue 102-292) which was observed as the sequence specific p53 protein. The p53 DNA binding domain is inhibited by chelation with a zinc ion. The DNA binding residues (100-300) are located in the central core which also contains the mutational spot within the p53 gene. The mutation of this protein is an underlying reason for the eventual

development of cancer (Whitford, 2008). The domain ubiquitinates as well as binds to p53, to initiate and control normal cell growth and development (Wei *et al.,* 1997). Permanent inactivation of the p53 domain is caused by high levels of MDM2 (murine double minute 2) which attach to the N terminal leading to blocked transcription and shows the likelihood that mutations will be passed to daughter cells. MDM2 negatively regulates the action of the p53 binding domain (McCoy *et al.,* 2003).

2.2.4 RB-binding domains

Rb-binding domain for the RBBP6 can be found at the C-terminal in higher eukaryotes with binding retinoblastoma gene function. The human gene is synthesised in the cell cycle with 928 amino acids binding to DNA virus proteins such as T-antigen (TAG) from SV 40, EA1 from adenovirus and E7 from papilloma virus (HPV) protein and regulates terminal differentiation in cells (Ko and Prives, 1996; Saijo *et al.*, 1995). The control of the G1 to S-phase transition is negatively regulated by the Rb to tumour formation in the cell cycle. In order to prevent carcinogenesis and when DNA is damaged, a signal is sent to the p53 protein in order to initiate apoptosis or for Rb to arrest the G1 onset (Ko and Prives, 1996). The enzymatic regulation of Rb is by cell cycle dependent phosphorylase through cdk4 and cdk6 (Bartek *et al.*, 1997). The onset of human cancers such as lung carcinomas, breast and prostate cancer has been implicated with the gene mutation (Sellers *et al.*, 1995).

2.2.5 RING finger domain

The human Really Interesting New Gene (RING) domain is located in the region of the chromosome. It is a protein sequence structure that regulates the important process of transferring E2-bound ubiquitin to protein substrates without thioester (Kandias *et al.*, 2009). The N-terminal of the RBBP6 contains the RING finger domain and contains a U- box with conserved residues that are hydrophobic. This domain contains 70 residues amongst which are eight cysteine rich residues which independently fold with the aid of two Zn^{2+} ions coordinated by four cysteine or histidine in a “cross brace” manner. It also implies that one of the ions is coordinated by the first and the third in line Cys/His pairs and the second and fourth Cys/His pairs coordinates the other ion. There are different classifications for RING finger according to the pattern of the zinc coordinating residue namely C3HHC3, C2H2C4, and C4C4 and most common C3HC4 RING fingers (Kappo *et al.*, 2012). The RING finger domain of RBBP6 has a C-X2-C-X1 1-C-C-X-[NS]-X2-C-X2-CX12-C-X2-C instead of the common C3HC4, which implies that they are either C4C4 or C3NC4-type RING fingers. This is determined by the type of residues involved in coordinating the two zinc ions (Pugh *et al.*, 2006). There are two types of RING finger structure with unique cross brace arrangement, where the X is representing amino acid cysteine (RING-HC) or a histidine (RING-HC) in the third position metal-chelate Zn^{2+} ion motif for binding. Through NMR spectroscopy and X-ray diffraction, many structures of E3-type RING fingers have been determined (Kandias *et al.*, 2009). The Real Interesting New Gene (RING) finger possess similar structure namely two large loops connected by a Zinc ion, an alpha helix and three short stranded β-sheets packed together. This structure

is close in resemblance with that of U-boxes; only that bridge of salt and hydrogen bonds replaces the Zinc ion ligation (Kappo *et al.*, 2012; Ohi *et al.*, 2003). Kappo and co-workers have reported that a dual classification of Zinc ligated RING finger and U-box has been accorded the RBBP6 cysteine rich domain at residues (249-335).

The important roles played by cysteine residues have been found to be beneficial as its disulphide bond bridges have been found to be important in protein stabilization. There is possibility that there are cysteine thiols chains that are not involved in enzymatic or structural activity, though this is at a cost to the organism (Bastida-González *et al*., 2016 and Raquejo *et al*., 2010). It has been postulated that if cysteine thiols are in abundance, it could become modified and eventually sacrificed (Raquejo *et al*., 2010). The Zn^{2+} ions bind to the domain in a “cross braced” fashion and can be replaced without any significant effect on the stability or the conformation by Cd^{2+} ions (Kappo *et al.*, 2006).

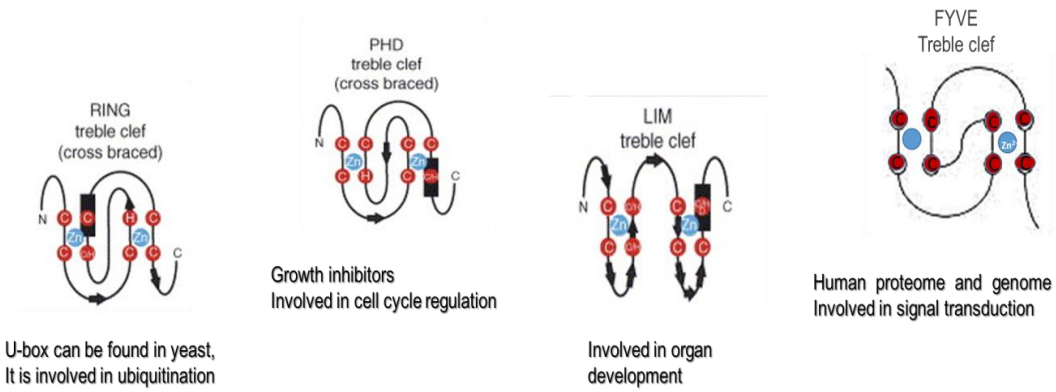


Fig 2.3: RING finger –like domains. The different forms of A. niger RING finger domain includes U-Box which is involved in Ubiquination, PHD is involved in cell cycle regulation, LIM in organ development and FYVE involved in signal transduction. (Adapted from Dominguez *et al*., 2004)

For ubiquitination activity to occur, most RING fingers and U boxes have evolved a way of dimerizing in solution to form homodimers. A 4-helix bundle in BRCA1/BARD1 heterodimer is formed when two monomers interact along the sheet of the N and C terminals while the interaction in the U box domains from CHIP and PUB14 occurs in between N terminal helices against the C-terminal helices. The helices are structurally packed loop.

Ubiquitin ligases are type of enzymes that facilitate protein recognition and degradation; of which RING finger is also a type, hence Matthew and Sunde (2002) suggested that there could be involvement of RING finger in the ubiquitination of protein and consequent degradation of proteins that are misfolded. The process of ubiquitination has been described as targeting a misfolded protein by adding to an ubiquitin tag the cell activator E1 to continue the degradation process. Other important enzymes include the ubiquitin conjugator E2 and the ubiquitin-protein ligase, E3. Ubiquitination as a process has been an important part of the cellular process of signal transduction, transcription and control of the enzymes. The substrate to be degraded needs to bind to the domain associated with the E2 enzymes; the motif stabilized by the zinc ion ensures that this is possible (Joazeiro and Hunter, 2000). This function is shared by the U-box and the RING finger to ensure that the ubiquitin is transferred to the substrate to be degraded. This is important for the substrate binding to the E3 ligase enzymes. This unique structure of the RING finger domains are observed in fungi such as *Pichia pastoris*, *Aspergillus niger* and *Saccharomyces cerevisiae* which all have the coordinating ligand for the zinc ion at the first and third pair of

cysteine residues lost and are now replaced by hydrogen bonds while bounding to only one zinc ion instead of two. The u -box are semblance of the same network (Kappo *et al.*, 2012). In *Aspergillus niger*, however, one of the second cysteine has been replaced by an aspartic amino acid (Kappo *et al.*, 2012). RBBP6 sequences in higher eukaryotes have an asparagine residue found at the position occupied by histidine in C3HC4 RING fingers. Kappo and workers have raised a question as to whether the substituted Histidine/Asparagine is involved in zinc coordination.This question with recent research has shown that direct asparagine-zinc ions (first shell ligands) is that they help form hydrogen bonds with the cysteine residue sulphur atoms to stabilise the binding sites of the zinc ion of the "second ligand" (Kappo *et al.*, 2012).

2.3 RING finger like domains

There are other RING finger–like (RFL) domains with similar cross brace structures such as the LIM, PHD, FYVE and U- Box domain.

2.3.1 LIM domain

This is one of the zinc fingers that exclusively interact with proteins. LIM domains bind two zinc atoms and play a role in protein-protein interactions. It is slightly different with a core structure of the RING domain slightly packed than that of LIM domains (Dominguez *et al.*, 2004; Matthews and Sunde, 2002).The difference between the RING finger domain and LIM is alpha helix structure and RMSD of $3A^{o}$ (Dominguez *et al.*, 2004; Capili *et al.*, 2001). Multi complexes protein formation and fundamental binding mechanisms processes are regulated by LIM

domains. It also functions as regulatory proteins, transcription factors and mixed lineage kinases (Matthews and Sunde, 2002).

2.3.2 PHD domain

Schneider and co-workers (1983) first discovered Plant homeodomain in *Arabidopsis homeobox* protein (Coscoy and Ganem, 2003). PHD is found in more than 400 eukaryotic proteins which are localised in the nucleus. It is also a specialised form of zinc finger motif with a sequence. Although there is similarity in the motif of RING finger and PHD domain; both differs in their quaternary folds and protein-protein interactions. The domain has also been found to be involved in chromatin remodelling and ubiquitination. It is found in E3 ligase, interacting with the ubiquitin conjugating enzyme (Matthews and Sunde, 2002). The identified Plant homeodomain (PHD) arises as a result of the protein been involved in plant root development (Du *et al*., 2007). Plant homeodomain structure consists of site 1 and site 2 zinc ligand. The N-terminal loop (first pair) and the beginning of the second loop (third pair) is located at the site 1 of the zinc ligand while the first two beta strands (second pair) and the end of the third beta strand (fourth pair) chelates between the site 2 ligand pair. The super imposition of the KAD-PHD and its counterpart IEEHV RING shows a RMSD of 1.3^{o}A. The PHD structure does not have the conserved alpha-helix found in RING finger as well as the difference between the position of the first zinc ligand with the second one (Kappo *et al.*, 2012).

The cellular kinase MEKKI shows PHD- dependent E3 ligase activities. The domain is involved in maintenance of chromatin structure, protein-protein

interaction and regulation of transcription (Bienz, 2006; Bottomley *et al*., 2005; Capili *et al*., 2001). The function of ubiquitin regulation of endocytosis has also been raised due to possibility of PHD domains being assigned a function in ubiquitin regulation. This is raised since it has an analogous E3-like function which is comparable to that of the RING finger domain (Coscoy and Ganem, 2003). The inactive ability of the MHC class 1 molecule with the mutation of its zinc coordinating residues has been associated with PHD domain ligase activity in-vivo (Coscoy and Ganem, 2003). Coscoy and co-workers (2001) and Hewitt and co-workers (2002) discovered that ubiquitination of MHC class 1 chains occurs with the presence of MIR1 or MIR2 causing a cystolic tail block of ubiquitin addition and the down grade of MHC 1. This fusion protein containing the MIR2 PHD in the presence of E1, E2, ubiquitin and cellular energy (ATP) can undergo the hallmark auto-ubiquitination *in vitro*-an hallmark of E3 ligase activity. The domains carry out the recruitment of E2 enzymes by recognition of the target protein in MIRs. The process of the homologues of RING finger in E3 classical ligase activity is similar to the above (Coscoy *et al*., 2001). The research on MIR1 and MIR2 have shown that these classes of E3 ligases ubiquitin rather prefers to interact with PHD instead of RING finger E3 ligases membrane bound (Coscoy and Ganem, 2003).

Lu and workers (2002) already validated the hypothesis and reported that PHD domain with an E3 activity is included in the structure of MEKK1 as Mitogen activated protein (MAP). Mitogen Activated Protein, a kinase enzyme phosphorylated several kinds of MEKS which is included in the structure of MEKK1. MEKK1 is a cellular mitogen-activated protein (MAP) kinase enzyme which phosphorylates several different MEKs. The activation of MAP is carried out

by osmotic shock resulting into phosphorylation of Extracellular-signal-regulated Kinases 1 and 2(ERKs) (Lu *et al*., 2002). A poly-ubiquitin process of degradation of the ERK1/2 occurs after activation and process inhibited by proteasome inhibitors. E3 ligase has been shown to aid in the structural affinity MEKK1 for ERKs with dependent on PHD motifs devoid of any mutation (Lu *et al*., 2002). The regulation and initiation of transcription, remodelling of ATP dependent chromatin complexes and maintenance of chromatin structure is also carried out by the Plant homeodomain (PHD), a zinc-finger motif. Many chromosomal proteins contain histone modified enzymes with PHD fingers and elongation factors (Bienz, 2006). It is majorly contained in chromatin remodelling proteins. The process of histones covalent modifications such as acetylation and methylation supports a major role in the tightly regulation of the genetic information contained in the DNA and in DNA repair and gene expression. Pedro (2009) carried out experiment to highlight the PHD-mediated targeted process of chromatin-remodelling to chromatin.

The recognition of the tri-methylation of lysine 4 of histone H3 (H3K4me3) by PHD finger of ING (Inhibitor of Growth) was correlated by initiation of transcription. The inhibitor of growth is a family of tumour suppressor proteins which plays a role in apoptosis and DNA repair. The NMR spectroscopy and X-ray Crystallography of this PHD finger of this ING family show as the specificity and affinity of the domain for the tri-methylated lysine 4 of histone H3. Intermolecular hydrogen bond exists between the aromatic side chains of the tyrosine and tryptophan residue to the trimethyl ammonium group of the Lys 4 peptide. The mutations of H3K4me3 due to cancer and the residue of the binding site being substituted causes the impairing ability to induce both the process of apoptosis and DNA repair *in vivo*.

The recognition of the H3K4me3 molecule has therefore been suggested as the hallmark of ING/YNG proteins (Pena *et al*., 2006; Pena, 2009).

PHD fingers have been thought to mediate protein –protein interaction with its canonical Zn^{2+} motif. Some PHD fingers do compete with RING finger domain surface for E2 ligases binding to also bind with nucleosome and nuclear partners. This might be a support towards the understanding that chromatin is a PHD finger nuclear ligand; structural basis formation of a complex. Pygo-Lgs/Bcl9-Arm and the Beta (β- catenin) in the Wnt signaling have been made possible by the understanding of the PHD finger of Pygo (Bienz, 2006, Pascual, 2000). A possible drug target for cancer may be suggested as made possible by the research on the interaction of Pygo PHD finger interface with Lgs/Bcl9 since colorectal cancer has been associated with the Wnt signaling (Bienz, 2006, Pascual, 2000). Mutation or alternative translocation of the PHD finger-proteins has been observed in various immunodeficiency syndromes, cancer and other human disease disorder that are neurological (Baker *et al*., 2008).

2.3.3 FYVE domain

FYVE domain was found in early endosomal antigen 1 and named after the first letter of the four protein where it was discovered namely Fab1p, YOTB, Vac1p and EEA1 (Stenmark *et al*., 1996, Gillooly *et al*., 2001). FYVE domain is made up of a C=-terminal and two central beta (β) strand that contains conserved eight cysteine residues which binds in a ‘cross braced’ structure the two zinc ions with a characteristic R(R}K)HHCRXCG motif near cysteine residue located at the third and fourth position. The FYVE domain is similar in structure with that of the “cross

brace" structure of the RING finger unlike the PHD that is different from the RING finger due to the arrangement at the different positions of the secondary structure (Matthews and Sunde, 2002; Stenmark *et al.*, 2002). There are several hydrophobic residues such as in arginine that is conserved among the FYVE domains near the C-terminus. One of the FYVE domain function is that of endosomal localization of EEA1 (Gillooly *et al.,* 2001). Gillooly and co-workers (2001) reported a structure determined by the X-ray crystallography as the ligand-free moiety of the FYVE domain and its homologue Vps27p. The results showed that FYVE domain consists of two zinc ions and a C terminal α-helix stabilising the two stranded β sheets that forms a basic pocket with the conserved arginine residues (Gillooly *et al.,* 2001).

The FYVE structure is similar to the treble clef superfamily of proteins commonly known as Protein Kinase C. FYVE domains were subsequently identified as phosphatidylinositol-3-phosphate (PtdIns3P*)* binding domains (Gillooly *et al.,* 2001), which have recognition site for the lipid PtdIns3P, which is part of the cell membranes of eukaryotes. It has been shown through NMR studies of the FYVE domain of EEA1 protein, that the domains are more specific for PtdIns3P as opposed to PtdIns5P. This is as a reason of the three basic residues Lys^{4}, Arg^{27} (and Arg^{28}) was being more influenced to directly bind to the 3' but not the 5' phosphate group. This in turn determines the specificity of the FYVE domains. The residue of the basic RRHHCRQCGNIF motif also participates in the binding and plays a role in ensuring the specificity of the FYVE domain for the structurally and most similar phosphoinositide to PtdIns3P. Gillooly *et al.* (2001) raised the observation that studies in NMR suggested that FYVE domain can dimerize which

increase its affinity for PtdIns3*P* apart from the homodimeric dimerization of EEA1 (Gillooly *et al*., 2001). Usually a FYVE domain function in cell membrane includes signal transduction, phagocytosis and vesicular trafficking (Matthews and Sunde, 2002; Stenmark *et al.*, 2002). EEA1 is one of the types of FYVE domain that has been widely studied with a C-terminal and coiled large protein. Rabenosyn-5 (Rab-5) is another FYVE domain which has been recently identified to play not just a role in endosome trafficking but has been proposed to be a homologue of Mammalian Vac1p which is also a homologue of EEA1. Out of the five FYVE domain found in yeast, the function for three of them (Vac1p, Vps27p and Fab1p) has been proposed as involved in (endocytic) vacuolar trafficking (Gillooly *et al.,* 2001; Nielsen *et al*., 2000; Peterson *et al*., 1999; Rothman *et al*., 1994).

2.3.4 U-boxes

U-boxes have been described as “non-zinc-binding type of RING fingers (Kappo *et al.*, 2012). The U-box is similar in structure to the RING finger as well as the function of mediating the ubiquitin-conjugation on protein substrates. The U-box motif though identified first as UFD2 (ubiquitin fusion degradation protein 2) in the yeast has progressively been shown to be included in other eukaryotes is having approximately 70 amino acid residues (Hatakeyama and Nakayama 2003). U-boxes have been described as “non-zinc-binding” RING fingers. But the chelating metal residues that is highly conserved in RING finger is lacking in U-box domain (Aravind and Koonin, 2000). One basic functional difference between RING fingers and U-boxes is that the latter interacts with chaperones, which mostly include notably with Hsp70, Hsp40, and Hsp90. U-boxes are also involved in

protein quality control. Kappo and co-workers (2012) place forward two bases for suggestion to classify RING finger -cysteine rich domain of RBBP6 as a U-box. This suggestion is based on their similarities in structure and their interaction with chaperones namely Hsp70, Hsp40, and Hsp90 though they differ in the zinc binding ions in the RING finger structure. Though similar in structure to the U-boxes, RING1b/Bmi1 heterodimer and the homodimer TRIM37 have been reported that they bind two zinc ions (Kappo *et al.*, 2012). The U-boxes in line with the RING finger help to recruit ubiquitin-conjugated E2 in order to transfer the enzyme E2 to the substrate.

The E3 substrate binding domain needs E2 enzyme to carry out its function. U-boxes have also been considered as a conserved E4 ligase also known as elongation factors. E4 functions as a catalyst not on the substrate but tagging addition of ubiquitin to already existing polyubiquitin chains. The process of U-boxes catalysing the poly-ubiquitination process renders the interaction with E4 ligases a problem because it does not initiate E3 ligase separately. It has been reported that chaperones such as the tetratricopeptide domain of Hsp70 interact with many U-boxes and ubiquitin unfolded proteins presented to it by Hsp70.

Chaperones are well known characterised C-terminus of Hsp70 interacting protein (CHIP) most well characterized chaperone being the C terminus of Hsp70 interacting protein (CHIP) (Kappo *et al.*, 2012).

The U-box domain has been shown to be involved in multi-ubiquitination of proteins as E4, a new class of ubiquitilating enzymes (Pringa *et al*. 2001; Aravind and Koonin 2000). While some lack their own substrate but aid in the transfer of

ubiquitin from a conjugating enzyme E2 during elongation of polyubiquitin chains, U-boxes have also been shown to act as E3 ligases such as mammalian homologue of yeast UFD2 (Hatakeyama and Nakayama 2003). RING finger, HECT (homologous to E6-AP carboxyl terminus) and U-box has been classified as a subclass of E3 enzymes based on their structure and features biochemically (Glickman and Ciechanover, 2002; Micel *et al*., 2013). Even though there is absence of zinc coordination which is critical to proper folding and stability in U boxes, it assumes the same three dimensional (3-D) typology as RING finger domains. U-boxes lacks the conserved cysteine residues required for metal chelation therefore does not bind zinc (Ohi *et al*. 2003; Andersen *et al*. 2004; Houben *et al*. 2005; Zhang *et al*. 2005;Tu *et al*. 2007). However, the stability of the protein is formed by hydrophobic residues such as salt- bridges and hydrogen bond (Andersen *et al*. 2004; Ardley and Robinson 2005; Aravind and Koonin 2000). Aravind and Koonin (2000) classified the RBBP6 RING finger domain as a U-box domain because of the presence of hydrophobic residues even though they are highly conserved in U box than RING finger domains (Aravind and Koonin, 2000). The presence of eight conserved cysteine residues will however favour their classification as RING finger domain rather than U-boxes. Micel and colleagues (2013) stated that E3 enzymes play a role of identifying protein meant for degradation. It carries out its role to facilitate the interaction between the E2, RING or U-box domains and the substrate domain as interactor adaptor molecules (Micel *et al*., 2013). Cooperative removal of irretrievably folded proteins aids the degradation of protein by proteasome. This aid provided by the U-box-containing proteins tagging chaperones suggests that ubiquitination mediated by chaperones

and protein quality control during cellular synthesis is a functional role of RBBP6/U-box. This post-translational process in U-box is important to prevent the aggregation of toxic protein which has been found to contribute to the development studies of U-box containing protein which functions as E3 enzymes. Furthermore, the identification and selections of protein for degradation is mediated by this U-box with an E3 ubiquitin ligases structure (Cyr *et al*., 2002).

The genes in *Arabidopsis* related to the U-box pathway are an evidence of the importance of E3 ubiquitin enzymes in the selective ubiquitin-proteasome pathway. Smalle and Vierstra (2004) and Stone and Callis (2007) reviewed various modification types of ubiquitin-proteasome related genes found in considerable numbers in plant genomes. The numbers of the E3 ligases in *Arabidopsis* has been reported to be expansive in numbers thereby confirming the enhanced use of protein turnover in the response of plants during growth and development. This interaction confers specificity to the ubiquitination of the targeted protein for degradation with high interest shown in organism growth and development (Patterson, 2002; Yee and Goring, 2008)

In plant, a form of U-box E3 ligase has been found. It existed in form of a bacterial effector, type III AvrPtoB effector used to suppress the response of plant and cell death to *Pseudomonas syringae* pv Tomato DC3000 (Yee and Goring, 2008). This further reduces the plant fight against the virulence of the bacterial (Abramovitch and Martin, 2005; de Torres *et al.,* 2006; Yee and Goring, 2008). The ligase activity evades the auto-response of plant cell death thereby inhibiting the function

in the host and supporting the virulence of bacterial (Janjusevic *et al.,* 2006; Abramovitch *et al.,* 2006; Yee and Goring, 2008).

In general, there are various themes or functional role carried out by plants, U-box. These U-box first themed as important in plant growth and development in root, cap, stem, leaf shape and fall, flowering and seed development and various gametophytic development and flowering (Rogers, 2005; Reape *et al.,* 2008; Love *et al.,* 2008; Yoo and Goring, 2008). Over-expression of AtChip and hypersensitive response associated with altered OsSPL11 and ACRE276 laid credence to this claim (Reape *et al.,* 2008; Love *et al.,* 2008; Yoo and Goring, 2008). PUB secondly has been observed as chaperones and co-chaperones and also as E3 enzymes which aids the plant response during homeostasis stress. Chaperones helps in the folding of misfolded and unfolded proteins (Hatakeyama et al., 2004). A question has been raised about E3 ligase been a promoter of subsequent alteration of the target protein, its activity, localization and interaction during poly-ubiquitination, degradation and/or non-ubiquitination (Hicke, 2001; Schnell and Hicke, 2003). The possibility of multiple ubiquitination roles for this E3 ligase during plant responses arises from the discovery of several targets of AtCHIP points (Luo et al., 2006; Yoo and Goring, 2008).

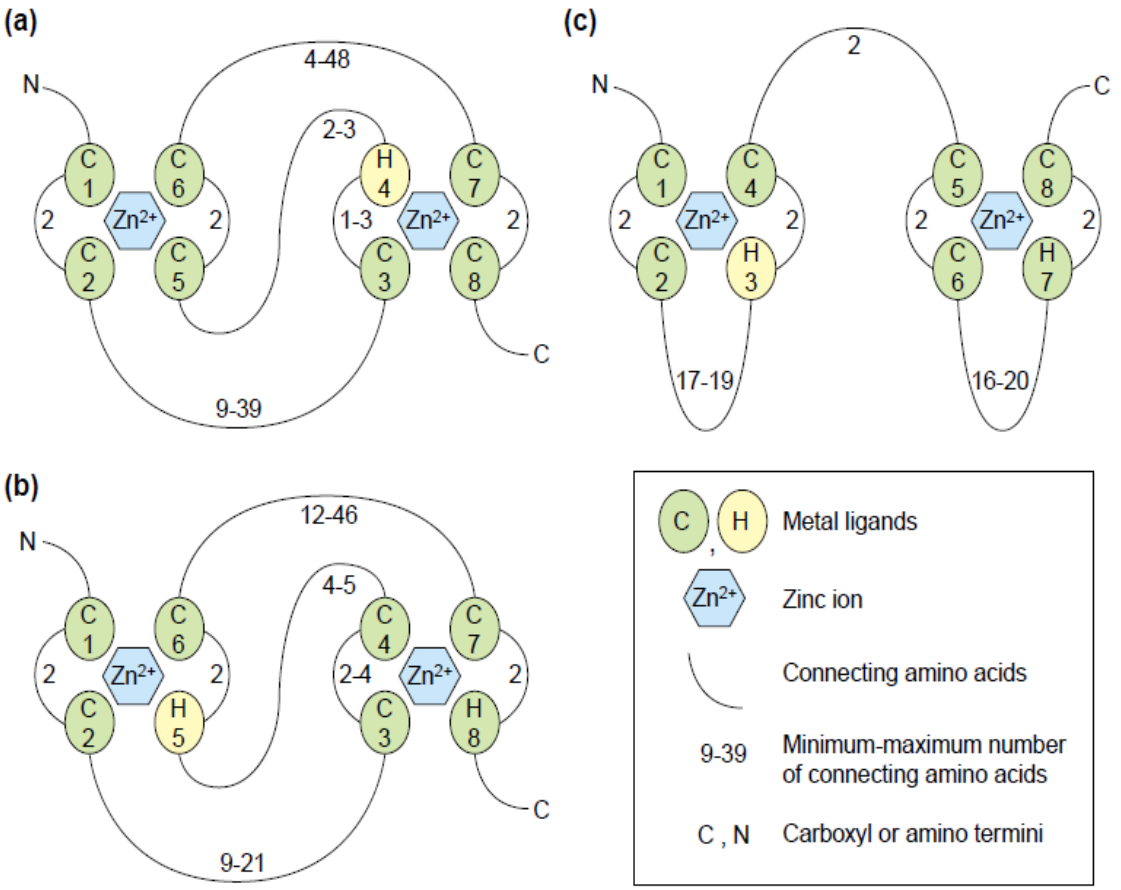


Figure 2.4: Diagram showing the structural presentation of RING, PHD and LIM domains (Image taken from Kosarev *et al.*, 2002). The figure above shows the similarities between and differences between the RING, LIM and PHD domains. The 3 domains are similar in structure in that the Cysteine (C) or Histidine (H) which acts as metal ligands are numbered, and these metal ligands coordinates two pairs of zinc ion. The diagram labelled (a) shows the structure of RING domain (RING- HC type). (b) Shows the cross- brace similar structural arrangement to that of the RING domain of PHD domains (c) shows the distinct zinc ligation of the LIM domain structure; Ligand pairs 1 and 2 is coordinated by the first zinc ion while the second ion is coordinated by pairs 3 and 4.

2.4 RING functions

2.4.1 Cell growth and embryonic development

RBBP6 plays an important role in the regulation of the cell cycle (Laurie *et al.*, 2007). During the developmental stage of the *Drosophila*'s eye, SNAMA, a family belonging to RBBP6, has been linked to the downstream hedgehog pathway that takes place (Jones *et al.*, 2006). The dE2F inhibition probably controls this

pathway by forming an interactive complex with RBF upon phosphorylation. In the development of *Drosophila,* the pathway could influence cell cycle exit or re-entry in a context dependent manner. These genes play a role in cellular function, DNA repair; apoptosis and differentiation are repressed by the action in the preceding cell cycle phase transitions processes.

It also regulates a pro-apoptotic molecule negatively (Mather *et al.*, 2005; DeGregori, 2005; Neumann, 2005). Maintenance of normal cell growth which has also been reported through tight regulation of p53 by PACT is by directly binding to p53 (Li *et al.*, 2007). Li and co-workers (2007) showed that the interaction between PACT and p53 could play a critical role in tumorigenesis and development of embryo and as well as in identification of PACT as negative regulator of p53. It also plays a role in cell growth retardation and attenuation of p53-Hdm2 interaction (Li *et al.*, 2007).

2.4.2 Apoptosis

Apoptosis is a term that describes the process of programmed cell death. Though superficially it appears as an unwanted process, thorough consideration has shown that it is vital to normal development even in the embryonic changes in structure as many changes require programmed cellular destruction. Activating a cascade of genes involved in cell growth is a cell response during apoptosis and cell cycle arrest. The pRB and p53 binding domains of the RBBP6 interacts with p53 and pRB respectively during this process. The role of preventing p53 degradation and increasing apoptosis in tumour cells is played by the RBBP6 inhibition of proteins that causes p53 degradation.

The mutated gene in human malignant cancer that is prevalent in a variety of primary sites and histogenesis is p53. Various triggers have been reported for p53 dependent apoptosis such as inappropriate oncogene activation, DNA damage or cytokine deprivation 4, heat shock and hypoxia (Bellamy, 1996). The p53 dependent response of normal proliferating cells in sustaining DNA damage is either by cell cycle arrest or apoptosis. Studies have shown that transcriptional activation of a cyclin–dependent kinase inhibitor called p21 on p53 triggers G1 arrest. Mice studies with p21 deficiency revealed that apoptosis dependent on p53 were retained in thymus and small intestinal epithelial tissues. The author also reported that there is similarity in the p21 arrest of growth and the p53 apoptosis dependent pathway respectively (Bellamy, 1996).

Nutlins, an inhibitor of E3 ubiquitin and antisense oligonucleotides have been found to trigger p53 mediated cell death in retinoblastoma (Laurie *et al.*, 2007) to inhibit expression of the Mdm2 gene (Bianco *et al.*, 2005; Wang *et al.*, 2001; Zhang *et al.*, 2005; Ntwasa, 2008) respectively. Li and co-workers (2007) demonstrated that early embryonic death occurs before embryonic day E7.5 due to the disruption of PACT in mice which was as a result of accumulation of p53 and widespread apoptosis. Data from the same study also demonstrated that the interaction between PACT-p53 could play a critical role in tumorigenesis and also identified that apoptosis results when p53 is negatively regulated by PACT. The pro-apoptotic and anti-apoptotic action of the RBBP6 are two independent pathways. The anti-apoptotic pathway is facilitated by the ubiquitination of Hmd2 through the repression of p53. The weight of the two pathways determines the final result (Motadi *et al.*, 2011; Chibi *et al.*, 2008). In Fig 2.5 below; to promote cell

proliferation, the degradation of p53 by ubiquitination is carried out by the interaction of the RING finger of RBBP6 with p53 mediated by Mdm2 (Motadi *et al.*, 2011). Increased apoptosis and prevention of p53 degradation is regulated by the inhibitory and regulatory role of the RBBP6 of mutant proteins in tumor cells cycle (Ntwasa, 2008).

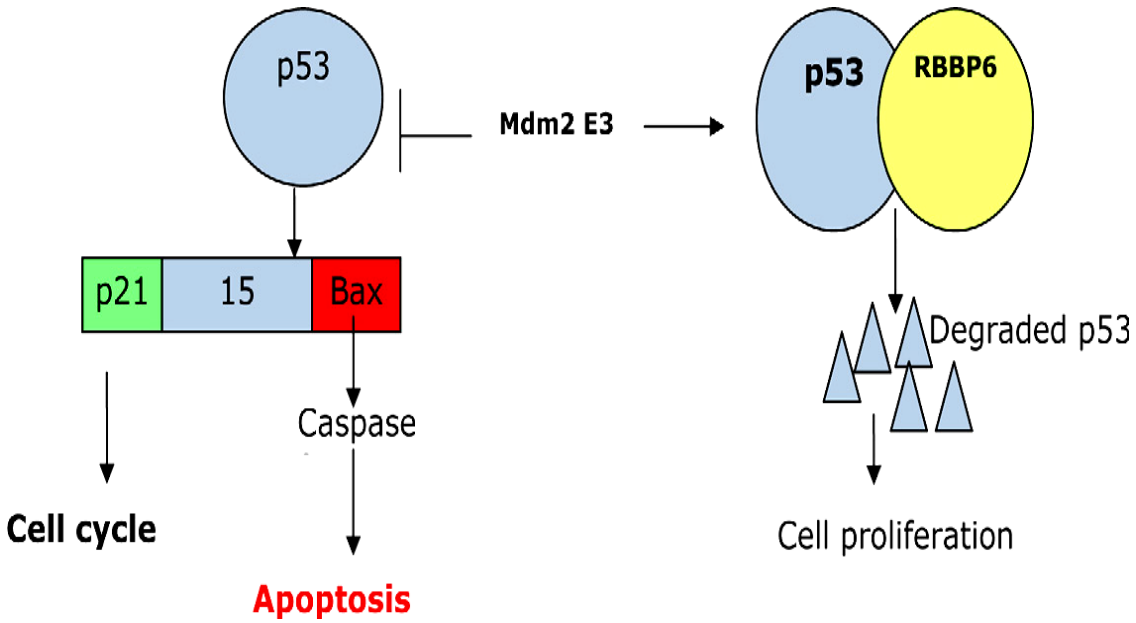


Figure 2.5: Diagram showing the RBBP6 interactions with p53, a tumour suppressor gene protein (Diagram adapted from Motadi *et al.*, (2011)).

2.4.3 Targets for anti-cancer therapy

The RBBP6 has played a potential role as anticancer therapy based on their regulatory activities such as transcription, during the cell cycle and their interaction through the RING finger domain with tumor suppressors, p53 and Rb, though little is still known about their function (Ntwasa, 2008; Pugh *et al.*, 2006). A mouse homologue called PACT that has been up-regulated in esophageal cancer is also being considered as promising target for immunotherapy (Li *et al.*, 2007). Scambia and coworkers (2006) have shown evidence that in case of non-retinal cancers such as sarcomas, breast and parathyroid cancers, pRB relevance has been observed in its regulation (Scambia *et al.*, 2006).

The development of drug discovery and control for cancer cells activity can also make use of the tumor suppressor proteins (Ntwasa, 2008). Earlier it has been reported that tumor suppressor protein alterations such as are the major causes of cancers. Aberrant and proliferation of cells that are uncontrolled give rise to breast cancer 1 and breast cancer 2 (BRCA1 and BRCA2), patched, E2F and many others (Chidambaram *et al.*, 1996; Du *et al.*, 1996; Du and Dyson, 1999; Greenberg, 2008). Though more evidence is needed on the role of RBBP6 in hedgehog signaling however, it has been observed that the RBBP6 is providing the necessary interventions platform to combat cancer and hedgehog signaling aberration diseases (McMahon *et al.*, 2003; Ntwasa, 2008). The numbers of pathological conditions in which RBBP6 proteins are involved are far less compared to their involvement in the hedgehog pathway of human tumors and abnormalities development (Ntwasa, 2008).

2.4.4 Ubiquitination

Ubiquitination mechanism is involved in various non-proteolytic cellular functions. It is an important process concerned with DNA repair, replication, virus budding intracellular trafficking and control of gene transcription carried out by attachment of single or poly (lysine 63) ubiquitin to protein to be degraded (Haglund and Dikic, 2005).The essential active role played in regulating a wide range of proteins at intracellular levels, which also includes the transcriptional regulators, is under the control of the ubiquitin–proteasome pathway. Ubiquitination is a key cellular controller of functions such as differentiation, cell progression, cell death and transduction (Fantappié *et al.*, 2003).

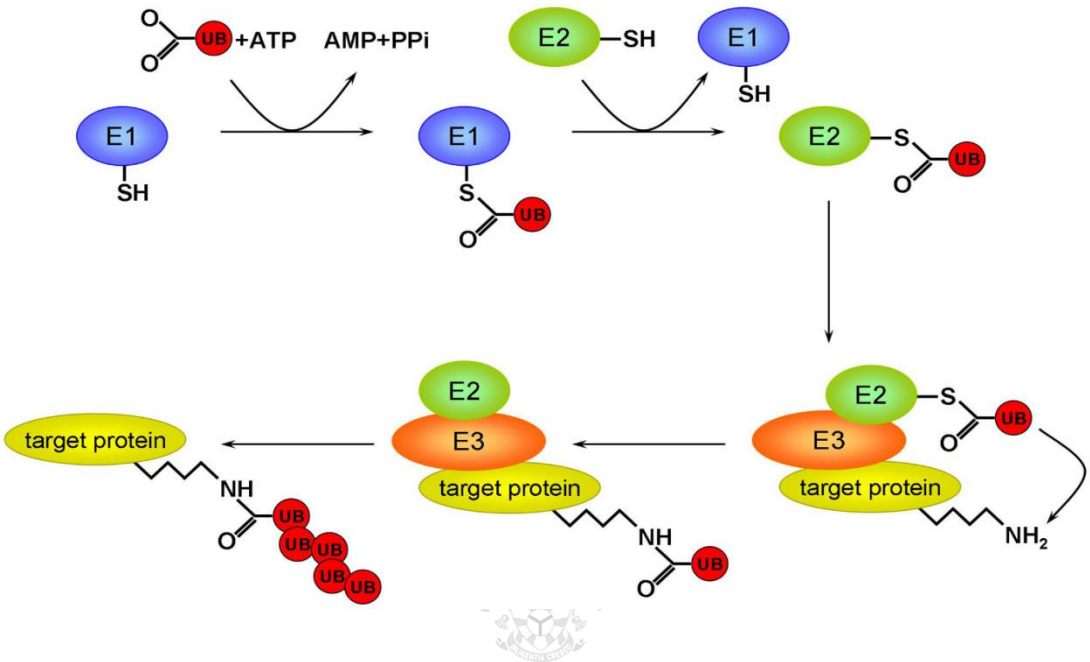


Fig 2.6: The ubiquitin pathway

(Adapted from http://www.labome.com/method/Identification-of-ubiquitinated-proteins.html). The three important enzyme- catalyst in the process of ubiquitination includes; the E1-ubiquitin activator enzyme, ubiquitin conjugating enzyme (E2) and the E3 ubiquitin ligase. The ubiquitination pathway starts with Adenosine triphosphate dependent E1 activator enzyme which forms a thio-ester bond between the ubiquitin C terminus and the E1 active site cysteine residue. The ubiquitin formed is transferred to the E2 Cysteine active site in a trans-thio esterification reaction (E2~Ub thioester intermediate). The intermediate thioester then binds to the E3 ligase along with the substrate, during this process the enzyme E3 carries out a dual role of catalyzing transfer of ubiquitin from the active site cysteine to the substrate lysine or N terminus and act as a matchmaker for the prospective E3 and substrate complex (Berndsen and Wolberger, 2014).

The RBBP6 has a RING finger domain along-side its conserved ubiquitin-like structure of the DWNN domain which can exist independently as a splice isoform as does the ubiquitin structure itself. The RING finger and the DWNN domain make it probable that the protein possesses an ubiquitin-ligase activity. This may involve interaction with hnRNPs and an ubiquitin-like moiety resulting in a decrease in affinity for mRNA (Ntwasa, 2008; Pugh *et al.*, 2006). However, it has been reported that the DWNN may have been a modified protein with an associated di-glycine peptide close to its final conserved proline which helps in conjugation of ubiquitin to other proteins. Another RING finger protein that mediates the ubiquitination and degradation of proteasome of p53 at low level is Mouse Double Mutant 2 (MDM2). However peculiar is the report that MDM2 homologue is absent in *Drosophila melanogaster* (Ntwasa, 2008).

The fact that RBBP6 containing RING finger protein could be an E3 ubiquitin ligase was investigated by Chibi and co-workers (2008). A screened RING finger domain with a full length of ^{35}S-labelled YB-1 multi-protein success initiated a further test with an unlabeled RING finger domain in an in-vitro ubiquitination assay. It was then deducted that in the absence of the RING finger and the YB-1 ubiquitination does not occurs. This further showed that RING finger is a catalyst for the in-vitro ubiquitination process. The study also reported that RBBP6 interact with the YB-1 protein resulting to ubiquitination and degradation further supports its ubiquitination role (Chibi *et al.*, 2008).

2.4.5 Transcription

Though several studies have been carried out on RBBP6, little is known about its biological role in transcription regulation. In an analysis carried out on an expressed sequence tag (EST), it was shown that the transcriptional regulation by RBBP6 is being modulated by two putative promoters called Promoter 0 (P0) and Promoter 1 (P1) (Pretorius *et al*., 2014) Though it has been previously reported that an immune system with two promoters is not unique however with ly49 genes, the function of multiple promoters have shown different interaction with MHC class 1 molecules and regulates NK-cell mediated target cell lysis negatively. Also indicated in further studies is that the activity of one promoter can be controlled by the other in what is called preferential promoter usage (Pretorius *et al.,* 2014).

2.5 Biophysical Methods of Characterization of Proteins

The increasing rate of demand for protein application in various fields ranging from biosensors, pharmaceutical and biomaterials requires modern and rapid methods of structural characterisation of proteins in dynamic environments such as aqueous and non-aqueous environment that simulate the biological systems. As discussed earlier, the use of NMR or X-ray crystallography are capable of providing three-dimensional structure of protein however they have their disadvantages. First, X-ray only works with high quality crystals relating to static picture and not the dynamic nature of the proteins in a dynamic environment while NMR on the other hand require cumbersome need for interpretation of its protein spectra which is complex and for now limited to protein with 30kDa size or proportion. Fourier transform infrared (FTIR) is fast becoming a popular method

based on molecular vibration around chemical bonds which include twisting, stretching and rotating. These vibrations around specific C=O and O-H corresponds to infrared region observed on the electromagnetic spectrum. The advantage of FTIR in relation to the spectra of protein is that most biological materials in wide variety of environment could have their spectra obtained whether in aqueous solutions, lipid membranes or single crystals. This can be achieved with minimal amount and it is inexpensive as compared with NMR, X-ray diffraction and CD spectroscopy (Dorn *et al*., 2014). Some companies apart from providing FITR instrumentation also provides software that can be used in spectral analysis. To visualize the protein band, there is need to digitally subtract the solvent absorption as in a water soluble protein from the spectrum of the protein sample. The infrared spectrum of the protein show as absorption regions are called amide modes (Haris and Severcan, 1999)

2.6 Application of Nuclear Magnetic Resonance (NMR) Spectroscopy

Pharmaceutical drug developments are made possible after careful understanding of the protein structure. One of the proven advantages of NMR is its use as a suitable hetero or homonuclear technique of evaluating the structural integrity of a protein. In alternative to labeled rich media which is expensive, 1D ^{1}H-NMR spectrum evaluation using unlabeled rich media instead could be used to evaluate the protein if it is well folded for protein production. This is carried out through high throughput expression and purification of protein. For a predictive protein folding, the methyl group in the amide region is important. There must be a large chemical shift dispersions range from 1.0ppm and -1.0ppm in the methyl group and also

8.5ppm in the amide region downfield (Scheich *et al.*, 2004). NMR-proton signals from the methyl groups are considered above the amide proton signals in cases of determining whether there is reliable and sensitive indication for a folded protein. This is because resonance signals from most protein samples are dilute and are not detectable from the amide region. However, the occurrence of protons from either of the regions sufficiently detects folded proteins.

2D NMR is another technique that probes the protein additionally to further confirm the folding state of the protein as well as to determine other factors such as the aggregation and stability of the protein of interest. There is a connection between the peaks and the chemical shifts of nitrogen and the hydrogen protons which are mostly present in proteins. The HSQC spectrum obtained will contain resonances in the form of “dots” which correspond to their own specific residue in the protein. The finger print of each amino acid making up the protein is represented by each peak. Backbone assignment with the use of triple resonance experiments is then used to identify these amino acids.

The double labeled with labeling isotopes such as ^{13}C glucose as the nitrogen and carbon source, ^{15}N ammonium chloride is used for 3D NMR studies. The use of triple resonance experiments such as CBCA (CO) NH (Fig 2.7) and HNCACB (Fig 2.8), each amino acid represented by each resonance or peak can be identified. These experiments make use of magnetic energy and each experiment has their own unique magnetization pathway.

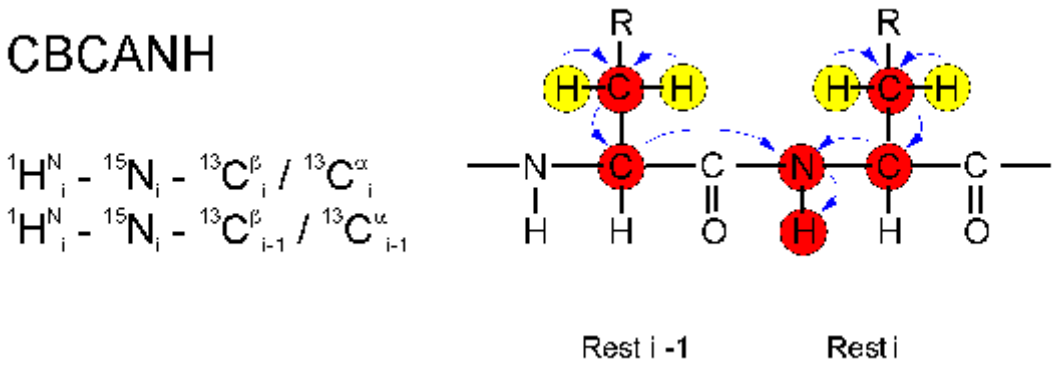


Figure 2.7: HNCACAB (or equivalent CBCANH) Triple Resonance Experiment. Image taken from http://www.nmr.chem.uu.nl/~klaartje/STRUCT_BIOL/assignment/assignment.html
The initiation of the magnetization energy starts from the amide proton to the attached nitrogen to the alpha carbon then to the beta carbon and back to the same path. Both alpha and beta carbons of the i and i-1 residue are labelled, therefore identifying four resonances.

2.7 AIM AND OBJECTIVES OF THE STUDY

Even though there were observed differences from the RING finger domains of other organisms. Kappo and co-workers (2012) reported that in *A. niger* the second cysteine amino acid has been replaced by an aspartic acid; which was deduced to have likely effect on the stability of the protein. Therefore, this research was aimed at expressing and purifying the *A. niger* RING finger domain to homogeneity and structurally characterizes the protein using heteronuclear Nuclear Magnetic Resonance (NMR) spectroscopy. This aim shall be accomplished through the following objectives:

1. *In-silico* analysis of *A. niger* RING finger domain.
2. Recombinant expression of the *A. niger* RING finger domain.
3. Homogeneity purification of the recombinant *A. niger* RING finger protein.
4. Determination of the concentration of the purified *A. niger* RING finger domain.
5. Structural characterization of the *A. niger* RING finger protein using NMR spectroscopy to ascertain the amiability of the protein to NMR studies.

CHAPTER 3

MATERIALS AND METHODS

3.1 Stock Solutions, Buffers and Media

2 x SDS PAGE Sample Buffer: 4% SDS, 0.125 M Tris-HCl, pH 6.8, 15 % Glycerol and 1 mg/mL Bromophenol blue. The buffer was stored at room temperature and 100 mM freshly prepared DTT was added prior to use.

4 x Stacking Buffer: 0.5 M Tris-HCl, adjusted to pH 6.8 with HCl. The buffer was stored at 4°C.

5 x SDS PAGE Electrophoresis Buffer: 25 mM Tris-HCl, 0.1 % SDS and 250 mM Glycine, pH 8.3. The buffer was stored at room temperature and diluted 5-fold when needed.

Ammonium Persulphate: 0.1 g was measured to make 10% stock solution prepared in deionized water. The solution was stored at 4°C.

Ampicillin: 1 g was measured to make 100 mg/mL stock solution which was prepared in deionized water. The solution was filter-sterilized using a 0.22 micron filter and stored at 80°C.

Bradford Dye: 0.1% Coomassie brilliant blue G250**,** 8.5% phosphoric acid and 4.75% ethanol. The solution was stored at 4°C and deionized water was used in diluting 5 times before use.

Cell Lysis Buffer: 1% Triton X-100, 100 µg/ml lysozyme, 1 mM PMSF, 1 mM DTT, 50 µM $ZnSO_4$.

Coomassie Staining Solution: 0.25 g Coomassie blue was measured into 100 mL of deionized water.

Destaining Solution: 40 ml of methanol, 10 mL of acetic acid in 50 ml deionized water to make 100 ml**.**

DTT: A 1 M stock solution was prepared in 0.01 M sodium acetate, pH 5.2. The solution was sterilized by filtration, divided into 1 mL aliquots and stored at 80°C.

Elution Buffer: 15 mM Glutathione and 50 mM Tris-HCl at pH 8.0.

Ethidium Bromide: 10 mg/ml stock solution was prepared in water, and stored in the dark at 4°C.

IPTG: A 1 M stock solution was prepared in deionized water. The solution was filter-sterilized, aliquoted and stored at -20°C.

Luria Agar: 10 g/L Tryptone powder, 5 g/l yeast extract, 5 g/l NaCl and 14 g/l bacteriological agar.

Luria Broth: 10 g/l Tryptone powder, 5 g/l yeast extract, 5 g/l NaCl and 2 g/l glucose.

PBS: 137 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4-$7H_20$ and 1.5 mM KH_2PO_4. The pH was adjusted to 7.4 with HCl and the solution was sterilized by autoclaving.

PBST: PBS containing 1% Triton X-100.

PMSF: A 10 mM stock solution was prepared in isopropanol. The solution was aliquoted and stored at 80°C.

Separating Buffer: 1.5 M Tris-HCl, adjusted to pH 8.8 with HCl.

Tris-EDTA (TE) buffer: 10 mM Tris-HCl and 1 mM b EDTA, pH 7.4 stored at 4°C.

3.2 Bacterial Strains used

1. *E. coli* BL21 (DE3) pLysS strain: B F^{-}*ompTgaldcmlonhsdS*$_B$($r_B^{-}m_B^{-}$) λ(DE3 [*lacIlacUV5-T7p07ind1sam7nin5*]) [*malB*$^{+}$]$_{K\text{-}12}$(λ^S) pLysS[*T7p20ori*$_{p15A}$](Cm^R)..

3.3 Preparation of competent *E. coli* BL21pLysS cells for transformation

Agar plates were streaked with *E. coli* (BL21) cells and incubated at 37^{o}C overnight. A single colony of *E. coli* (BL21) cells from the overnight plate was incubated in a test tube containing 5 mL YT broth with shaking at 37^{o}C overnight. The following morning, the 5 mL broth was transferred into 50 ml tube containing 45 ml broth. This was further incubated at 37°C until an OD_{600} of 0.6 was achieved. The culture was centrifuged at 5000 x g for 10 minutes. The supernatant was discarded and 10 ml $MgCl_2$ was added and incubated on ice for 20 minutes. The mixture was centrifuged at 5000 x g for 10 minutes and the supernatant was discarded. Thereafter, 10 ml $CaCl_2$ was added to the pellet and left on ice for 3-4 hours. The mixture was again centrifuged; the supernatant was discarded and 3 ml $CaCl_2$ containing 30% glycerol was added to the pellet and mixed gently. Aliquots of 100 µl each was prepared and stored at -80°C until further use.

3.4 Transformation of *E. coli* BL21 cells with *A. niger* pGEX- 6P-2-RING expression constructs

Transformation was carried out by carefully introducing 100 µl of thawed chemically competent *E. coli* BL21 cells to 2 µl of the plasmid of *A. niger* Pgex-6p-2-RING DNA to make transformation mixture in a 2 ml micro centrifuge tube labeled as T (Test). Another 2 ml micro centrifuge tube was set up as control tube. To this tube, 100 µL *E. coli* BL21 cell was added with no plasmid DNA. The mixtures were incubated on ice for 20 minutes after which the cells were then heat shocked on a heating block at 42^{o}C for 1 minute. Thereafter, 900 µl of YT broth was added and then incubated at 37°C for 1 - 2 h. The mixture was centrifuged and 100 µl from each sample was streaked on agar plates with Ampicillin. From the remaining 900 µl, 800 µl was removed and discarded while the remaining 100 µl pellet mixture was streaked out on an agar plate with ampicillin and incubated overnight at 37 °C.

3.5 Small-scale protein expression

Four colonies of *E. coli* BL21 cells were randomly picked from the test plate from the transformation product and inoculated into 5 ml Luria broth with 5 µl ampicillin in four different tubes which were then incubated for 4 hours at 37^{o}C with vigorous shaking. Thereafter, 1 ml was removed from each culture, transferred to a clean 15 ml tube and 0.5 mM Isopropyl beta- D-thiogalactoside (IPTG) was added to serve as the induced culture. Another set of four tubes were setup without the addition of 0.5 mM Isopropyl beta- D-thiogalactoside (IPTG), this were labelled as un-induced cultures.

Both un-induced and induced cultures were incubated for further 2 hours at 37^{o}C with vigorous shaking after which the cells from both cultures were then harvested by centrifugation at 5000 x g for 10 minutes. Twenty microliter from each sample was then analyzed by SDS-PAGE electrophoresis. The remaining 3 µl of the best expressing culture was used to make a glycerol stock which was later used for large scale recombinant protein expression.

3.6 Recombinant production of *A. niger* RING finger domain

3.6.1 Large scale expression

100 µl was taken from the remaining 3 ml culture of the best expression from the previous expression screening product and inoculated in 100 ml of LB with 100 µl of ampicillin overnight at 37°C with shaking. The overnight 100 ml culture was scaled up to 2L and further incubated at 37°C for 16 hours with vigorous shaking. The OD (optical density) at 550 nm was checked intermittently until it reached 0.6, then the culture was then induced with 0.5 mM IPTG (Isopropyl-β-D-thio galactopyranoside) and incubated at 25°C overnight. The cells were harvested by centrifugation at 5000 x g for 15 minutes at 4°C and stored at -80°C if not lysed immediately. Before the induction, the culture was then supplemented with 100 mM of filter-sterilized $ZnSO_4$ solution to allow for the incorporation of Zn^{2+} ions into the protein.

3.6.2 Extraction

The frozen cell pellets were taken out from -80°C, thawed on ice and re-suspended in 15 ml of cell lysis buffer containing (PBS, 1 % Triton X- 100, , 100

µg/ml Lysosome, 1mM PMSF, 1mM DTT, 50 µM $ZnSO_4$). It was then vortexed until the cell were observed to have mixed together very well and sonicated in a 50 ml Eppendorf tube for 5 minutes at 5 seconds interval. The cells were also subjected to three cycles of freeze-thawing. The total bacterial lysate with debris were further centrifuged at 5000 x g for 30 minutes at 4^{o}C. The clear supernatant containing soluble GST-*Aspergillus niger* RING finger domain fusion protein was collected into fresh 50 mL Eppendorf tubes. Sodium Azide 0.02% concentration was added to the sample and stored at -80°C.

3.6.3 Affinity Purification

Glutathione agarose column was prepared by weighing out the required amount of glutathione agarose beads which was suspended in distill water. This was allowed to swell overnight at 4°C so that it could pack well. Firstly, the column was washed with 5 column volumes of 1 M NaCl. There after the column was equilibrated with 5 column volumes of 50 mM Tris containing 20 mM NaCl. The bacterial lysate was loaded onto the column and the flow through was collected.

The column was then wash with 10 column volumes (10 cv) of equilibrating buffer containing 50 mM, 20 mM NaCl at pH 8 and the last 1 ml was collected clean wash. The protein was eluted with elution buffer containing 50 mM Tris Stock, 20 mM NaCl stock , 0.02% Nan3, 1 Mm DTT, 1 Mm PMSF and 0.23 g of 15 Mm glutathione was added. The column was then washed with 5 column volume of 2 M NaCl to remove any traces of bound protein from the column. The column was then stored at 4°C.

3.6.4 Precision Protease Cleavage and Recovery of RING Domain

The different fractions from *A. niger* GST- RING finger domain fusion were pooled together and poured carefully into a snake skin pleated tubing. One hundred microliter (100 µl) of 3C protease was added and dialysed overnight at 4°C in a 1000 mL precision protease cleavage buffer (50 mM Tris, 100 mM NaCl pH 7.0 and 1 mM DTT). This was incubated at 4°C with continuous stirring for 18 – 24 hours. *A. niger* RING domain fusion protein with cleaved fractions was analysed by SDS- PAGE electrophoresis. The sample was run through glutathione agarose column after cleavage; this was collected in the flow through. GST- 3C protease and Bound GST were eluted.

3.7 SDS- Polyacrylamide Gel Electrophoresis

Fifteen percent (15%) SDS polyacrylamide gels were prepared according to the protocol set out in Table 3.2. Ten microliters of 2X sample buffer was mixed with twenty microliters (20 µl) of the protein sample to be analysed, boiled for 5 minutes with heat block equipment at 95°C and then centrifuged for 1 minute at 15, 000 x rpm. The mixture was electrophoresed in 1 x SDS buffer (25 mM Tris-HCl, pH 8.3, 192 mM Glycine, 0.1% SDS) at a constant voltage of 120 V/cm using a gel electrophoresis system. The gel was allowed to run until the Bromophenol blue dye front reached the bottom of the separating gel and then it was stopped. The gel was stained by incubation in Coomassie staining (0.25 g Coomassie blue, 45 ml methanol, 10 ml glacial acetic acid and 45 mL deionized water) for 15 minutes and was de-stained overnight in a destaining solution (50 ml methanol, 10 ml glacial acetic acid and 40 ml of water).

Table 3.1 Summarised composition of a 15 % SDS PAGE gel

	Separating gel	**Stacking gel**
Deionised water	2 ml	3 ml
Tris	2 ml at pH 8.8	630 µl at pH 6.8
40% Bis Acrylamide 37:5:1	4.2 ml	830 µl
Ammonium Persulphate	150 µl	50 µl
10% SDS	342 µl	50 µl
TEMED	16 µl	5 µl

3.8 *In-silico* characterization of *A. niger* RING finger domain

The full protein sequences of *A. niger* were retrieved from the NCBI database. The physicochemical parameters of the region corresponding to the *A. niger* RING finger domain were analyzed using ProtParam on the ExPAsy website. The amino acid composition of these sequences were computed using excel. Multiple sequence alignment for region corresponding to the *A. niger* RING finger domain was aligned manually. The molecular phylogenetic tree for *A. niger* RING finger domain was built using the phylogeny.fr. The secondary structure was predicted using PSIPRED v3 and JPred4 (http://www.compbio.dundee.ac.uk/jpred4). Sequences corresponding to the *A. niger* RING finger domain were predicted for 3D structure using Swiss model web-based server and visualised using UCSF Chimera. QMEAN score provided by the SWISS-MODEL workspace was used to determine the overall quality of the model (Benkert *et al*., 2009), a scoring function that shows that a predicted 3D structure is within the score usually observed in native proteins of corresponding size was obtained more so, validation of the model was done by Ramachandran plot. The STRING database was used to identify the protein interaction partners. Furthermore, the hydrophobicity of the protein was assessed using ProtScale tool on the ExPASy workspace https://web.expasy.org/protscale/ (Wilkins *et al*., 1999), which evaluated the physicochemical behaviour of the antigenic sequences in addition to recognized numerous highly hydrophobicity regions.

Moreover, the antigenic prediction tool on the immunomedicine group was used to determine the antigenic peptides in this protein (Álvarez *et al*., 2016). This server forecast the regions within a protein sequence which are possible to be antigenic sites by eliciting an antibody response. The accuracy of this online server for antigenic site prediction was 75%. To attain consensus epitopes, the continuous and discontinuous B-cell epitopes were predicted using bioinformatic algorithm, since determination of the B-cell epitopes using experimental methods is time-consuming and capital intensive. Here, ElliPro (Ponomarenko *et al*., 2008) was used to have insight into the linear (continuous) and discontinuous B-cell epitopes. ElliPro is an online server that predicts antibody epitopes in protein antigens for a give sequence or structure (Ponomarenko *et al*., 2008). The peptides with the highest score from the results were viewed with an open-source molecular viewer Jmol.

3.9 Biophysical Characteristics of *A. niger* RING finger domain

3.9.1 Identification of *A. niger* RING functional groups using Fourier transform Infrared spectroscopy

An infrared spectrophotometer is mainly used to estimate the structure of organic compounds. This instrument shines infrared light onto the molecules, which absorb infrared radiation equivalent to the interatomic vibrational energy of the atoms that comprise the molecules. It then estimates the structure and quantifies the compound by investigating this IR absorbance.

3.10 Nuclear Magnetic Resonance Spectroscopy

Protein analysis was carried using Nuclear Magnetic Resonance Spectroscopy at 25°C in 50 mM sodium phosphate buffer, 150 mM NaCl, 1 mM DTT, 0.02% Sodium Azide, pH 6.0. Unless otherwise specified, NMR data was collected on a 600 MHz UNITYINOVA spectrometer.

CHAPTER 4

RESULTS AND DISCUSSION

4.0 *In-silico* characterization of *Aspergillus niger* RING finger domain

4.1. Introduction

Bioinformatics has emerged as an important multidisciplinary area of research in basic and applied life sciences in recent years (Chowdhary *et al.,* 2016). *In silico* studies using bioinformatics tools are employed by scientists to predict structural and functional properties of proteins in order to reduce time spent on experiments and also to reduce the risks associated with experimental failure (Aghamollaei *et al*., 2016).

4.2 Multiple Sequence Alignment and Phylogenetic Analysis

The alignment of all selected sequences was analysed using BIOEDIT. Results from this analysis, reveals the conserved region and pattern of the protein sequences (Figure 4.1). In like manner, phylogenetic analysis based on the sequences is presented in Figure 4.2. The figure revels that *A. niger* RING FINGER Domain has a close relationship with OJJ43319.1 and KLU90121.1. The most familiar task in bioinformatics is the alignment of three or more amino acid sequence. Multiple sequence alignment shows conserved and other variable sites within a family, it is also needed for further analyses of protein families including homology modeling. These alignments can be used to generate other models (Gribskov *et al*., 1987) like Markov models (Bucher *et al*., 1996; Haussler *et al*., 1993) which check databases for other related members of the family. The information gathered from the generation of these data will give us the knowledge about evolutionary and structural relationships within the family.

In this research work, the protein sequence of *Aspergillus niger* was generated from NCBI, thereafter, the NCBI online blast tool was consulted in order to generate the multiple sequence alignment of *Aspergillus niger* RING finger domain and organisms that assume common evolutionary relationship and share common ancestry.

4.3 Secondary structure prediction

The secondary structure prediction was performed using the PSIPRED v3 and JPred 4 online server. Both online server predictions were in agreement. The server revealed that *Aspergillus niger* RING finger domain contains 2 α-helices, and 3 random coils (Figure 4.3 and Figure 4.4).

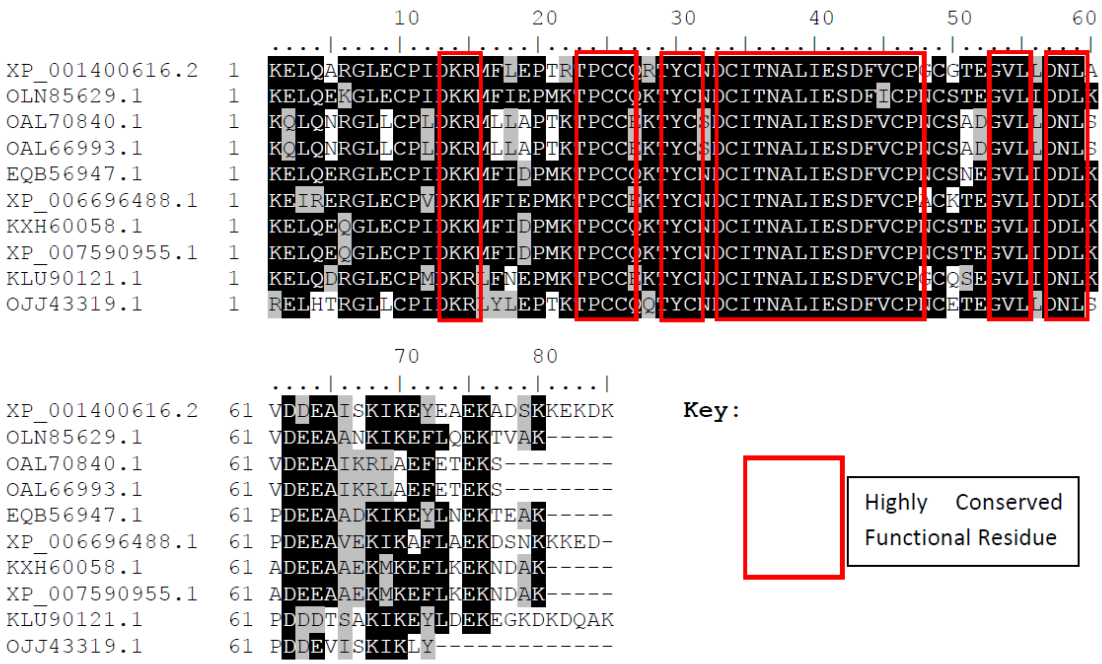


Figure 4.1: Multiple sequence alignment of *A. niger* RING finger domain

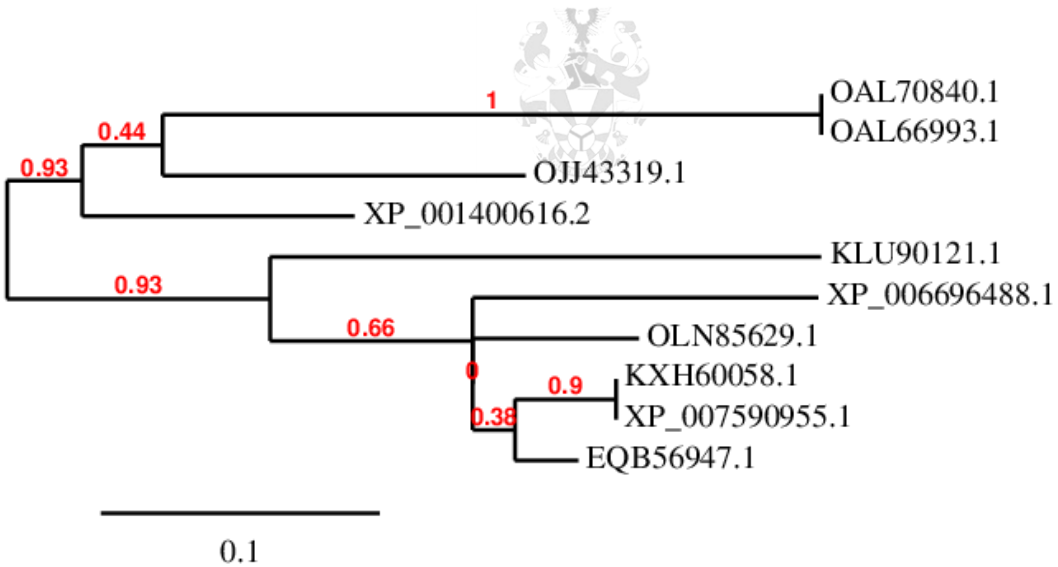


Figure 4.2: The phylogeny tree of the *A. niger* (XP_001400616.2) which is the organism of interest in this research work. *A. niger* (XP_001400616.2) has close relationship with other protein especially OJJ43319.1 and KLU90121.1

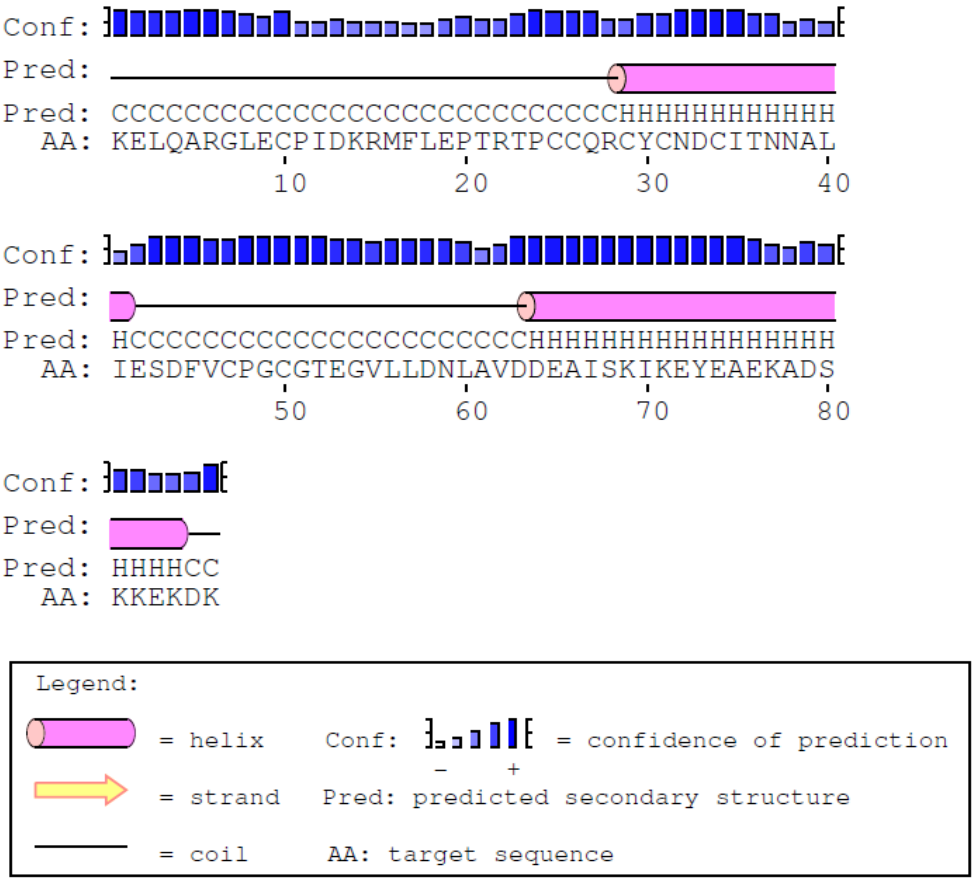


Figure 4.3: **Graphical representation of the predicted secondary structure of *Aspergillus niger* RING finger domain using online server psipred**. Diagram shows the presence of two α-helices (in the cylindrical shape colored in pink)

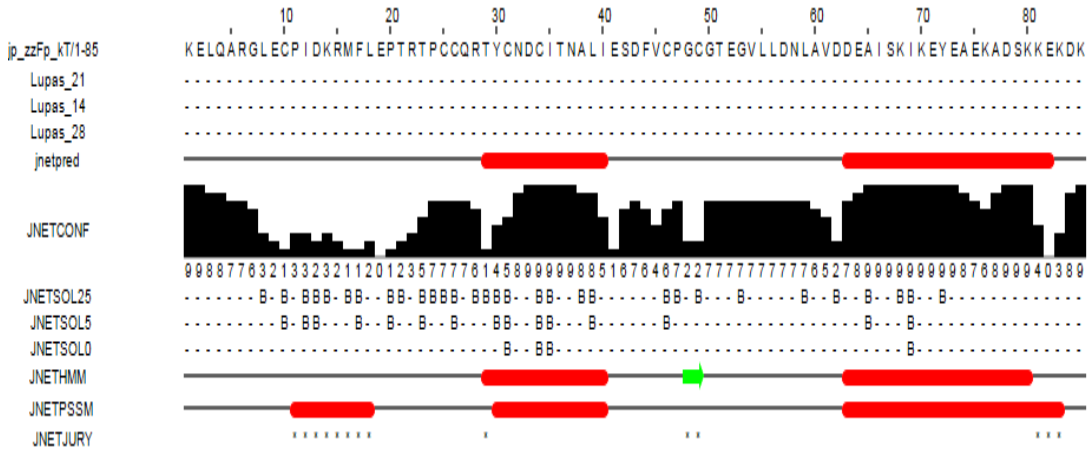


Figure 4.4: **Graphical representation of the predicted secondary structure of *Aspergillus niger* RING finger domain using online server JPred 4**. Diagram shows the presence of two α-helices (in the cylindrical shape colored in pink)

4.4 Tertiary structure prediction

The 3D modeled structure of the protein was generated using SWISS-MODEL web-based server. 3ztg.1.A was picked and used as template in building this protein because 3D structure of *A*. *niger* protein has been document in the SWISS-MODEL library. Thereafter, QMEAN score was used to ascertain the quality of the RING finger protein computational model and it scored the 3-D model -3.13. The resulting structure from the server was visualized on Chimera as presented in Figure 4.5. The result shows the topology consisting of 2 α-helices and 3 random coils. Prediction of the surface representation of the *A. niger* protein was also generated using Chimera and it shows that majority of the residues which makes up the hydrophobic core are completely buried within the protein structure (Figure 4.6).

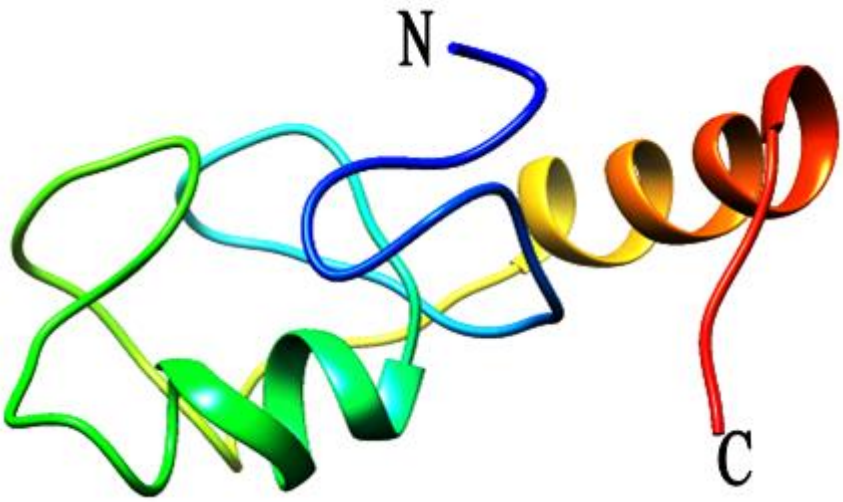


Figure 4.5: **Predicted cartoon representation of the homology model of *Aspergillus niger* RING finger domain using SWISS-MODEL online server**. This figure shows the depicts two standard helices and the positions of the N and C-terminals of the protein are also shown. The figure was visualized using UCSF chimera molecular visualization application.

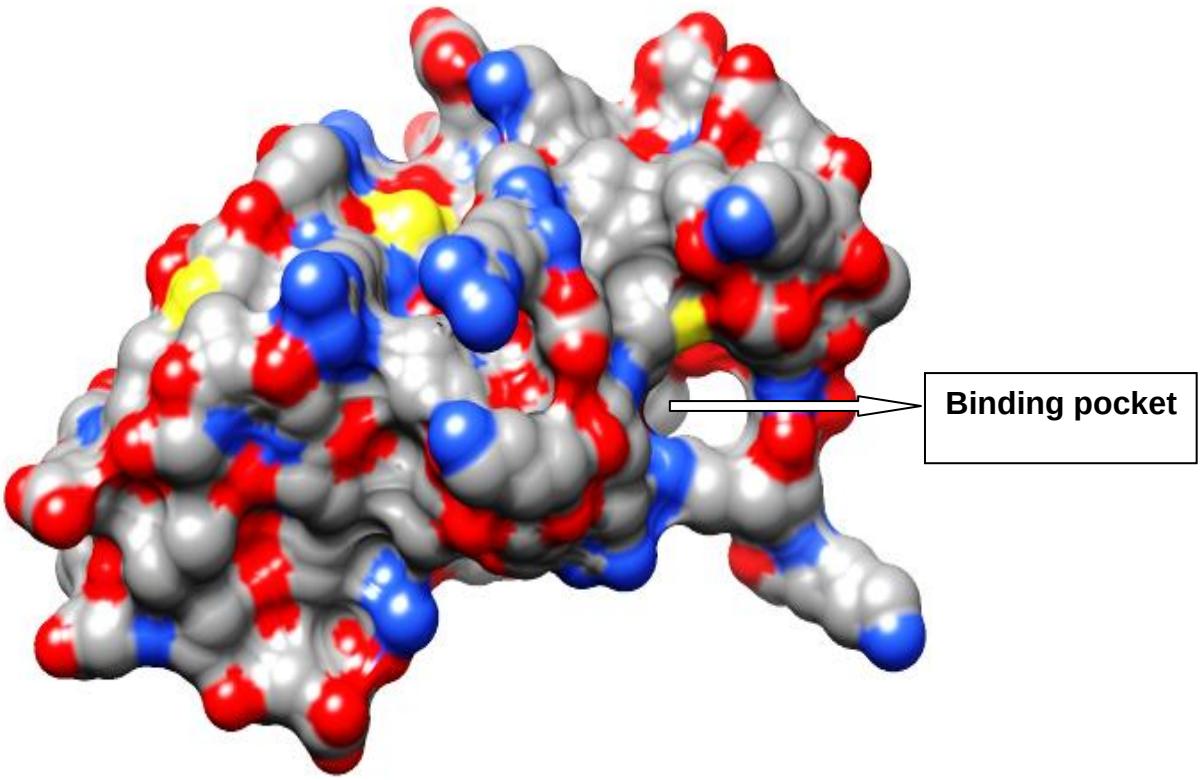
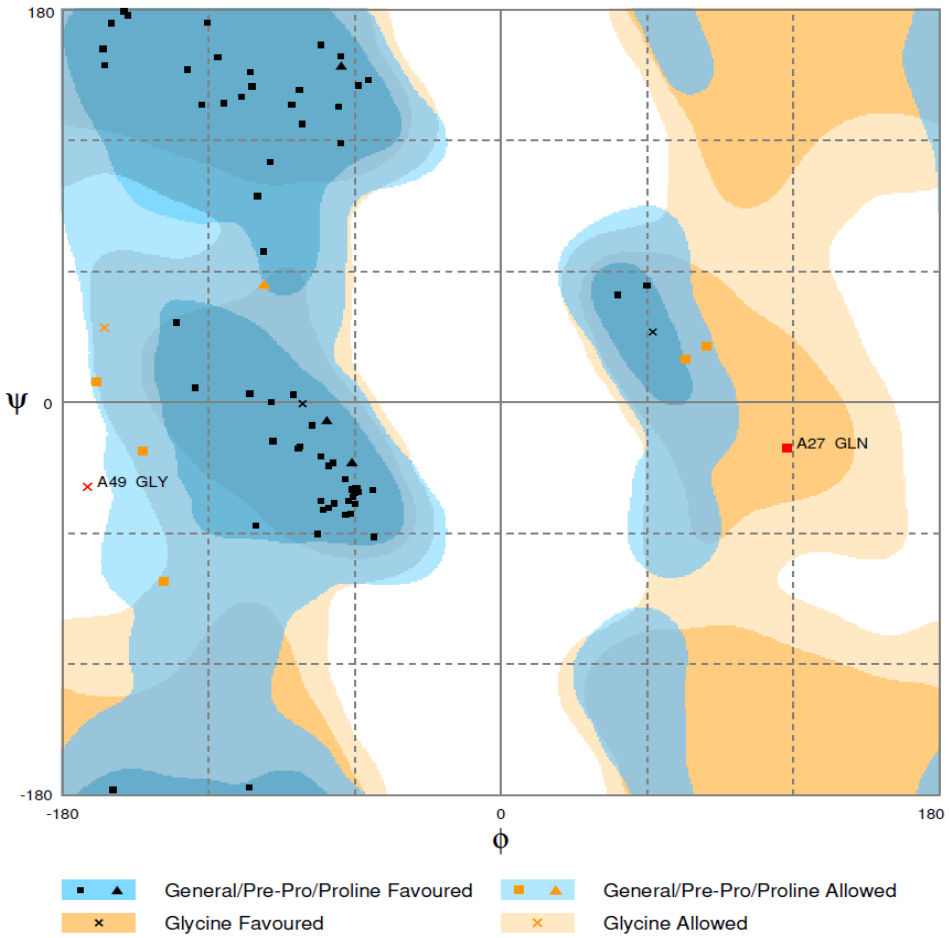


Figure 4.6: **Molecular representation of the homology model of *Aspergillus niger* RING finger domain using Chimera**. This diagram shows the binding pocket and hydrophobic cores of the protein.

4.5 Structural validation of predicted tertiary structure of *A. niger* RING domain

The quality of predicted model was validated for any stereochemical clashes using Ramachandran plot from Rampage server. RAMPAGE analysis showed that the model has 88.0% of the residues in the favoured region, 9.3% of the residues are in the allowed region and 2.7 % of the residues in the outlier region (Figure 4.7 A and Figure 4.7 B). Additionally, validation of the RING finger Domain structure by Z score shows the quality of a structure when compared to a non-redundant set of PDB structures of the same size. The image shown in Figure 4.8 shows that the modeled structure's score, indicated by the red "star", falls within the range of scores of the reference structures of the same size; the protein is said to be of absolute good quality (Benkert *et al*., 2011). Similarly, Figure 4.9 shows the local quality estimate of the protein. This result indicates that the protein is rigid because the predicted local similarity to target is very close to 0.8. Any values higher than 0.5, is taken as being rigid and well-structured.



Number of residues in favoured region (~98.0% expected) : 66 (88.0%)
Number of residues in allowed region (~2.0% expected) : 7 (9.3%)
Number of residues in outlier region : 2 (2.7%)

RAMPAGE by Paul de Bakker and Simon Lovell available at http://www-cryst.bioc.cam.ac.uk/rampage/

Figure 4.7 A: Structure validation of the predicted 3D model of *Aspergillus niger* RING finger domain. The Ramachandran plot shows that the final model had 88.0% residues in favored regions, 9.3% in allowed regions and 2.7% in outlier regions. Structure validation of predicted

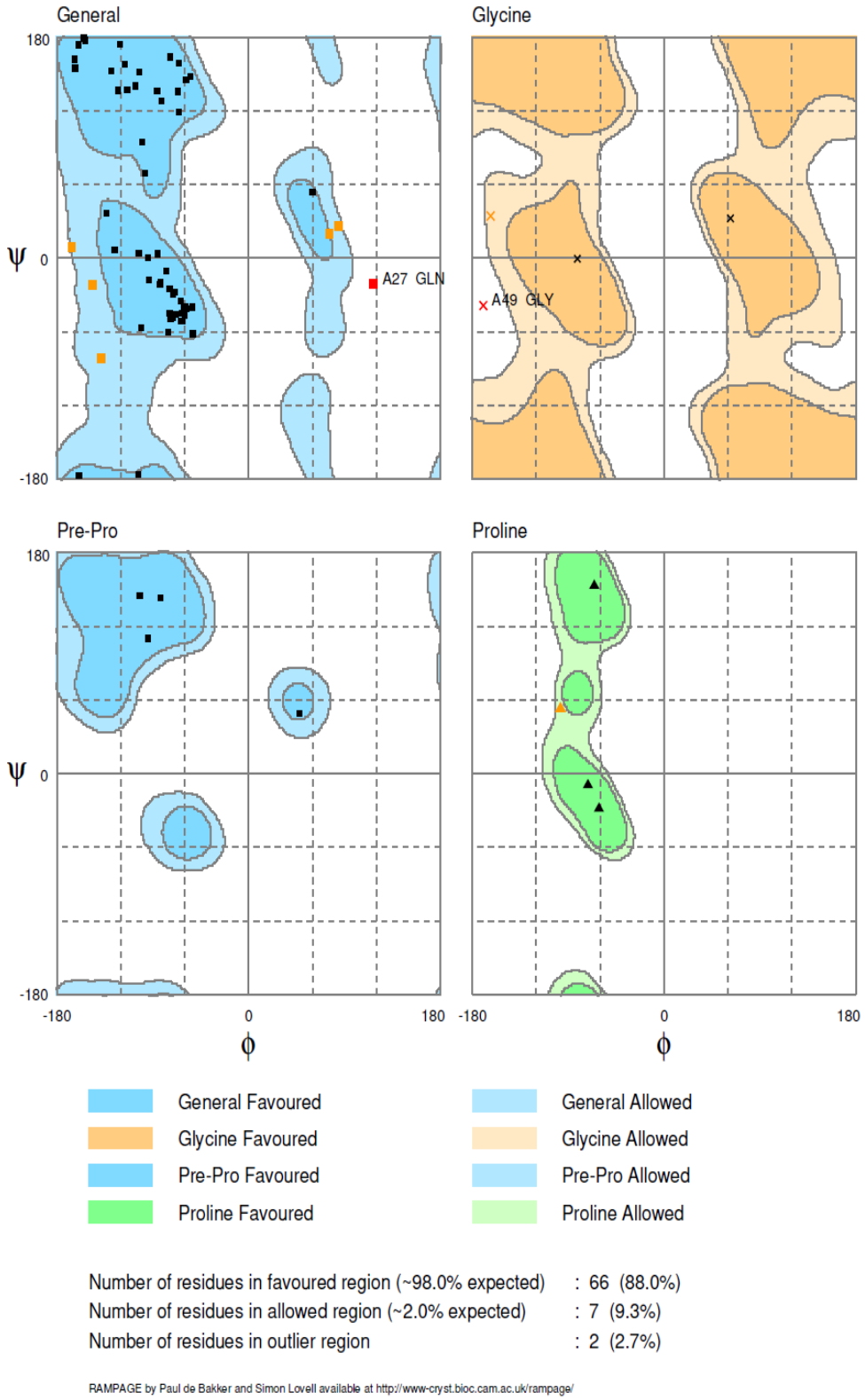


Figure 4.7 B: Ramachandran plot of *Aspergillus niger* RING finger domain protein hits region. It is widely accepted that a presence of over Ninety percent (90%) residues in the most favored regions of Rampage's Ramachandran plot indicates excellent quality models.

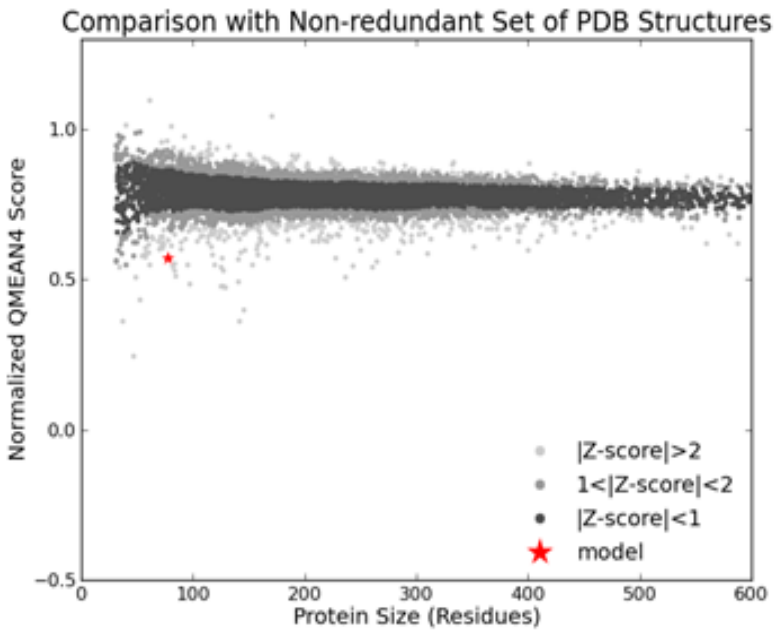


Figure 4.8: **Validation of the RING finger Domain structure by Z score.** The modelled structure's score, indicated by the red "star", which falls within the range of scores of reference structures of the same size.

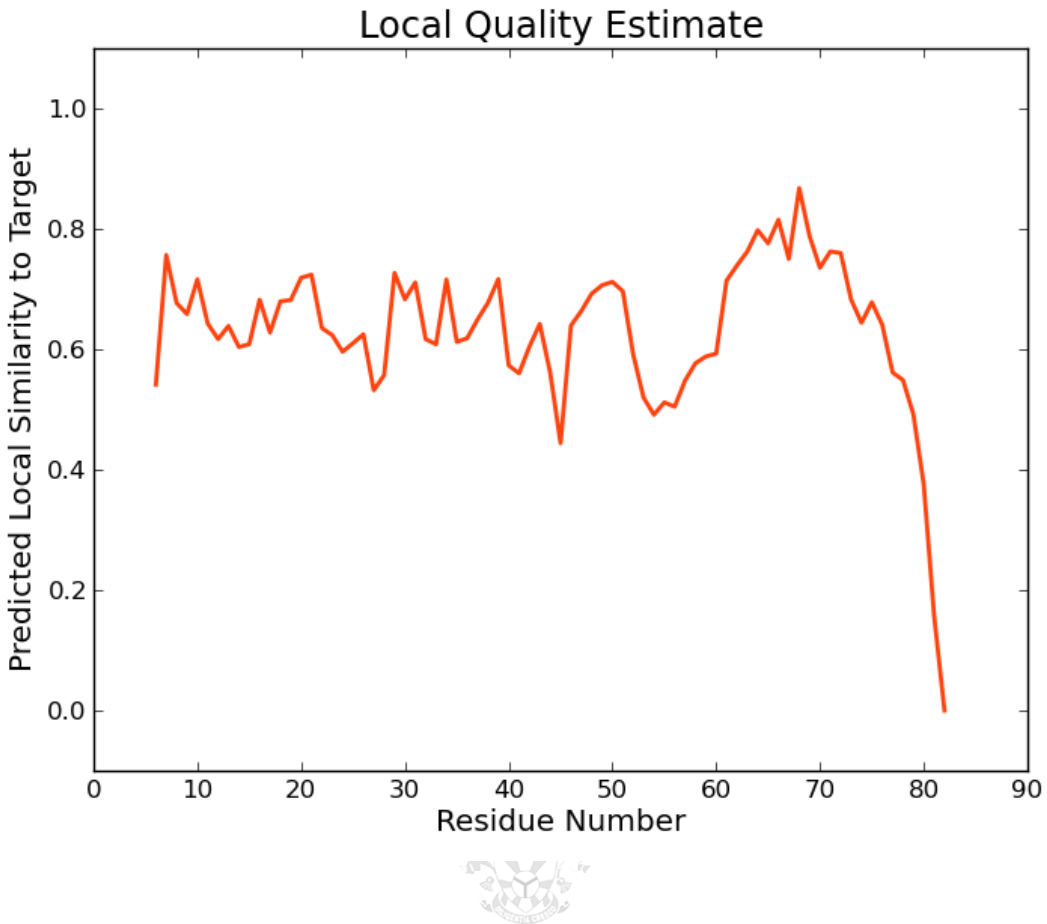


Figure 4.9: **Graph showing the predicted local quality estimate**. This shows that the protein is rigid because the predicted local similarity to target is very close to 0.8.

4.6 Protein interaction partner analysis

STRING was used to compare the protein-protein interaction of *A. niger* with other protein and their similarities. The result shows evolutionary relationship in terms of functions of *A. nige*r (An12go4760) in mRNA splicing and transcription and (An01g0450) which is one of the important processes for cell regulation.

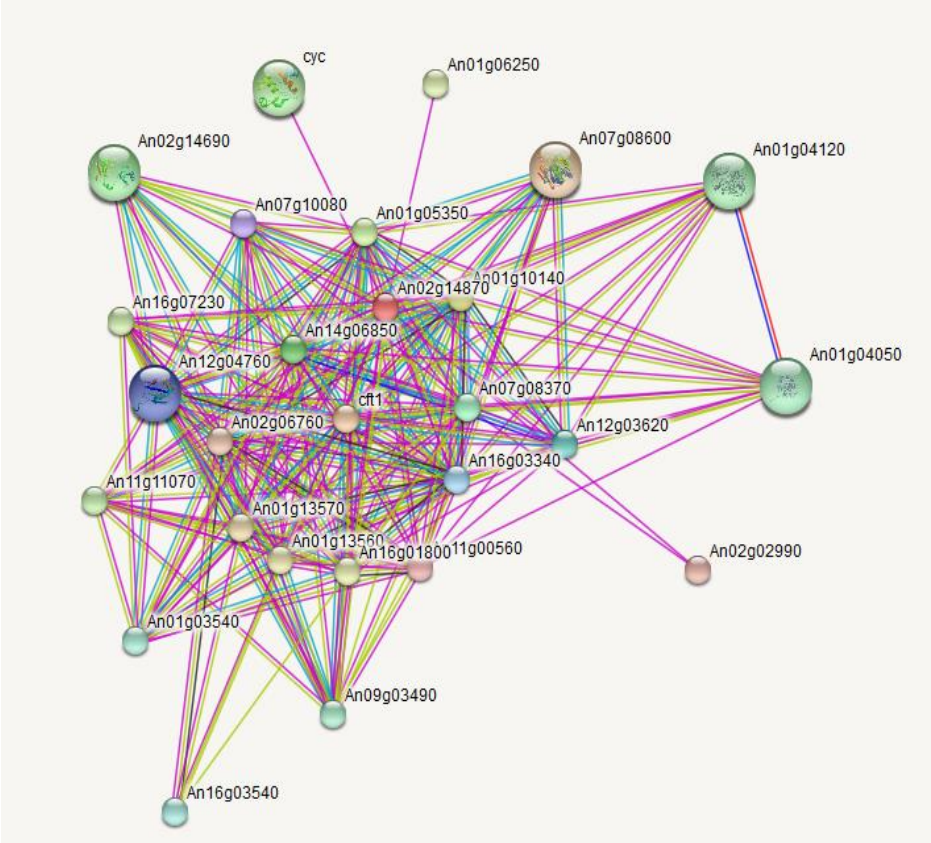


Figure 4.10: Diagram showing proteins interactions analysis using the STRING software. Different *Aspergillus* species such as An12g04760 have been characterized to have the function related to mRNA transcription and splicing.

Table 4.1: Comparison of string characteristics between organisms of interest and other close relatives

String no	Characterized function	Similar to *A. niger* RING finger
An12g04760	• promotes the transport of mRNAs from the nucleus to the cytoplasm • processing promotes the stability of mRNAs • processing enhances the translation of mRNAs into proteins. • intricately coupled to the transcription and splicing machineries	Transcription of Proteins
An01g04050	Cell cycle and DNA processing	Transcription

4.7 Predictions of antigenic epitopes on the *A*. *niger* RING protein

Kyte and Doolittle method is utilized in forecasting regions of a protein that are inside or outside of a membrane, since the best possible antigenic epitopes are hydrophilic, positioned on the surface of the protein and flexible. Therefore, the hydrophobic nature of *A*. *niger* RING protein as analyzed by Kyte and Doolittle, according to Adekiya and co-workers (2017) regions below Zero are within the interior of the protein, which is indicative that they are hydrophobic in nature, while regions above zero are hydrophilic, which is most likely to be exposed on the outside of a folded protein (Figure 4.11).

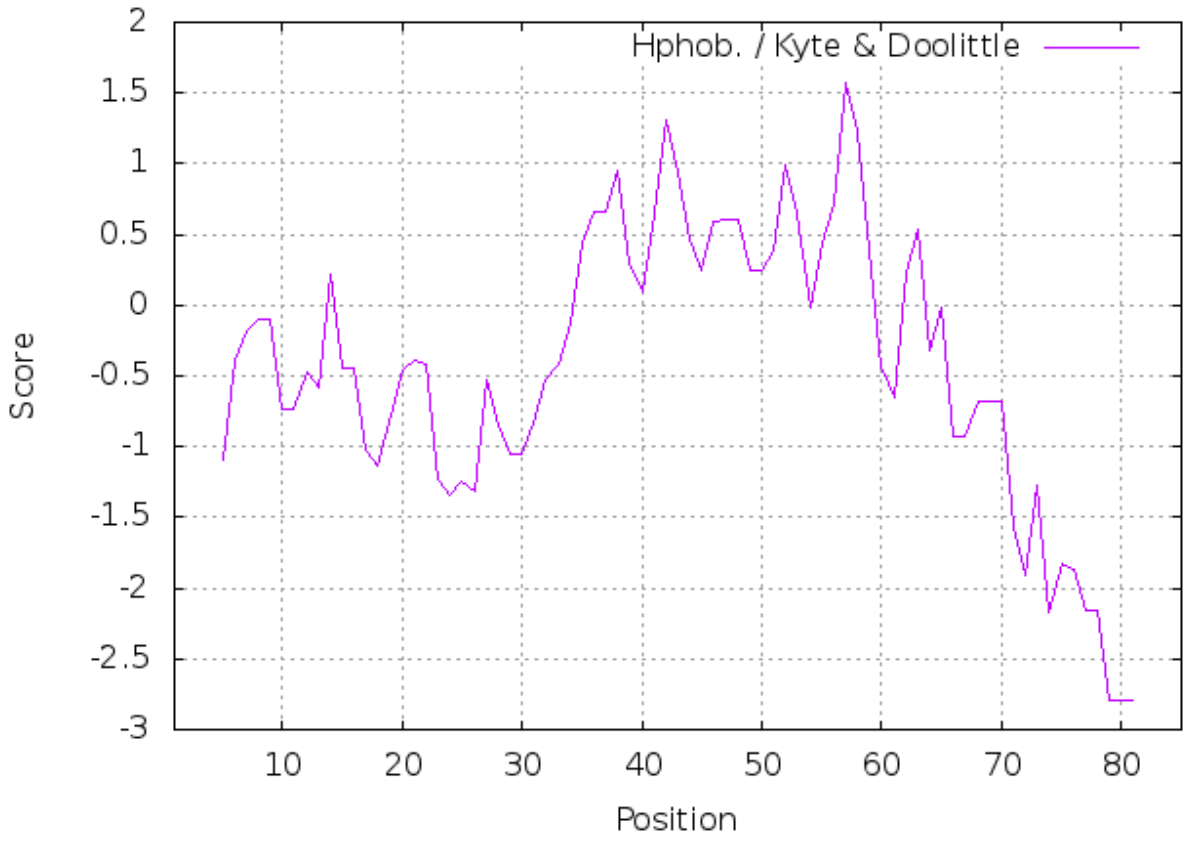


Figure 4.11: Kyte-Doolittle hydropathy plot for *A. niger* RING protein. Plots above 0 signify hydrophobic regions in the protein while plots below 0 signify hydrophilic regions.

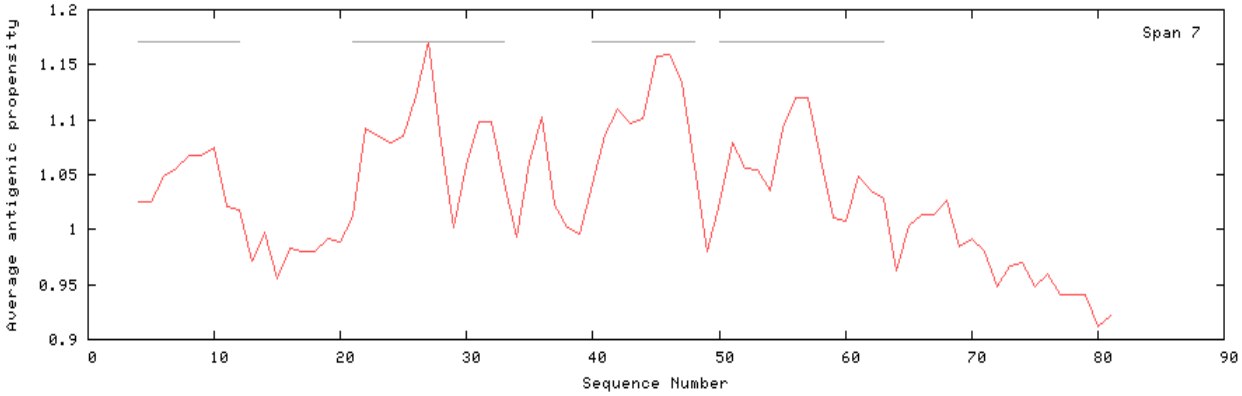


Figure 4.12: Antigenic graphical plot of *A. niger* RING protein. The forecasted antigenic peptides with potential antigenic properties showing the start and end positions respectively. This gives a graphical view of the antigenic regions in this protein. The antigenic propensity score for this protein is 1.0276 which is indicative that the protein has a potential antigen because the score is greater than the average score of 1.0 for potentially antigenic proteins. The *A. niger* RING protein sequence is made up of four antigenic epitopes, which are described with both the start and end positions respectively as shown in table 4.2. Consequently, PyMol was used to display the predicted epitopes in different colours on the 3-D structure (Figure 4.13).

Table 4.2: predicted antigenic peptides on *A*. *niger* RING finger protein with antigenic propensity of 1.0264

Number	Start Position	Sequence	End Position
1	4	QARGLECPI	12
2	21	TRTPCCQRTYCND	33
3	40	IESDFVCPG	48
4	50	GTEGVLLDNLAVDD	63

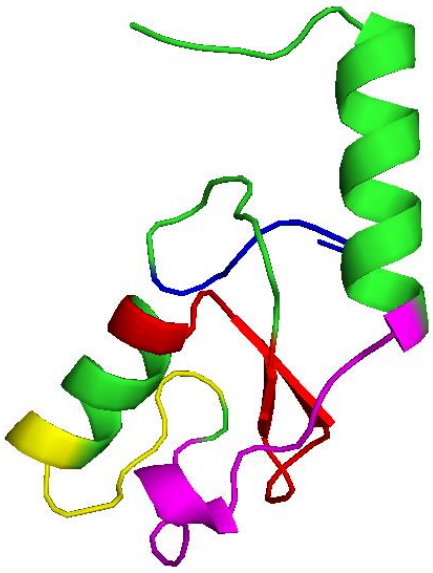


Figure 4.13: Localization of the predicted epitopes. PyMol was used to map the epitopes on the cartoon structure of the *A*. *niger* RING protein.

Furthermore, both the continuous and discontinuous B-cell antigenic epitopes were predicted. Having a deeper knowledge into B-cell antigenic epitopes can assist in the design of vaccine targets and immunodiagnostic reagents (Yao *et al*., 2013). Using computational predictive technique on the ElliPro server, eight potential continuous and discontinuous epitopes were predicted from the *A*. *niger* RING protein. Table 4.3A shows predicted continuous B-cell epitopes and table 4.3B shows discontinuous B-cell epitopes, however, figures 4.14A and 4.14b displays the epitope residues with the highest scores.

Table 4.3A: Predicted linear epitopes and scores

Number	Start Position	End Position	Sequence	Score
1	70	81	KEYEAEKADSKK	0.811
2	40	45	IESDFV	0.719
3	47	54	PGCGTEGV	0.586
4	56	64	LDNLAVDDE	0.564

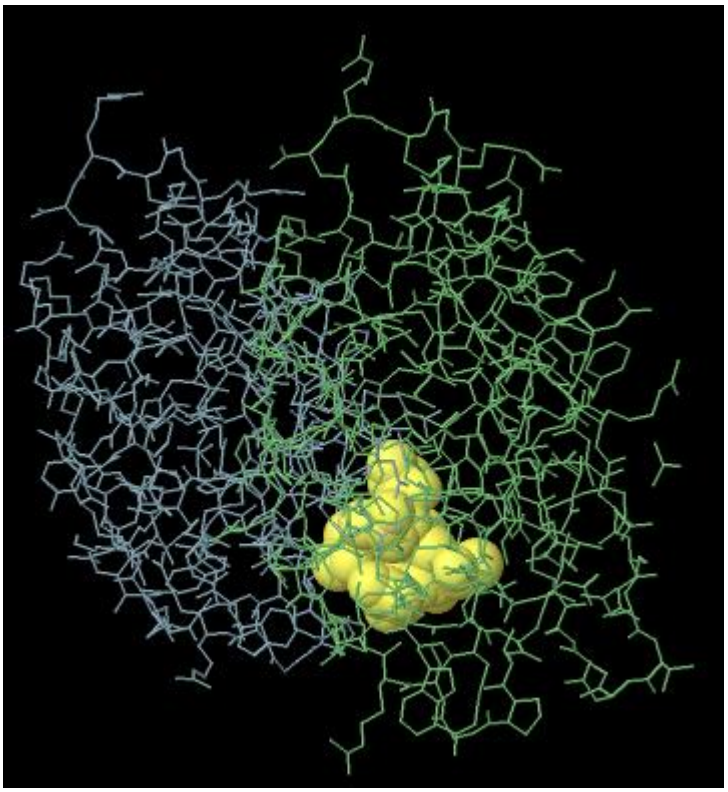


Figure 4.14A: **3-D structure mapping for the first of the four predicted continuous epitopes.** This was visualized by Jmol and the epitope residues are in yellow.

Table 4.3b: Predicted discontinuous epitopes and scores

Number	Residues	Score
1	Y72, A74, E75, K76, A77, D78, S79, K80	0.854
2	S67, K70, E71	0.706
3	A60, V61, D62, D63, E64	0.589
4	P24, C25, C26, R28, I40, E41, S42, D43, F44, V45, C46, P47, G48, C49, G50, T51, E52, G53, V54, L55, L56, D57, N58, L59	0.559

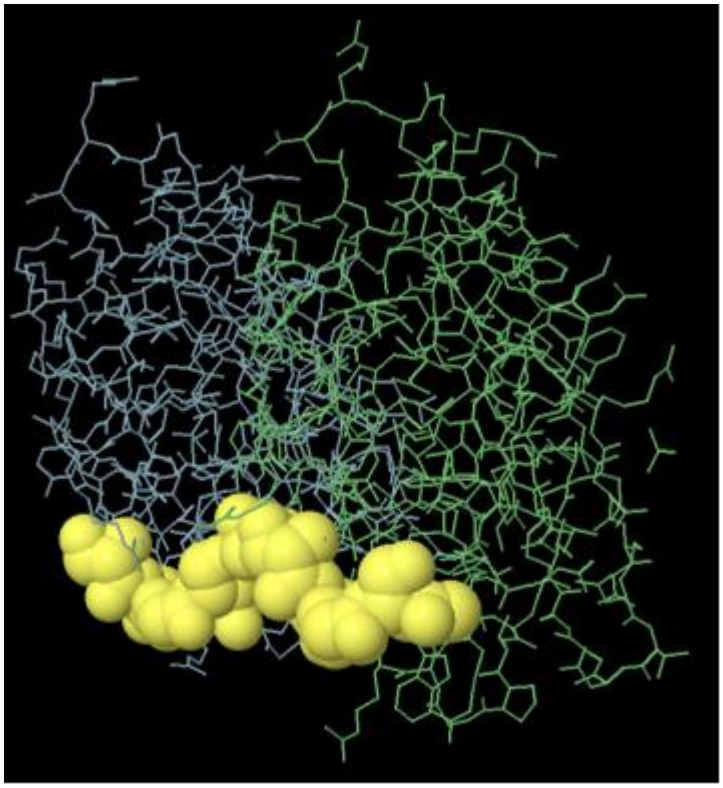


Figure 4.14B: 3-D structure mapping for the first of the four predicted discontinuous epitopes. This was visualized by Jmol and the epitope residues are in yellow.

4.8 Expression and purification of the RING finger domain from *Aspergillus niger*

4.8.1 Small Scale Protein Expression

The RING domain sequence was cloned into the *Bam* HI and *Xho*I sites of the pGEX-6P-2 multiple cloning cassettes as shown in Figure 4.15. PGEX-6P-2 contains a strong tac promoter which is inducible by the addition of Isopropyl β-D- thio galactopyranoside (IPTG). *E. coli* BL21 cells were transformed with the pGEX-6P-2-RING construct (Figure 4.15) and the resulting transformed bacterial cells were screened for the expression of GST-RING domain fusion protein.

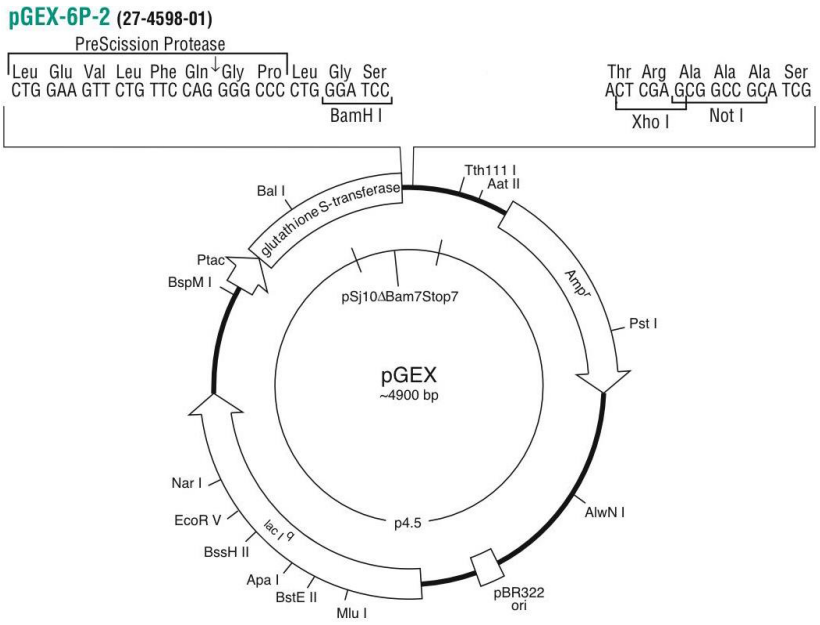


Figure 4.15: A diagrammatic representation of the pGEX-6P-2-RING domain constructs. This illustrates diagrammatically the BamH1 and Xho1 sites having inserted RING domain of the pGEX-6P-2 expression vector.

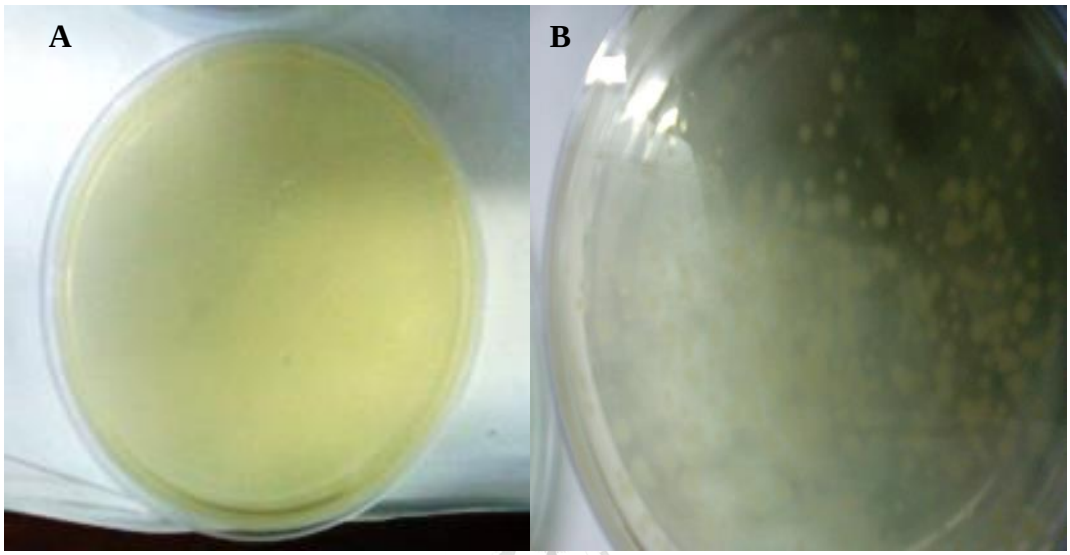


Figure 4.16: Control and test plates showing results for transformation. A. Control Plate: no growth was observed on the control plate. **B**. Test Plate: colonies of bacterial growth were observed on the test plate which shows the positive transformants.

SDS-PAGE analysis of the total bacterial lysates from the four randomly chosen colonies is shown in Figure 4.17. Result showing from Lanes 1 - 8 contains alternating pairs of un-induced (lane 1,3 ,5 and 7) and induced samples (lane 2, 4, 6, and 8) while lane M, is the molecular protein marker. Prominent bands identified at the GST fusion protein at ~37 kDa indicate that the protein was well expressed at the expected band. 100µL from the induced culture in lane 4 was used to inoculate a large-scale expression of GST-RING fusion protein as described in Section 3.5.1. During recombinant protein production, expression screening provides information on the best expressing colonies which can be used for further large scale protein expression.

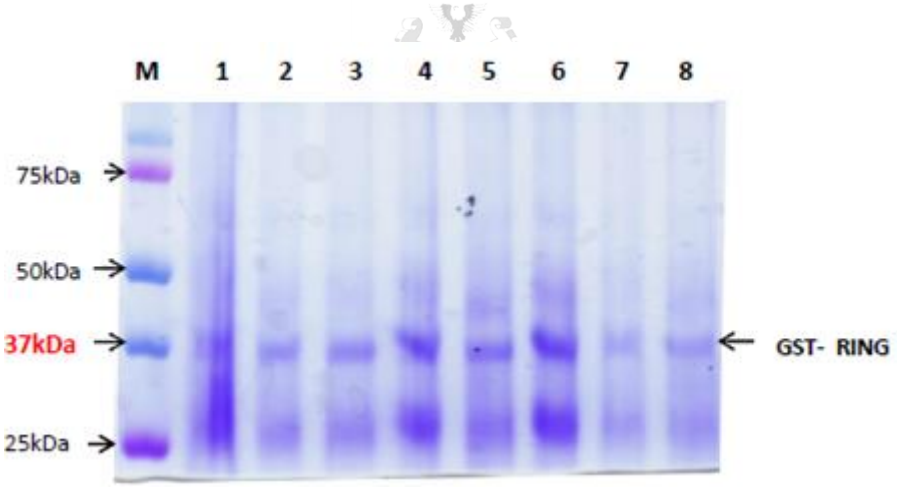


Figure 4.17: Small Scale expression screening of colonies transformed with pGEX-6P-2- RING domain of *A. niger*. Lanes 1,3,5,7 represents samples induced with 0.5mM IPTG while lanes 2,4,6,8 represent Un-induced samples.

4.8.2 Large Scale Expression

100 µL from the best expressing colony from the small scale expression screening was used for the large scale expression. SDS-PAGE analysis of the large scale expression is shown in Figure 4.18. The result in Figure 4.18 shows that a very high yield of the fusion protein was obtained. The protein was loaded at different volumes (5 µL, 10 µL, 15 µL), and prominent bands were also identified for the GST fusion protein running at the expected size ~37 kDa, which further confirmed that the protein was well expressed.

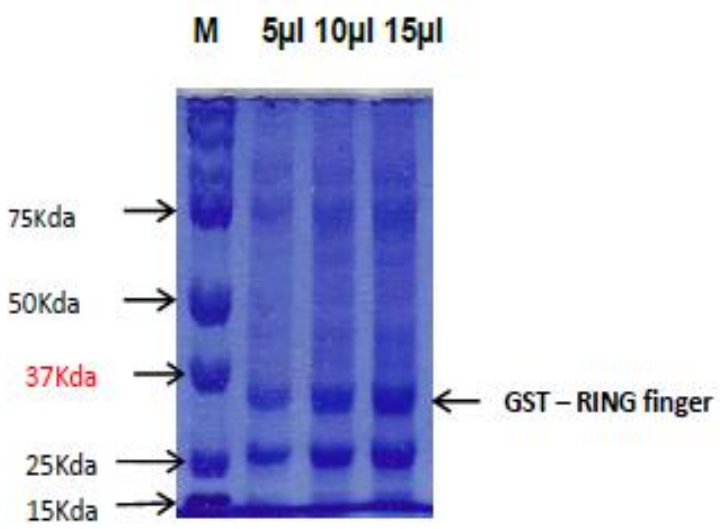


Figure 4.18: Large scale expression of colonies transformed with PGEX-6P-2- RING domain of *A. niger*. Different concentration of the protein sample was loaded on the gel while the protein marker was labelled M. 5µl of the protein sample was loaded on lane 1 ,10µl of the protein sample was loaded on lane 2 while 15µl of the protein sample was loaded on lane 3.

4.8.3 Extraction and affinity purification pGEX-6P-2- RING domain of *Aspergillus niger*

Extraction and affinity purification of recombinant pGEX-6P-2- RING domain of *Aspergillus niger* (fusion protein) were carried out as described in Sections 3.6.2 and 3.6.3. The SDS-PAGE analysis after affinity chromatography was shown in Figure 4:19, In which Lane M represents the molecular weight marker, LY represents the total bacterial lysates, FT and CW indicates the flow through and clean wash from the GST agarose column respectively. The GST-RING domain fusion protein elution from the affinity column is indicated by E1, E2 and E3 respectively.

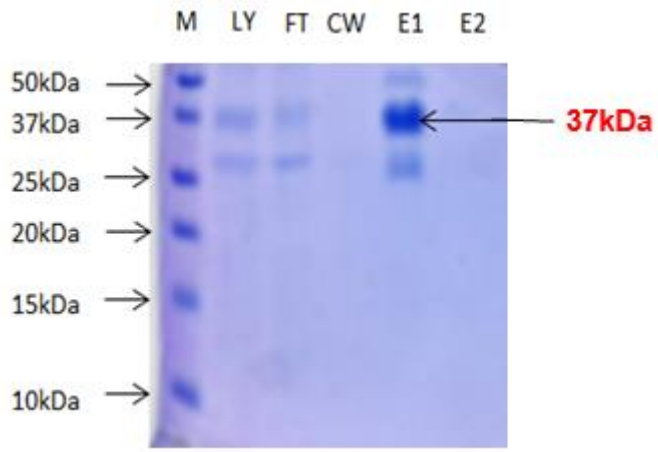


Figure 4.19: Affinity purification of *A niger* GST-RING on a GST-Agarose column. M represents the marker, LY represents lysate, CW represents clean wash, E1 represents elution 1 and E2 represents elution 2.

4.8.4 Precision Protease Cleavage and Recovery of RING Domain

The fusion protein was subjected to 3C protease cleavage as described in Section 3.6.4. The cleaved protein samples were analyzed on SDS-PAGE as presented in Figure 4.20 as it shows the complete 3C protease cleavage of *Aspergillus niger.* Lane M represents the molecular weight marker. Line 2 and 3 indicates GST-RING finger protein after cleavage using Snake pleated dialysis tubing incubated at 4°C with continuous stirring for 18 - 24 hours respectively.

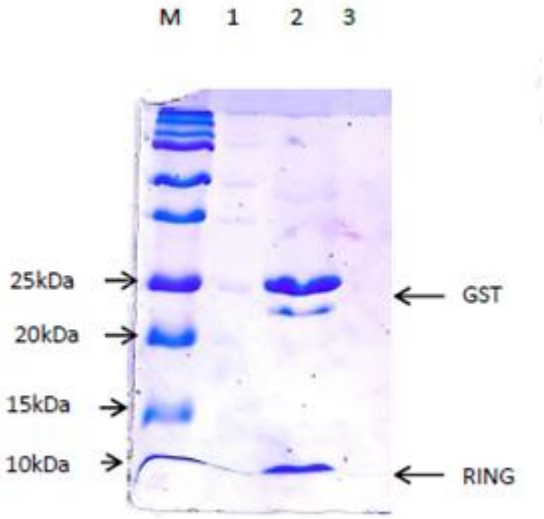


Figure 4.20: Cleaved and affinity purification of cleaved *Aspergillus niger* RING finger domain protein

4.9 Protein concentration determination

Determination of the protein concentration of *Aspergillus niger* RING finger was carried out using NanoDrop® *ND2000* Spectrophotometer (NanoDrop Technologies Inc) at a wavelength of 280 nm. The protein concentration of *Aspergillus niger* RING finger determined at a wavelength of 280 nm was 1.020 mg/mL as shown in Figure 4.21. The presence of impurities in protein can influence the concentration of a protein.

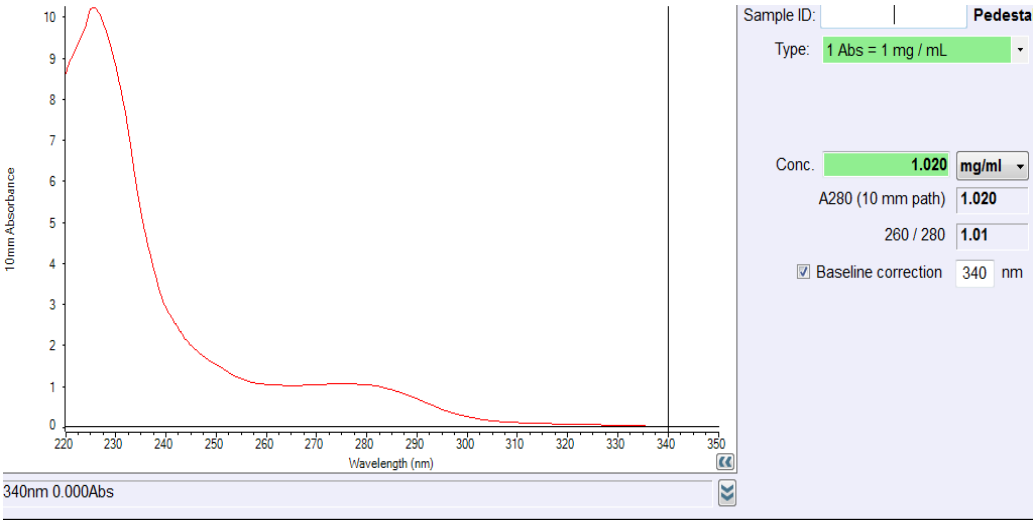


Figure 4.21: Concentration of *A. niger* RING finger domain obtained using NanoDrop® ND2000 Spectrophotometer (NanoDrop Technologies Inc) at a wavelength of 280nm with a concentration 1.020 mg/ml.

4.10 Biophysical characterization of *A. niger* RING Finger Domain

4.10.1 Identification of *A. niger* functional groups using Fourier transform Infrared spectroscopy

The identification of the functional groups present in the *A. niger* RING finger domain protein was elucidated using Fourier Transform infrared spectroscopy. The identified functional group peaks are shown in Figure 4.19. The observed peak that is close to 3300 cm^{-1} is identified to be N-H stretch vibrations for the amine group with substituted intensity results from a side chain COO- while the peak close to 1650 cm^{-1} is identified to be Amide 1 band, this indicates the presence of variable intensity Alkene stretch and a stretch carbonyl group (C=O) with strong intensity. Similarly, the peak close to 2200 cm^{-1} indicates the presence of the Alkyne stretch and variable intensity. The peak near 1850 cm^{-1} reveals the presence of cyclic stretch with strong intensity and anhydride stretch with two bands intensity. These peaks identified using FTIR gives information about the secondary structures of the *A. niger* RING finger domain.

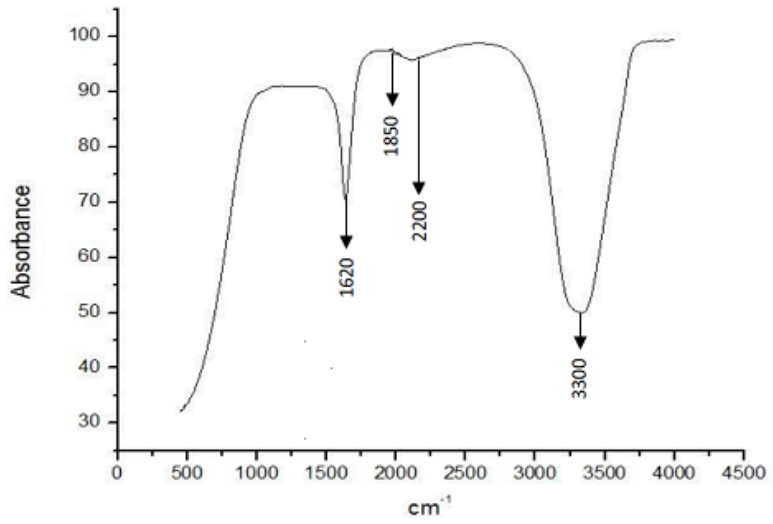
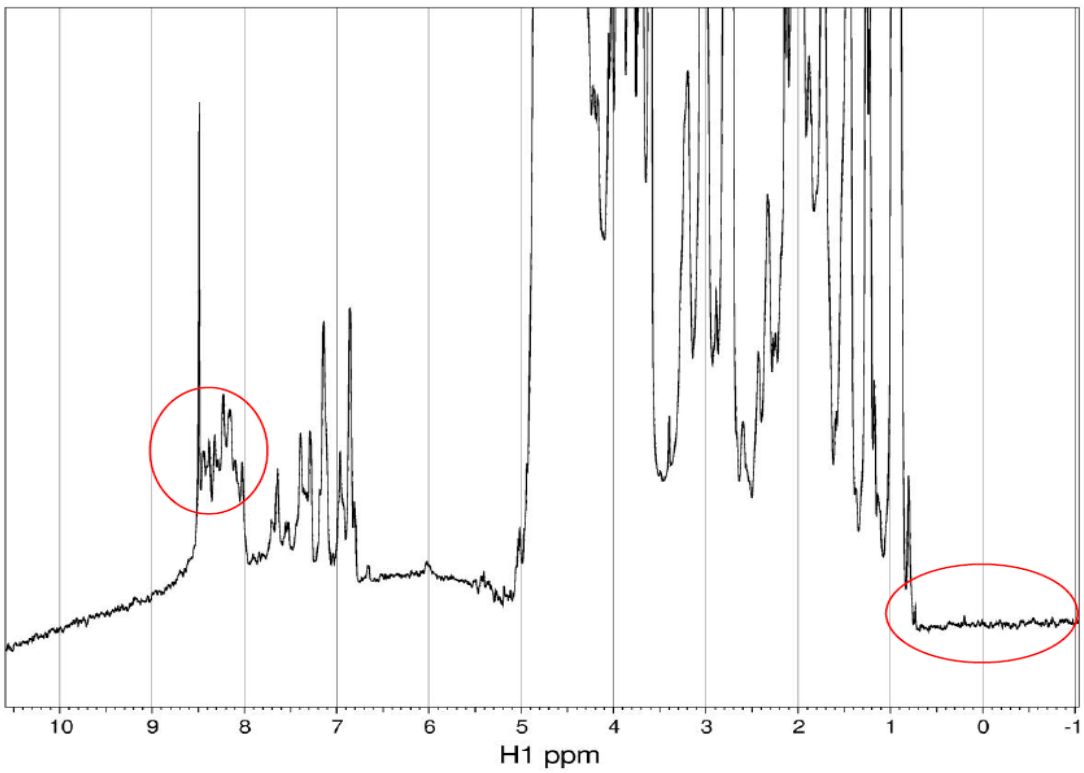


Figure 4.22: **Identification of functional groups present in *A. niger* RING finger domain using FTIR spectroscopy.**

4.10.2 Nuclear magnetic resonance (NMR)

In this research, inexpensive unlabeled rich media was used to evaluate the protein after throughput expression and purification of the RING finger protein. The NMR scale is standardized with tetramethylsilane (TMS) to make a zero reference to obtain a signal called chemical shift with a range 0 – 12 ppm (parts per million). The unit of measurement is parts per million and resonance frequency of the spectrophotometer is in MHz. The determination of whether there is reliable and sensitive indication for a folded protein is based more on the NMR-proton signals from the methyl groups than the amide proton signals. However, the occurrence of protons from either of the regions sufficiently detects folded proteins. The most electron withdrawing group are the Methyl (CH_3) while the aromatic phenyl group are the most electron donating group. The methyl and phenyl group resonates at lowest and highest chemical shifts respectively.

For a predictive protein folding, the methyl group in the amide region is imp5ortant. Scheich and co-workers reported that there must be a large chemical shift dispersions range from 1.0 ppm and -1.0 ppm in the methyl group and also 8.5 ppm in the amide region downfield (Scheich *et al.,* 2004). In Figure 4.23 there was large chemical shift dispersion in the region of 8.5 ppm and slight chemical shift dispersions in the region of 1.0 ppm and -1.0 ppm. The result obtained indicates the folded nature of this protein.



1

Figure 4.22: 1D ^{1}H spectrum of full length RING domain at pH 6.0, 25 °C recorded at 700 MHz. There was a large chemical shift dispersion range of the methyl group in the 3.5-0 ppm region. There is the presence of amide protons (H^{N}) in region 8.6 to 6.7 ppm. The protein indicated by the appearance of amide protons in the region above confirms the foldedness of the protein. Alpha protons (α-protons (H^{α}) can be observed in the region 6.3 – 4.4 ppm while beta protons (H^{β}) were indicated in the 4.2 – 3.3 ppm.

CHAPTER 5

CONCLUSION

5.0 CONCLUSION

The Retinoblastoma binding protein 6 can be found only in eukaryotes and is a 250 kDa nuclear protein which contains a conserved N-terminal RING finger domain. Studies have shown that the RING finger domain interacts with Rb and p53 domains *in vivo* and *in vitro*; these domains have been reported to be involved in protein degradation (Li *et al.*, 2007; Pugh *et al*., 2006; Yoshitake *et al.*, 2004). Even though there were observed differences from the RING finger domain of other organisms, Kappo and co-workers (2012) reported that the second cysteine amino acid in *A. niger* has been replaced by aspartic acid; which was deduced to have likely effect on the stability of the protein. In this research work, *A. niger* GST-RING finger was best over-expressed and analyzed on an SDS PAGE electrophoresis.

The mode of storage (freezing) of the lysates and the rounds of freeze thawing may have significant effects on the degradation of the *A. niger* RING finger protein. However, Xu and co-workers suggested that GST fusion protein lysates should be stored at temperatures between -20°C to -80°C, in order to sustain their activity and stability. Loss of activity and denaturation could occur with varying exposure of proteins to salt and physiological pH conditions during rounds of thawing and freezing (Pikal-Cleland *et al*., 2000; Xu *et al*., 2017). This phenomenon was also observed in this work, the best purified protein that retained its concentration was the sample stored at temperature of -80°C.

Results from the purification of the GST tagged *A. niger* RING finger domain showed that the protein was not purified to homogeneity due to the presence of some contaminants believed to be varying length of proteolysed *A. niger* GST-tagged proteins. However, Xu and co-workers (2017) observed that a GST-tagged HPV16

E6 protein, contains many contaminants on elution while using the single step GST affinity chromatography but on a GST affinity chromatography preceded by a good buffered cation-exchange chromatography, a high purity was obtained (Xu *et al*., 2017). *A. niger* GST RING was purified using affinity chromatography though it is recommended that cation exchange chromatograph should be used in future studies in order to get better purified protein.

U-box and RING finger has been argued to be similar but U-boxes lack cysteine residues, and yet folds into a 3D folding state using hydrophobic bonds without binding to the zinc metal chelation even though zinc coordination is critical to proper folding and stability in U boxes. The stability of the protein is by hydrophobic residues forming interactions such as salt-bridges and hydrogen bonds (Ardley and Robinson 2005; Andersen *et al*., 2004; Aravind and Koonin 2000). The replacement of cysteine residues in *A. niger* RING finger by aspartic acid is similar to the replacement also observed in asparagine/histidine replacement in human RING finger and the loss of cysteine in the structure of U-box (Kappo *et al*., 2012). Kappo and co-workers have reported that a dual classification of Zinc ligated RING finger and U-box has been accorded the RBBP6 cysteine rich domain at residues (249-335). It was further reiterated that the Zn^{2+} ions that binds to the domain in a “cross braced” fashion can be replaced without any significant effect on the stability or the conformation by Cd^{2+} ions (Kappo *et al.*, 2006). Therefore the substitution observed in *A. niger* RING finger may not have any significant effect on the folding and stability of the protein. Further studies using cadmium exchange experiment may be required to better understanding zinc coordination in the *A. niger* RING finger domain.

REFERENCES

Abramovitch R.B, Janjusevic R, Stebbins CE, Martin GB. 2006. Type III effector AvrPtoB requires intrinsic E3 ubiquitin ligase activity to suppress plant cell death and immunity. *Proceedings of the National Academy of Sciences, USA* 103, 2851–2856.

Abramovitch R.B, Martin GB. 2005. AvrPtoB: a bacterial type III effector that both elicits and suppresses programmed cell death associated with plant immunity. Federation of European Microbiological Societies, Microbiology Letters 245, 1–8.

Adekiya T.A., Aruleba R.T., Khanyile S., Masamba, P., Oyinloye B.E., and Kappo A.P. (2017). Structural Analysis and Epitope Prediction of MHC Class-1-Chain Related Protein-A for Cancer Vaccine Development. *Vaccines*, *6*(1), 1

Álvarez C.A., Gomez F.A., Mercado L., Ramírez R. and Marshall S.H. (2016). *Piscirickettsia salmonis* imbalances the innate immune response to succeed in a productive infection in a salmonid cell line model. *PLoS ONE* 11, e0163943.

Andersen P., Kragelund B.B., Olsen A.N., Larsen F.H., Chua N.H., Poulsen F.M. and Skriver K. (2004). Structure and biochemical function of a prototypical Arabidopsis U-box domain. *Journal of Biological Chemistry* 279, 40053-40061.

Aravind L. and Koonin E.V. (2000).The U box is a modified RING finger—a common domain in ubiquitination. *Current Biology* 10(4), 132-134.

Ardley H. C. and Robinson P.A. (2005). E3 ubiquitin ligases. *Essays Biochemical* 41, 15-30.

Asao H., Sasaki Y., Atria T., Tanaka N., Endo K. , Kasai H., Takeshita T., Endo Y., Fujita T., and Sugamura K. (1997) Hrs is associated with STAM, a signal

transducing adaptor molecule. *The Journal of Biological Chemistry*. 272**,** 32785-32791.

Baker L.A., Allis C.D and Wang G.G.(2008). PHD fingers in human diseases: Disorders arising from misinterpreting epigenetic marks. Mutation *Research* 647, 3–12.

Bartek J., Bartkova J., and Lukas J. (1997).The retinoblastoma protein pathway in cell cycle control and cancer. *Experimental Cell Research* 37(1), 1-6.

Bastida-González F., Celaya-Trejo Y., Correa-Basurto J., and Zárate-Segura P. (2016). Predicted 3D Model of the Rabies Virus Glycoprotein Trimer. *Biomedical Research International*. http://dx.doi.org/10.1155/2016/1674580

Benkert P., Biasini M., and Schwede T. (2011).Toward the estimation of the absolute quality of individual protein structure models. *Bioinformatics* 27(3), 343–350.

Benkert P., Künzli M. and Schwede T.(2009). QMEAN server for protein model quality estimation. *Nucleic Acids Research* 37, 510–514.

Berndsen C.E. and Wolberger C. (2014). New insights into ubiquitin E3 ligase mechanism. *Nature Structural and Molecular* Biology 21(4), 301-307.

Bianco R., Ciardiello F. and Tortora G. (2005). Chemosensitization by antisense oligonucleotides targeting MDM2. *Current cancer drug targets* 5(1), 51-56.

Bienz M. (2006). The PHD finger, a nuclear protein-interaction domain. *Trends in Biochemical Sciences* 31, 35-40.

Bombarda E., Morellet N., Cherradi H., Spiess B., Bouaziz S., Grell E., Roques B.P., and Mely Y. (2001). Determination of the pK (a) of the four Zn^{2+}-coordinating residues of the distal finger motif of the HIV-1 nucleocapsid protein: consequences on the binding of Zn2+. *Journal of Molecular Biology 310*, 659-672.

Bottomley M.J., Stier G., Pennacchini D., Legube G., Simon B., Akhtar A., Sattler M., and Musco G. (2005). NMR structure of the first PHD finger of autoimmune regulator protein (AIRE1). Insights into autoimmune polyendocrinopathy-candidiasis ectodermal dystrophy (APECED) disease. *Journal of Biological Chemistry* 280, 11505-11512.

Capili A.D., Schultz D.C., Rauscher I.F., and Borden K.L. (2001). Solution structure of the PHD domain from the KAP-1 corepressor: structural determinants for PHD, RING and LIM zinc-binding domains. *The European Molecular Biology Organization Journal* 20, 165-177.

Chibi M., Meyer M., Skepu A., Rees D.J.G., Moolman-Smook J.C., and Pugh D.J. (2008). RBBP6 interacts with multifunctional protein YB-1 through its RING finger domain, leading to ubiquitination and proteosomal degradation of YB-1. *Journal of Molecular Biology* 384*,* 908-916.

Chidambaram A., Goldstein A.M., Gailani, M.R., Gerrard B., Bale S.J., DiGiovanna, J.J. and Dean, M. (1996). Mutations in the human homologue of the Drosophila

patched gene in Caucasian and African-American nevoid basal cell carcinoma syndrome patients. *Cancer Research 56*, 4599-4601.

Coscoy L., and Ganem D. (2003). PHD domains and E3 ubiquitin ligases: viruses make the connection. *Trends in Cell biology* 13, 7-12.

Coscoy L., Sanchez D.J. and Ganem D. (2001). A novel class of herpes virus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition. *Journal of Cell Biology* 155, 1265–1273

Cyr D.M., Höhfeld J and Patterson C., (2002). Protein quality control: U-box-containing E3 ubiquitin ligases join the fold. *Trends in Biochemical Sciences* 27*,* 368-375

d'Azzo A.., Bongiovanni A., and Nastasi T. (2005). E3 ubiquitin ligases as regulators of membrane protein trafficking and degradation. Traffic (Copenhagen, Denmark) 6, 429-441.

DeGregori J. (2005). E2F and cell survival: context really is key .*Developmental cell* 9, 442-444.

Dominguez C., Folkers G.E., and Boelens R. (eds) (2004). Biological introduction: RING domain protein. *Contributions to Handbook of Metalloproteins. Wiley and Sons* 3**,** 338-351.

Dorn M, e Silva M Barbachan, Buriol L.S., and Lamb L.C.(2014). Three-dimensional protein structure prediction: Methods and computational strategies. *Computer Biology Chemistry Elsevier Limited* 53**,** 251–76.

Driscoll P.C. (2001). Solving the FYVE domain--PtdIns (3)P puzzle. *Nature Structural Biology* 8, 287-290.

Dude T., and Lim C. (2003).Principles governing Mg, Ca, and Zn binding and selectivity in proteins. *Chemical Reviews* 103, 773-788.

Dul B.E.and Walworth, N.C. (2007). The plant homeodomain fingers of fission yeast Msc1 exhibit E3 ubiquitin ligase activity. *The Journal of Biological Chemistry 282*, 18397-18406.

Du W. and Dyson N(1999).The role of RBF in the introduction of G1 regulation during Drosophila embryogenesis. *The European Molecular Biology Organization Journal* 18, 916-925.

Du W., Vidal M., Xie B.E.and Dyson N (1996).RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in Drosophila. *Genes and development 10*: 1206-1218.

Durfee T., Mancini M.A., Jones D.Elledge S.J., and Lee W.H. (1994). The amino-terminal region of the retinoblastoma gene product binds a novel nuclear matrix protein that co-localizes to centers for RNA processing. *The Journal of Cell Biology* 127**:** 609-622.

Fantappié M.R., Osman A.., Ericsson C., Niles E.G., and LoVerde P.T. (2003). Cloning of Schistosoma mansoni Seven in Absentia (SmSINA) + homologue cDNA,

a gene involved in ubiquitination of SmRXR1 and SmRXR2. *Molecular and Biochemical Parasitology* 13**,** 45-54.

Garcia-Boronat M., Diez-Rivero C.M., Reinherz E.L. and Reche P.A. (2008). PVS: a web server for protein sequence variability analysis tuned to facilitate conserved epitope discovery. *Nucleic Acids Research* 36, W35-41

Gillooly D.J., Simonsen A., and Stenmark H. (2001). Cellular functions of phosphatidylinositol 3-phosphate and FYVE domain proteins. *The Biochemical Journal* 355, 249-258.

Greenberg R.A. (2008). Recognition of DNA double strand breaks by the BRCA1 tumor suppressor network. *Chromosoma 117*, 305-317.

Haglund K. and Dikic I. (2005). Ubiquitylation and cell signalling**.** *The European Molecular Biology Organization Journal*. *24*, 3353–3359

Haris P.I and Severcan F. (1999). FTIR spectroscopic characterization of protein structure in aqueous and non-aqueous media. *Journal of Molecular Catalysis B: Enzymatic* 7, *207–221*

Hatakeyama S. and Nakayama K.I. (2003). U-box proteins as a new family of ubiquitin ligases. *Biochemical and Biophysical Research Communications* 302, 635-645.

Houben K., Wasielewski E., Dominguez C., Kellenberger E., Atkinson R.A., Timmers H.T., Kieffer B., and Boelens, R. (2005). Dynamics and metal exchange properties

of C4C4 RING domains from CNOT4 and the p44 subunit of TFIIH. *Journal of Molecular Biology* 349, 621-637.

Joazeiro C.A..and Hunter T. (2000). Ubiquitination--more than two to tango. *Science* 289, 2061-2062.

Jones C., Reifegerste R., and Moses K. (2006). Characterization of Drosophila mini-me, a gene required for cell proliferation and survival. *Genetics*, 173**,** 793-808.

Kale S.K. and Deshmukh A.G.(2016).3D structure prediction of lignolytic enzymes lignin peroxidase and manganese peroxidase based on homology modelling. J*ournal of Biological Sciences.*5 (1): 1-11.

Kandias N.G., Chasapis C.T., Bentrop D., Episkopou V., and Spyroulias G.A. (2009). High yield expression and NMR characterization of Arkadia E3 ubiquitin ligase RING-H2 finger domain. *Biochemical and biophysical research Communications* 378, 498-502.

Kappo M.A., Eiso A.B., Hassem F., Atkinson R.A., Faro A., Muleya V. and Pugh D.J. (2012). Solution structure of RING finger-like domain of retinoblastoma-binding protein-6 (RBBP6) suggests it functions as a U-box. *Journal of Biological Chemistry* 287, 7146-7158.

Ko L.J., and Prives C. (1996). p53: puzzle and paradigm. *Genes and development 10*, 1054-1072.

Kosarev P., Mayer K.F., and Hardtke C.S. (2002). Evaluation and classification of RING-finger domains encoded by the Arabidopsis genome. *Genome Biology 3*, 12-18.

Krebs B. B. De Mesquita J.F. (2016) Amyotrophic Lateral Sclerosis Type 20 - In Silico Analysis and Molecular Dynamics Simulation of hnRNPA1. PLoS ONE 11(7), e0158939. doi: 10.1371/

Kutateladze T.G., Ogburn K.D., Watson W.T., de Beer T., Emr S.D., Burd C.G., and Overduin M. (1999). Phosphatidylinositol 3-phosphate recognition by the FYVE domain. *Molecular Cell* 3, 805-811

Laurie N.A. and Dyer M.A., (2007).Targeting MDM2 and MDMX in retinoblastoma. *Current cancer drug targets*, *7*, 689-695.

Li L., Deng B., Xing G., Teng Y., Tian C., Cheng X., Yin X., Yang J., Gao X., Zhu Y., Sun Q., Zhang L., Xang X., and He F. (2007). PACT is a negative regulator of p53 and essential for cell growth and embryonic development. Proceedings of the *National academy of sciences* 104, 7951-7956.

Luo J., Shen G., Yan J., He C., and Zhang H. (2006). AtCHIP functions as an E3 ubiquitin ligase of protein phosphatase 2A subunits and alters plant response to abscisic acid treatment. *The Plant Journal 46*, 649–657.

Mather A., Rakgotho M., and Ntwasa M. (2005). SNAMA, a novel protein with a DWNN domain and a RING finger-like motif: A possible role in apoptosis. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression 1727*, 169-176.

Matthews J.M. and Sunde M.(2002). Zinc fingers--folds for many occasions. *International Union of Biochemistry and Molecular Biology life 54*, 351-356.

McCoy M.A., Gesell J.J., Senior M.M., and Wyss D.F. (2003). Flexible lid to the p53-binding domain of human Mdm2: implications for p53 regulation. *Proceedings of the National Academy of Sciences* 100, 1645-1648.

McMahon A.P., Ingham P.W., and Tabin C.J. (2003). Developmental roles and clinical significance of Hedgehog signaling. *Current topics in Developmental Biology 53*, 1-114.

Micel L.N., Tentler J.J., Smith P.G., and Eckhardt S.G. (2013). Role of Ubiquitin Ligases and the Proteasome in Oncogenesis: Novel Targets for Anticancer Therapies. *American Society of Clinical Oncology* 1231

Miura S., Takeshita T., Asao H., Kimura Y., Murata K., Sasaki Y., Hanai J. I., Beppu H., Tsukazaki T., Wrana J. L. et al. (2000) Hgs (Hrs), a FYVE domain protein, is involved in Smad signalling through cooperation with SARA. *Molecular Cellular Biological 20*, 9346-9355

Motadi L.R., Bhoolas K.D., and Dlamini Z. (2011). Expression and function of retinoblastoma binding protein 6 (RBBP6) in human lung cancer. *Immunobiology 216*, 1065-1073.

Neumann C. (2005). Hedgehogs as negative regulators of the cell cycle. *Cell cycle 4*, 1139-1140.

Nicholas P. Greene, Allister Crow, Colin Hughes, and Vassilis Koronakis (2015) Structure of a bacterial toxin-activating acyltransferase. PNAS | Published online May 27, 2015 www.pnas.org/cgi/doi/10.1073/pnas.1503832112

Nielsen E., Christoforidis S., Uttenweiler-Joseph S., Miaczynska M., Dewitte F., Wilm M., Hoflack B., and Zerial, M. (2000) Rabenosyn-5, a novel rab5 effector, is complexed with hVPS45 and recruited to endosomes through a FYVE Finger domain. *The Journal of Cell Biology 151*, 601-612

Ntwasa M. (2008). The retinoblastoma binding protein 6 is a potential target for therapeutic drugs. *Biotechnology Molecular Biology Reviews 3*, 24-31.

Ohi M.D., Vander Kooi, C.W., Rosenberg J.A., Chazin W.J., and Gould K.L. (2003). Structural insights into the U-box, a domain associated with multi-ubiquitination. *Nature Structural & Molecular Biology 10*, 250-255.

Pascual J., Martinez-Yamout M., Dyson H.J. and Wright P.E. (2000) Structure of the PHD zinc finger from human Williams-Beuren syndrome transcription factor. The Journal of Molecular Biology. *304*, 723–729

Patterson C. 2002. A new gun in town: the U box is a ubiquitin ligase domain. Science STKE 2002, *Current Biology* 4, *220-233*

Pavletich N.P., Chambers K.A., and Pabo C.O. (1993). The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots. *Genes & development 7*, 2556-2564

*Pena Pedro PhD (2009).*The plant homeodomain and its role in chromatin recognition. University of Colorado Health Sciences Center. 168 pages; 3361676.http://search.proquest.com/docview/305102151

Peterson M.R., Burd C.G. and Emr S.D. (1999). Vac1p coordinates rab and phosphatidylinositol 3-kinase signalling in Vps45p-dependent vesicle docking/fusion at the endosome. *Current Biology* 9**,** 159-162

Pretorius A., Kaur M., Wamalwa M., Essack M., Bajic V.B. and Rees D.J.G. (2014). Functional analysis and characterization of the human RBBP6 promoters based on a combination of molecular biology and in silico approaches provide additional evidence for RBBP6 role in apoptosis. *Journal of BioSciences* 1*,*

Ponomarenko J., Bui H.H., Li W., Fusseder N., Bourne P.E., Sette A., and Peters B. (2008). ElliPro: a new structure-based tool for the prediction of antibody epitopes. *BMC Bioinformatics*,*9*, 514.

Pugh D.J., Eiso A.B., Faro A., Lutya P.T., Hoffmann E. and Rees, D.J.G. (2006). DWNN, a novel ubiquitin-like domain, implicates RBBP6 in mRNA processing and ubiquitin-like pathways. *BioMed Central Structural Biology 6*, 1-12.

Requejo R., Hurd T.R., Costa N.J and Michael P (2010). Cysteine residues exposed on protein surfaces are the dominant intramitochondrialthiol and may protect against oxidative damage. *Federation of European Biochemical Societies Journal* 277 (2010) 1465–1480). doi:10.1111/j.1742-4658.2010.07576.x

Rothman J.E and Warren G (1994).Implications of the SNARE hypothesis for intracellular membrane topology and dynamics.

Sakai Y., Saijo M., Coelho K., Kishino Niikawa N. and Taya Y. (1995). cDNA sequence and chromosomal localization of a novel human protein, RBQ-1 (RBBP6), that binds to the retinoblastoma gene product. *Genomics* 30(1), *98-101.*

Scambia G., Lovergine S.,and Masciullo V (2006).RB family members as predictive and prognostic factors in human cancer. *Oncogene* 25 (38), *5302-5308*.

Scheich C., Leitner D., Sievert V., Leidert M., Schlegel B.,Simon B., Letunic I., Büssow K. and Diehl A. (2004). Fast identification of folded human protein domains expressed in E. coli suitable for structural analysis. *BioMed Central structural biology* 4(4): doi: 10.1186/1472-6807-4-4.

Schnell J D and Hicke L(2003) Non-traditional functions of ubiquitin and ubiquitin-binding proteins. *Journal of Biological Chemistry* 278, *35857–35860.*

Scott R.E.,Giannakouros .,T.Gao S., and Peidis P. (2003). Functional potential of P2P-R: A role in the cell cycle and cell differentiation related to its interactions with proteins that bind to matrix associated regions of DNA. *Journal of cellular biochemistry* 90 (1), *6-12.*

Sellers W.R., Rodgers J.W and Kaelin W.G. (1995). A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. *Proceedings of the National Academy of Sciences* 92 (25), *11544-11548*.

Simons A., Melamed-Bessudo C., Wolkowicz R., Sperling J., Sperling R., Eisenbach L. and Rotter V. (1997). PACT: cloning and characterization of a cellular p53 binding protein that interacts with Rb. *Oncogene* 14 (2), *145-155*.

Stenmark H., Aasland R., and Driscoll P.C. (2002). The phosphatidylinositol 3-phosphate-binding FYVE finger. *Federation of European Biochemical Societies, Letters* 513 (1), *77-84.*

Tu D., Li W., Ye Y., and Brunger, A.T. (2007). Inaugural Article: Structure and function of the yeast U-box-containing ubiquitin ligase Ufd2p. Proceedings of the *National Academy of Sciences of the United States of America*. *104*, 15599-15606.

Wang H., Nan L., Yu D., Agrawal S., and Zhang R. (2001). Antisense Anti-MDM2 Oligonucleotides as a Novel Therapeutic Approach to Human Breast Cancer in Vitro

and in Vivo Activities and Mechanisms. *Clinical Cancer Research* 7 (11), *3613-3624*.

Wilkins M.R., Gasteiger E., Bairoch A., Sanchez J.C., Williams K.L., Appel R.D. and Hochstrasser D.F. (1999). Protein identification and analysis tools in the ExPASy server*. Methods in Molecular Biology* 112, 531–552.

Witte M.M. and Scott R.E. (1997). The proliferation potential protein-related (P2P-R) gene with domains encoding heterogeneous nuclear ribonucleoprotein association and Rb1 binding shows repressed expression during terminal differentiation. *Proceedings of the National Academy of Sciences* 94 (4), 1212-1217.

Xiong J., Yuan D., Fillingham J.S., Garg J., Lu X., Chang Y., Liu Y., Fu C., Pearlman R.E. and Miao W. (2011). *Gene network landscape of the ciliate Tetrahymenathermophila. PloS one* 6 (5), *20124.*

Xu Z., Devlin K.I., Ford M.G., Nix J.C., Qin J and Misra S (2006). Structure and interactions of the helical and U-box domains of CHIP, the C terminus of HSP70 interacting protein*. Biochemistry* 45*, 4749-4759*.

Yao B., Zheng D Liang S. and Zhang C. (2013). Conformational B-Cell Epitope Prediction on Antigen Protein Structures: A Review of Current Algorithms and Comparison with Common Binding Site Prediction Methods. *PLoS ONE* 8(4): e62249

Yee D., and Goring D. R., (2008).The diversity of plant U-box E3 ubiquitin ligases: from upstream activators to downstream target substrates. *Journal of Experimental Botany, Volume* 60, *No. 4, pp. 1109–1121, 2009*

Yoshitake Y., Nakatsura T., Monji M., Senju S., Matsuyoshi H., Tsukamoto H., Hosaka S., Komori H., Fukuma D., Ikuta,Y., Katagiri T., Furukawa Y., Ito H., Shinohara M., Nakamura Y. and Nishimura Y. (2004). Proliferation potential-related protein, an ideal esophageal cancer antigen for immunotherapy, identified using complementary DNA microarray analysis. *Clinical cancer research* 10 (19), *6437-6448.*

Zhang R., Wang H., and Agrawal S. (2005). Novel antisense anti-MDM2 mixed-backbone oligonucleotides: proof of principle, in vitro and in vivo activities, and mechanisms. *Current cancer drug targets* 5 (1), 43-49.

Zhang M., Windheim M., Roe S.M, Peggie M., Cohen P., Prodromou C and Pearl LH (2005). Chaperoned ubiquitination--crystal structures of the CHIP U box E3 ubiquitin ligase and a CHIP-Ubc13-Uev1a complex. *Molecular Cell 20*, 525-538.

Zheng Q. and Zhao Y. (2007). The diverse bio functions of LIM domain proteins: determined by subcellular localization and protein-protein interaction. *Biological Cell*. 99: 489-502.

APPENDIX I: GENERAL CHEMICALS AND ENZYMES

Chemical	Company
Acryl: bis-acrylamide	Sigma
Acetic Acid	Merck
Ammonium Persulphate	Sigma
Ampicillin	Sigma
Bacteriological Agar	Merck
Bromophenol Blue	Sigma
Calcium Chloride	Merck
Coomassie Brilliant Blue	Merck
Dithiothreitol (DTT)	Sigma
Ethanol	Merck
Ethidium Bromide	Merck
Ethylene diamine tetra Acetic Acid	Merck
Glacial Acetic Acid	Merck
Glucose	Merck
Glutathione	Sigma
Glutathione Agarose	Sigma
Glycerol	Merck
Glycine	Merck
Hydrochloric Acid	BDH
Isopropyl β-D-thiogalactopyranoside (IPTG)	Sigma
Lysozyme	Sigma
Magnesium Chloride	Merck

Magnesium Sulphate	Merck
Methanol	Merck
Phenyl Methyl Sulphonyl Fluoride (PMSF)	Sigma
Potassium Chloride	Merck
Sodium Azide	Sigma
Sodium Chloride	Merck
Sodium Dodecyl Sulphate (SDS)	BDH
Sodium Hydroxide	Merck
TEMED (*N,N,N',N'*-Tetra methyl ethylene diamine)	Sigma
Tris (Tris [hydroxymethyl] amino ethane)	Merck
Triton X-100 (iso-octylphenoxypolyethoxyethanol)	Capital Lab
Tryptone powder	Merck
Yeast extracts	Biolab
Zinc Sulphate	Merck

APPENDIX II: GENERAL EQUIPMENT

Equipment	Company
Mini centrifuge	Eppendorf
Centrifuge 5804 R	Eppendorf
Ultrasonic Homogenizer	Mrc
Electrophoresis Machine	BIOCAM
Heat Block	Techne
Precisson^{TM} Protease	Separation Scientific (Pty) Ltd
Incubator	Inforst HT Ecotron
Shaker	Inforst HT Ecotron

APPENDIX III: PREDICTION OF PROTEIN PARAMETERS

Appendix A: Parameters for shortened RING domain

ProtParam

Physicochemical properties of the RING finger domain shorten form obtain from ProtParam

Properties	Values
Number of amino acids	86
Molecular weight	9661.99 Da
Theoretical Isoelectric point	4.72
Formula	$C_{409}H_{665}N_{113}O_{138}S_9$
Total number of negatively charged residues (Asp + Glu)	18
Total number of positively charged residues (Args + Lys)	13
Computed instability index (II)	27.21
Aliphatic index	71.51
Grand Average of hydropathY (GRAVY)	-0.615

APPENDIX IV*: Aspergillus niger* RING finger domain

The amino acid long chain sequence of the RING finger domain from *A. niger* excluding the mRNA sequence. The part of the sequence highlighted in RED was used for this research.

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10         20         30         40         50         60
MSSSVHFKFK SQKEPSRVTF DGTGISVFEL KREIINQSRL GDGTDFELSI YNEDTGEEYD

        70         80         90        100        110        120
DDTSIIPRST SVIARRLPAS RPGKGGAARY VSGKMPVNAR SAPRNEQYPL TRASPNTSNG

       130        140        150        160        170        180
VLELNNAQTE EEKINALFNL QANQWKEQQQ EMANATPVPF GRGRGRPVNV PDHPPPPGYL

       190        200        210        220        230        240
CYRCREKGHW IQACPTNNDP KFDGKYRVKR STGIPRSLQT KVEKPESLTL DGSNEDPRNT

       250        260        270        280290        300
GVMVNADGDF VIAKPDKAAW ELYQEKAKAS AAAAAEAAAA EYSKELQARG LECPIDKRMF

       310        320        330        340        350        360
LEPTRTPCCQ RTYCNDCITN ALIESDFVCP GCGTEGVLLD NLAVDDEAIS KIKEYEAEKA

       370380        390        400        410        420
DSKKEKDKQQ ANDIGPGIDK TAIQGKGPVA TTESLPPIPP SQPASNKRPA EDETPRTAVG

       430        440        450        460        470        480
GSSPSSLSKK LKTNDKADSQ TPQATGPVTG FPAFPFGQQM PFGSFGFMPN QGMPAMPLSD

       490        500        510        520        530        540
PMNAMGVPSS GGFPPNMDQS WNPMNAMNFN PAPGMYGDNV NSYATPNLYN GVGEPSMNMF

       550        560        570        580        590        600
HMPQMMGLQP NSGLAQGPGI GRFSNQQRTT FSTPFAREED TAYFRQPVNP QRHQARHRRI


RPSDYREL
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