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Identification of proteins that interact with DWNN domain of SNAMA a member of a novel protein superfamily

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Title of M Sc dissertation:
Identification of proteins that interact with DWNNdomain of SNAMA a member of a novel protein superfamily.

A dissertation submitted in fulfillment of the requirements for the Degree Master of Science in the School of Molecular and Cell Biology at the University of the Witwatersrand

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Declaration

I declare that *Identification of proteins that interact with DWNN domain of SNAMA a member of a novel protein superfamily* is my own work, that has not been submitted for any degree in any other institution, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Name of candidate: Patrick Mpho Rakgotho

Signed on this day:……

Signature of candidate:…….

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ABSTRACT

SNAMA is a 142 kDa *Drosophila melanogaster* protein, which consists of the uncharacterized conserved domain with no name (DWNN), zinc and RING finger-like motifs. The primary structure of SNAMA suggests that it might play an important role in cell cycle regulation and apoptosis. Previous studies revealed that homozygous SNAMA mutants underwent ectopic apoptosis which resulted in recessive lethality. SNAMA orthologues such P2P-R, PACT and RBBP6 are involved in cell cycle regulation, whereas Mpe1 is involved in mRNA processing. The aim of this study was to map out the role of SNAMA by isolating proteins which interact with it. DWNN was inserted into pGEX6P-2, phylexzeo plasmid (bait) and the *Drosophila* 0-12 hours cDNA library inserted in pJG4-5 (prey). The bait and the prey plasmid were used to transform appropriate yeast cells to probe for interacting proteins in yeast two hybrid assays, whereas the pGEX6P-2 was used for heterologous overexpression of DWNN in *E. coli*. Immunoprecipitation assays were also carried out with the crude protein extract from embryos, adult wild type, SNAMA mutant flies and the overexpressed protein using antibodies against SNAMA, *Drosophila* p53 Human DWNN and GST. The hybrid assay did not produce any interactors. Some of the proteins obtained from the immunoprecipitations were isolated and sequenced. The proteins identified were hsp82, Hsp70 and CG2985-PA. Data obtained from the immunoprecipitations suggest that SNAMA like Dmp53 might be involved in cell cycle regulation.

LIST OF ABBREVIATIONS

BDGP	Berkeley Drosophila Genome Project
CIDE	Cell death inducing DFF-like effector
CPF	Cleavage and Polyadenylation Factor
DFF	DNA fragmentation factor
DMF	Dimethyl formamide
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
DWNN	Domain with no name
FOG	Friends of GATA
GST	Glutathione-S-transferase
GATA	Cluster of tetranucleotide G A T A
HUB-1	Homologous to ubiquitin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IAP	Inhibitor of apoptosis
LB	Luria-bertani
LiPEG	Lithium acetate Tris EDTA Poly ethylene glycol 330
LiTE	Lithium acetate Tris EDTA solution
MDM2	Murine double minute-2
NCBI	National Center for Biotechnology Information
P2P-R	Proliferation Potential Protein-Related
PACT P53	Associated Cellular-protein-Testis derived
PBS	Phosphate buffered saline
PI-3	Phosphatidylinositol
PMSF	Phenylmethylsulphonylfluoride
PVDF	Polyvinylidene Fluoride
RBQ1/RBBP6	Retinoblastoma Binding Protein 6
RING	Really Interesting New Gene
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SR	Serine rich
SUMO-1	Small ubiquitin related modifier-1
TAE	Tris-acetate EDTA buffer
TE	Tris-EDTA
TEMED	N,N,N,N ’ -Tetramethylethylenediamine
TFB	Transformation Buffer
Ubl	Ubiquitin like protein
Ubp	Ubiquitin based protein
YNB	Yeast Nitrogen Base
YPD	Yeast Peptone Dextrose
X-gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside

Identification of proteins that interact with DWNN domain of SNAMA a member of a novel protein superfamily

SECTION 1

1. INTRODUCTION

Domain with no name (DWNN) is a conserved domain that characterises a superfamily of proteins found in most eukaryotes but not in prokaryotes. Genomic searches using the conserved domain revealed that the *SNAMA* has one gene in lower eukaryotes such as mosquitoes (*Anopheles gambiae*), fruit flies (*Drosophila melanogaster*) and nematodes (*Caenorhabditis elegans*). In humans, the gene has two transcripts, which encode the 13 kDa DWNN domain only and a 200 kDa multidomain complex protein (Mbita, 2004).

The *Drosophila* homologue is called *SNAMA* (Mather *et al*., 2005). *SNAMA* is a Xhosa word, which means something that sticks. It has a predicted molecular weight of 142 kDa and consists of zinc and RING (**R**eally **I**nteresting **N**ew **G**ene) finger-like motifs closely associated with the N-terminal DWNN (figure 1). Even though the exact role of SNAMA is unknown, its primary structure suggests that the protein might play a role in transcription and cell cycle regulation. Zinc finger proteins tend to promote protein-protein and protein-DNA interactions (Matthews *et al*., 2000; Hart *et al*., 1996). Adams *et al.* (2000) reported that about half of the transcription factors in *Drosophila* are zinc finger proteins.

Othologues of SNAMA such as P53 Associated Cellular-protein Testis derived (PACT) (Simons *et al*., 1997), Retinoblastoma Binding Protein6 (RBBP6) (Sakai *et al*., 1995) and Proliferation Potential Protein-Related P2P-R (Witte and Scott, 1997), were reported to interact with p53 and possibly regulate its activity (Scott *et al*., 2003), thus suggesting that SNAMA might play a similar role in flies. P53 is a tumor suppressor, which prevents growth and survival of stressed cells. It is a sequence-specific transcription factor, which mediates activation and repression of cell cycle genes (Ryan *et al*., 2001).

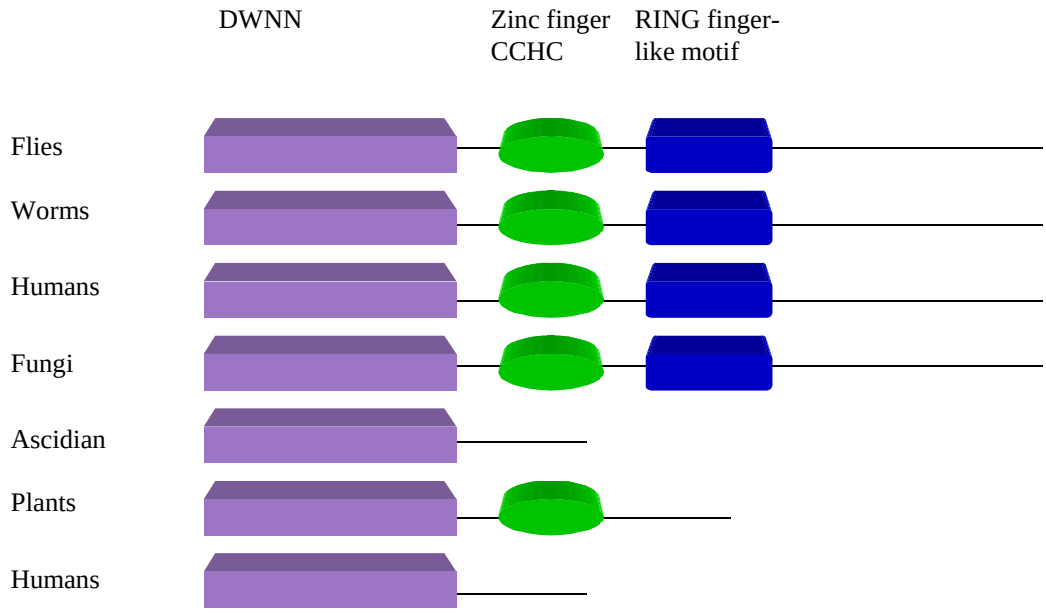


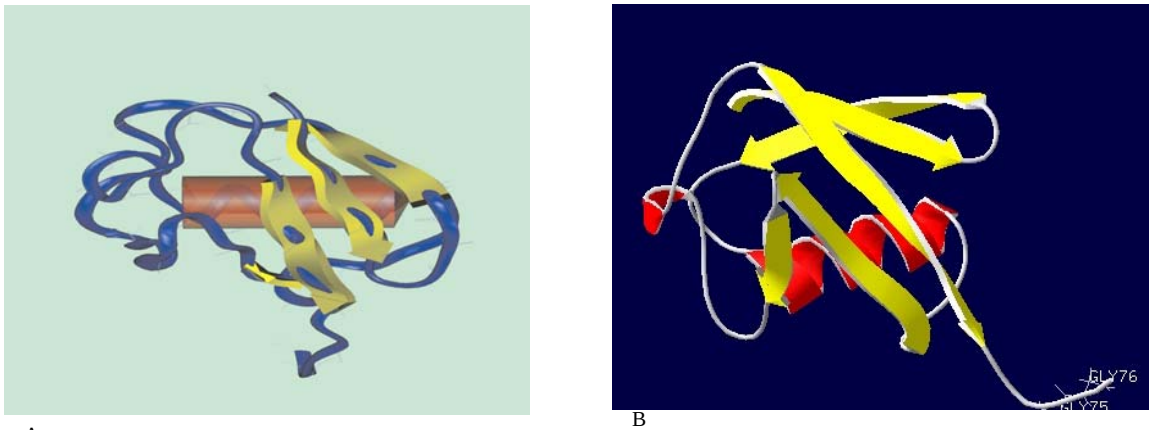
Figure 1: **DWNN superfamily domain organization.**

Homozygous *SNAMA* knockout mutants underwent ectopic apoptosis during embryonic stage and never developed further into adulthood (Mather *et al*., 2005). The human homologue of this protein was isolated in cells, which were resistant to cytotoxic T cell killing due to the disruption of the DWNN gene, thus implying

a certain role of the DWNN protein in apoptosis (Pugh *et. al*., 2006). Taken together these facts strengthen the hypothesis that SNAMA plays an important role in pathways involved in cell cycle regulation and apoptosis.

1.1 DWNN has a ubiquitin like fold

Structural prediction suggests that the domain will have an ubiquitin like fold. The alpha Helix and the four beta sheets of DWNN were found to correspond to the arrangement observed in ubiquitin (figure 2). Even though sequence identity between the DWNN and *Drosophila* ubiquitin is only 22% (figure 3), the structural prediction suggests that the domain might undergo ubiquitin like interactions (Mather *et al*., 2005).



A B

Figure 2: A. Computer generated model of DWNN based on data of (B) ubiquitin structure. DWNN image was created using Insight II modeller (Accelrys Inc), whereas ubiquitin structure was created with PDB viewer (Guex and Peitsch, 1997). Both DWNN and ubiquitin have the β-β-α-β-α-β structure (Vijay-Kumar *et al*., 1987)

```
DWNN_1       .MSCVHYKFSSKLNYDTVTFDGLHISLCDLKKQIMGREKL.KAADCDLQI      48
DWNN_2       .MSCVHYKFSSKLNYDTVTFDGLHISLCDLKKQIMGREKL.KAADCDLQI      48
SNAMA        ..MSVHYKFKSTLNFDTITFDGLHISVGDLKREIVQQKRLGKIIDFDLQI      48
Mpe1p        MSSTIFYRFKSQRNTSRILFDGTGLTVFDLKREIIQENKLGDGTDFQLKI      50
Ubiquitin    .....MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIF      45
Consensus     mscvhykfksklnydtitfdglhislcdlkkeiiqreklgkaadcdlqi

DWNN_1       TNAQTKEEYTDDNALIPKNSSVIVRRIPIGG...                      79
DWNN_2       TNAQTKEEYTDDNALIPKNSSVIVRRIPIGG...                      79
SNAMA        TNAQSKEEYKDDGFLIPKNTTLIISRIPIAHPTK                      82
Mpe1p        YNPDTEEEYDDDAFVIPRSTSVIVKRSPAIKS..                      82
Ubiquitin    AGKQLEDGRTLSDYNIQKESTLHLVLRLRGG...                      76
Consensus    tnaqtkeeytddnalipknssvivrripigg
```

Figure 3: Sequence alignment of DWNN orthologues and ubiquitin. DWNN1 (AAI10042) is a human 13kDa transcript, DWNN2 (XP219296) is a 200kDa transcript and Mpe1 (NP012864) is the yeast orthologue (Vo *et al*., 2001). The sequences were aligned using DNAMAN programme version 4.03 (Lynnon Biosoft).

Protein such as Small Ubiquitin related Modifier-1 (SUMO-1); an ubiquitin-like (ubl) protein has low homology to ubiquitin and modifies proteins by a process called sumoylation. It is vital in protein trafficking and stabilisation

(Yeh, *et. al*., 2000). SUMO-1 regulates p53 stability by covalent ligation, and induction of conformational change (Rodrigues *et al.*, 1999). The DWNN in SNAMA lacks the C-terminal di-glycine residues which characterises ubiquitin and ubl proteins, thus suggesting that it might belong to another set of ubiqiutin-like proteins such as Homologous to Ubiquitin-1 (HUB1) (Figure 4). Luders *et al*. (2003) reported that HUB 1 interacts and covalently attaches to other proteins even though it lacks the C-terminal di-glycine residues. HUB1 though has conserved c-terminal di-tyrosine and also interacts with other proteins via its N-terminal region.

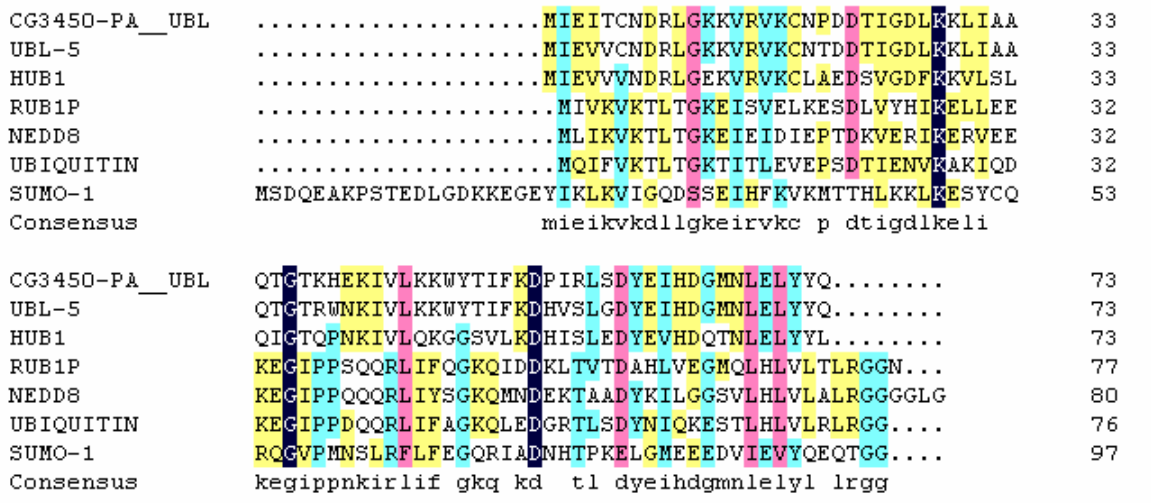


Figure 4: **Alignment of ubiquitin and ubiquitin like domains**. Ubiquitin [AAH14880; Vijay-Kumar *et al*., 1987] and most of its related proteins (SUMO1 [NP001005781; Saitoh *et al*., 1997], Rub1p [NP010423; Hochstrasser, 1996], NEDD8 [AAH04625; Kumar *et al*., 1993] are characterised by the N-terminal di-glycine residues. HUB1 [Q6Q546; Luders *et al*., 2003] undergo ubiquitin like interactions even though it lacks the di-glycine residues). HUB1 homologues are ubiquitin like protein 5 (ubl-5) [AAP36019; McNally *et al*., 2003] and CG3450 [AAF57398] found in humans and flies respectively.

Ubiquitin is a 76 amino acid protein, which tags proteins destined for degradation by the proteasome pathway. It exists as a monomer, or usually as

fusion molecule tagging proteins for degradation (Varshavsky, 1997). Proteins targeted for degradation are covalently bound via the lysine residues to the glycine at the C- terminal end of ubiquitin and eventually degraded by the 26S proteasome (Ciechanover, 1994; Varshavsky, 1997; Hershko and Ciechanover, 1998). The proteasome pathway is a major intracellular proteolytic pathway for maintaining protein turnover in eukaryotes (Figure 5). Ubiquitination involves three enzymes; ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin protein ligase (E3) (Obin *et al*., 1996; Shang *et al*., 1997). The initial step in the pathway is ATP dependent and results in the activation of ubiquitin. The activated ubiquitin is then transferred to E2. The E2 enzyme either catalyses the attachment of the ubiquitin to the substrate directly, or require the third enzyme E3 (Gilchrist *et al*., 1997; Jahngen-Hodge *et al*., 1997; Shang *et al*., 1997). The ubiquitinated protein is eventually degraded by the 26S proteasome with the liberation of the ubiquitin moeity.

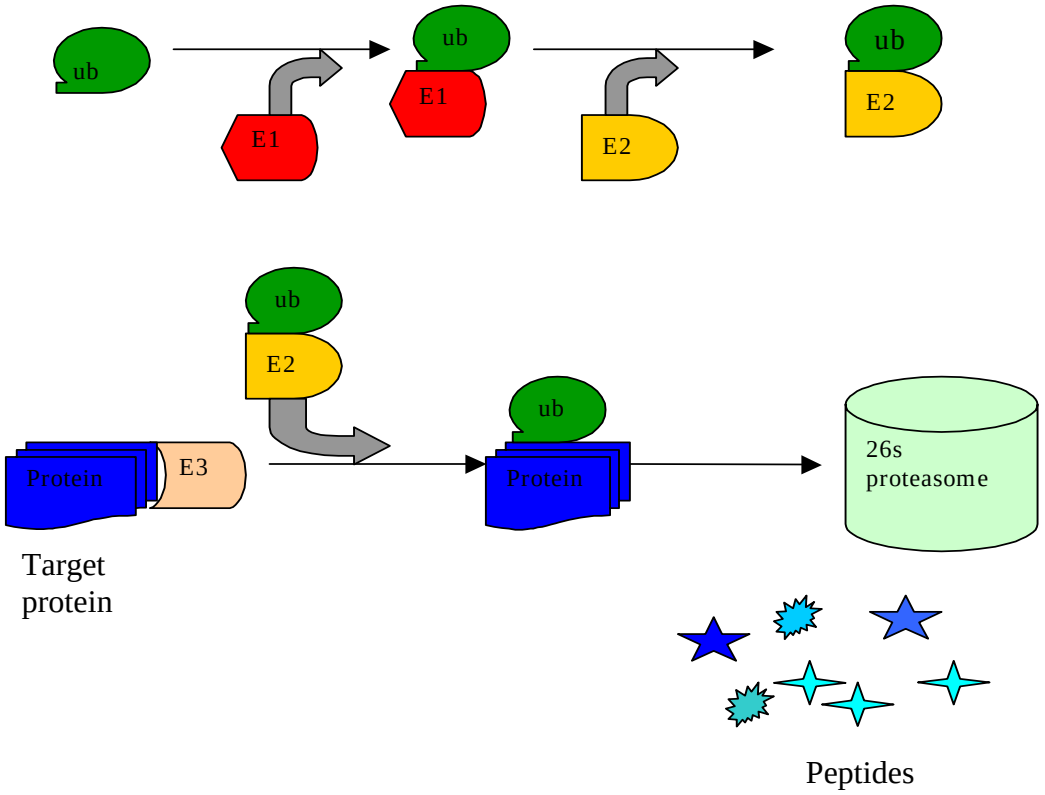


Figure 5: **The Ubiquitin-Proteosome Pathway.** Illustration adapted from Hershko and Ciechnover, (1998).

1.2 DWNN associated domains

1.2.1 RING finger

The RING finger is a modified zinc finger motif (Freemont *et al*., 1991). It is highly conserved and most common motifs are characterised by a core C3HC4 sequence. Known RING finger containing proteins have ubiquitin ligase activity and thus might play a role in protein trafficking and turnover via the proteasome pathway. The pathway is vital in regulating cellular proteins, like degradation of

short-lived regulatory proteins such as transcription and DNA repair factors, kinases, phosphatases and tumor suppressors. It also plays an important role during morphogenesis and organelle biogenesis (Hatakeyama *et al*., 2001).

The RING finger motifs catalyse the ligation of activated ubiquitin with the target protein. Mutations in the RING finger of some proteins are linked to developmental abnormalities (Joazeiro and Weissman, 2000) and diseases such as Parkinsons (Satijn and Otte, 1999).

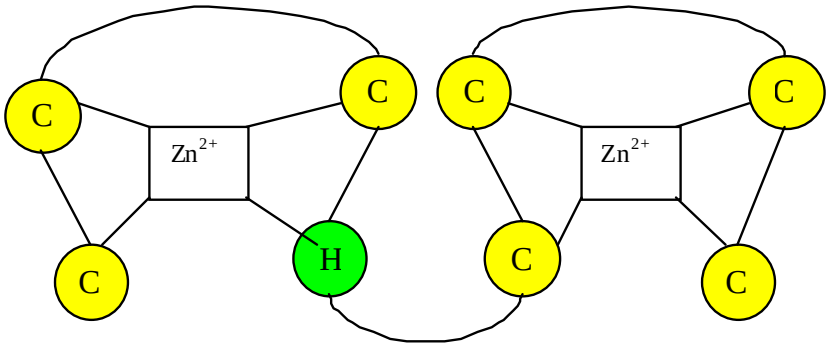


Figure 6: The cross-brace model of RING finger motifs. Conserved cysteine residues highlighted. The lines represent other amino acids. Adapted from Freemont *et al*. (1991).

Homology searches revealed SNAMA and all other orthologues have an unusual RING finger-like motif because the histidine in position 4 is substituted by a serine (figure 6). This arrangement of amino acids suggests that the motif might belong to a new set of uncharacterised RING fingers (Mather *et al*., 2005).

1.2.2 Cysteine rich domains

Zinc fingers are cysteine/histidine rich motifs widely found within many proteins in eukaryotes and are characterised by their ability to bind zinc ions. They are unusually small self-folding domains of which a zinc atom is crucial component of its tertiary structure; they are present in structures of most regulatory proteins where they act as nucleic acid binding domains (Kawagashira *et al*., 2001). In *Drosophila* most zinc fingers have been implicated in arrays of protein-protein interactions and many developmental processes (Hart *et al*., 1996). These proteins constitute a major group of transcription factors and play important roles in gene expression and cellular signal transduction (Otsuka *et al*., 1996). Zinc fingers are extremely common and easily distinguishable by their nature and spacing of the zinc coordinating residues. These motifs tend to mediate interactions with DNA, RNA and proteins (Lai *et al*., 2000). Most common and abundant is the Cys_2His_2 (CCHH) zinc finger which is characterised by the X-**Cys**-$X_{2,4}$-**Cys**-X_3-PheX_5-Leu-X_2-**His**-$X_{3\text{-}5}$-**His** sequence. The CCHH zinc finger motif was first discovered in studies of the *Xenopus laevis* transcription factor IIIA (TFIIIA) and the protein was shown to contain bound zinc ions (Miller *et al.*, 1985). Zinc fingers are most common motifs in eukaryotic proteins (Laity *et al.*, 2001; Jantz *et al.*, 2004). The $\beta\beta\alpha$ fold characterises these motifs. This prototype fold was observed in the transcription factor Zif268, which has three zinc fingers (Laity *et al*., 2001).

Other transcription factors such as GATA (Cluster of tetranucleotide **G**uanine **A**denine **T**hymidine **A**denine) family contain several zinc fingers located at both

ends of the protein. The Cys_4 (CCCC) fingers located at the N-terminal of these proteins tends to be specifically for interaction with co-factors, whereas the classical zinc fingers (CCHH) interacts with DNA (Mackay *et al.*, 1998). GATA-1 has 3 zinc fingers, where half were shown to promote interactions with other transcriptional factors such as FOG (**F**riends **O**f **G**ATA) (Mackay *et al.*, 1998; Fox *et al.*, 1999).

SNAMA contains one of the unusual zinc fingers, the CCHC. These are the retroviral type zinc fingers (Tzafati *et al.*, 1995). In eukaryotes these motifs were reported to promote protein-protein interactions (Matthews *et al.*, 2000). The motifs interact with zinc fingers of other proteins as observed in proteins that interact with the transcription factor GATA, such as FOG (Fox *et al.*, 1999). FOG is a transcriptional co-factor, which interacts with GATA resulting in the activation and gene expression. GATA factors and its co-activators pannier and serpens control ultimate fate of cells during development in *Drosophila* (Fox *et al.*, 1998). Several other CCHC containing proteins such as LIM domains (Specialised double zinc-finger motifs) (Kuroda *et al.*, 1996), U-shaped (Matthews *et al.*, 2000) and CBP (cAMP-response element-binding protein-binding protein) (Newton *et al.*, 2000) were also reported to interact with other proteins. Unlike the DNA binding zinc finger proteins with several motifs close to each other, the CCHC fingers exist in most proteins as single motifs. Although these zinc fingers are known to predominantly promote protein-protein interaction, some yeast zinc finger proteins such as MPE1 (YKL059c), are involved in single stranded nucleic acid processing. Even though the exact role in

the processing is still unclear it is one of the proteins in the yeast's cleavage and polyadenylation factor complex (Vo *et al*., 2001).

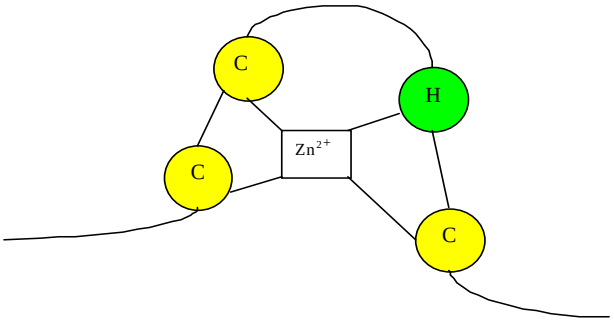


Figure 7: **The prototype fold of the retroviral type zinc finger motifs CCHC.** Adapted from Williams *et al*. (2002).

Other zinc fingers such as the CCCH have only been found in a handful of proteins such as tristetraprolin (TTP) (DuBois *et al*., 1995). The exact role of these unusual zinc fingers in cell activities is not clearly known. Generally most zinc finger proteins tend to have a regulatory role. TTP was reported to be the physiological regulator of tumor necrosis factor-α and granulocyte-macrophage colony-stimulating factor (Carballo *et al*., 2000); it also regulates their mRNA stability in normal cells (Lai *et al*., 2000).

Diversity of the zinc finger containing proteins makes them interesting. Despite their diversity, research has shown that they can be divided into subclasses based on their mode of function. Their specificity renders them effective tools that may be important in future applications. Recently, several artificial zinc finger motifs have been synthesised and were observed to mimic

activities of their natural counterparts (Hurt *et al.*, 2003; Libri *et al.*, 2004; Wolfe *et al.*, 2003; Nguyen-Hackeley *et al.*, 2004). This opens a new field of research where DNA binding and protein binding zinc fingers might be used in targeted drug delivery and gene therapy.

1.2.3 PACT interacts with p53

The region downstream of the DWNN domain is highly homologous to PACT (p53 associated cellular protein, testis derived). PACT is a murine polypeptide, which was initially identified as a p53 associated protein that has the ability to bind wild type p53 and Retinoblastoma (Rb) and interferes with their DNA binding sides (Simons *et al*., 1997). PACT has an N-terminal RING finger and highly basic C-terminus. The protein has an alternatively spliced region adjacent to the serine rich (SR) region. The alternatively spliced variant of PACT was later reported to be proliferation potential protein-related (P2P-R) (Scott *et al*., 2003).

P53 is a 393 amino acid nuclear phosphoprotein, sequence specific DNA-binding transcription factor which acts as a tetramer (Lane and Hall, 1997). It is a transcription factor with the primary structure divided into four distinct domains; Transactivation (N-terminal), DNA binding (Core), the oligomerization and regulation domains (C-terminal) (Hupp *et al*., 2000; Prives, 1994). It is a tumor suppressor protein, which regulates transcription of genes required for cell-cycle arrest and apoptosis (Cadwell and Zambetti, 2001; Jin *et al*., 2000). The protein also plays an important role in protecting the integrity of the genome following

DNA damage and other physiological stress (Prives, 1994). The C-terminal regulatory domain of p53 contains the phosphorylation and acetylation sites, which modulates protein-protein interactions with SUMO-1 (Hupp *et al*., 2000), whereas the N-terminal transactivation domain interacts with Murine double minute-2 (MDM2) and p300 (Hupp *et al*., 2000). The tumor suppression ability of p53 is also cell type dependent, as it is known to induce senescence in G1 and cell cycle arrest in G2 (Gregorc *et al*., 2003; Lohrum and Vousden, 2000; Moll *et al*., 2001). P53 prevents cell progression in late G1 phase by modulating the function of p21/WAF-1, which inhibits G1 cyclin dependent kinases thus preventing the cell from progressing to the next stage of cell division (Kim *et al*., 2002). Various forms of cellular stress such as ionization radiation and DNA damage leads to activation and stabilization of the protein (Peters *et al*., 2002). DNA damaging agents induce p53 stabilization, by phosphorylation of certain serine residues within the N-terminal and C-terminal domains (Michael and Oren, 2003). Phosphorylation is carried out by a specific set of enzymes called Phosphatidylinositol-3 (PI-3) kinases (Latonen *et al*., 2002). The protein has been implicated to play a role in tumourigenesis due to its high levels of expression in tumor cells, where the DNA binding domain region of this protein is predominantly mutated (Halazonetis *et al*., 1993; Slingerland *et al*., 1993). P53 is maintained at low levels in normal cells, by rapid protein turnover (Rodriguez *et al*., 1999). Regulation of this is a highly complex process, which involves p53 being the inducer of its master regulator MDM2. MDM2, an ubiquitin ligase, tags p53 which leads to its degradation (Fuchs *et al*., 1998). Wild type p53 in some tumors is responsible for activation and overexpression of MDM2 (Perry *et al*., 2000). The oncogenic potential of the MDM2 gene product is attributed to

the fact that it binds to the transactivation domain and thus inhibits p53-mediated transactivation of antiproliferative target genes (Bottger *et al*., 1999). Genome wide searches revealed that *Drosophila melanogaster* lacks MDM2 homologue. Expression of MDM2 did induce apoptosis in flies (Sutcliffe *et al*., 2003; Jin *et al*., 2000) and SNAMA may be a candidate protein which might fulfill the role of MDM2 in flies.

Drosophila melanogaster p53

Dmp53 was discovered through homology searches using the human homologue. Although the overall homology with the human p53 is low, there was significant similarity in the transactivation and DNA binding domains located in the N-terminal region and the C-terminal located oligomerization domain (figure 8) (Ollmann, 2000). Bourdon et al (2007) have recently shown that the human and the Drosophila p53 have several isoforms and they have different expression patterns. Unlike the human p53 Dmp53 lacks MDM2 binding sites thus implying evolutionary divergence (Sutcliffe *et al*., 2003).

Experimental evidence showed that overexpression of Dmp53 results in apoptosis but not cell cycle arrest. Therefore, the evolutionary role of p53 appears to be cell death induction, whereas in postmitotic retinal cells the protein protected cells against UV irradiation (Jassim *et al*., 2003). It only induces apoptosis in proliferating cells as is the case in retinal cells, whereas in wing disk cells it plays a prominent role in promoting apoptosis (Lee *et al*., 2003).

Loss of function mutants flies also revealed that Dmp53 interacts with the RHG (*Reaper, Head-involution defective[hid], Grim and Sickle*) locus proteins and induces apoptosis. Known *Drosophila* apoptosis inducers reaper and sickle are downstream effectors of Dmp53-mediated apoptosis in flies. Death inducing signals like ionising radiation and DNA damage tends to mediate reaper dependent cell death. Reaper has a Dmp53-binding region within the ionisation radiation-inducing domain (Steller, 2000). Dmp53 appears to preserve genomic stability by regulating cell death in developing cells (Sogamme *et al*., 2003), whereas it also protects postmitotic cells against hid mediated apoptosis (Jassim, 2003). The regulation mechanism of Dmp53 is unknown, but Jin *et al*. (2000) suggested that the protein might be regulated by a mechanism used by PEST containing proteins. Expression of this protein in normal cells is very low like in humans, thus implying that it has a strong regulatory mechanism.

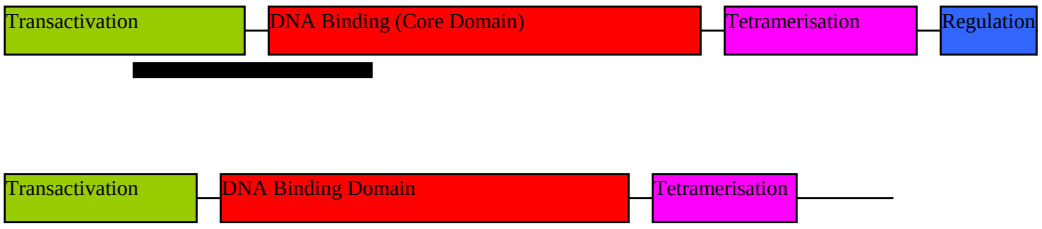


Figure 8: **Domains arrangement of human p53 [hp53] (upper panel) and *Drosophila melanogaster* p53 [Dmp53] (lower panel).** The N-terminal region of hp53 contains binding sites for MDM2 (black box). Green box is the transactivation, red box the DNA binding and the blue box the regulation domain. Adapted from Hupp *et al*. (2000) and Jin *et al*. (2000).

1.3 SNAMA family members

Blast searches with SNAMA revealed that the protein is conserved and found in most eukaryotes. The protein sequences were aligned and used to construct the pylogenetic tree (figure 9). Even though most of the proteins in the phylogenetic tree are uncharacterised, the score on the tree suggests that the proteins evolved from a common ancestor. Database searches also revealed that SNAMA is similar to previously characterised proteins RBBP6 (Sakai *et al*., 1995), PACT (Simons *et al*., 1997), P2P-R (Witte and Scott, 1997) and Mpe1 (Vo *et al*., 2001). P2P-R is a nuclear protein, which is highly expressed in proliferating cells (Gao *et al*., 2002). The protein also has the ability to restrict mitotic progression at prometaphase and induce mitotic apoptosis when overexpressed (Gao and Scott, 2002). Mpe1 was reported to play a role in RNA processing. This function was also observed with P2P-R.

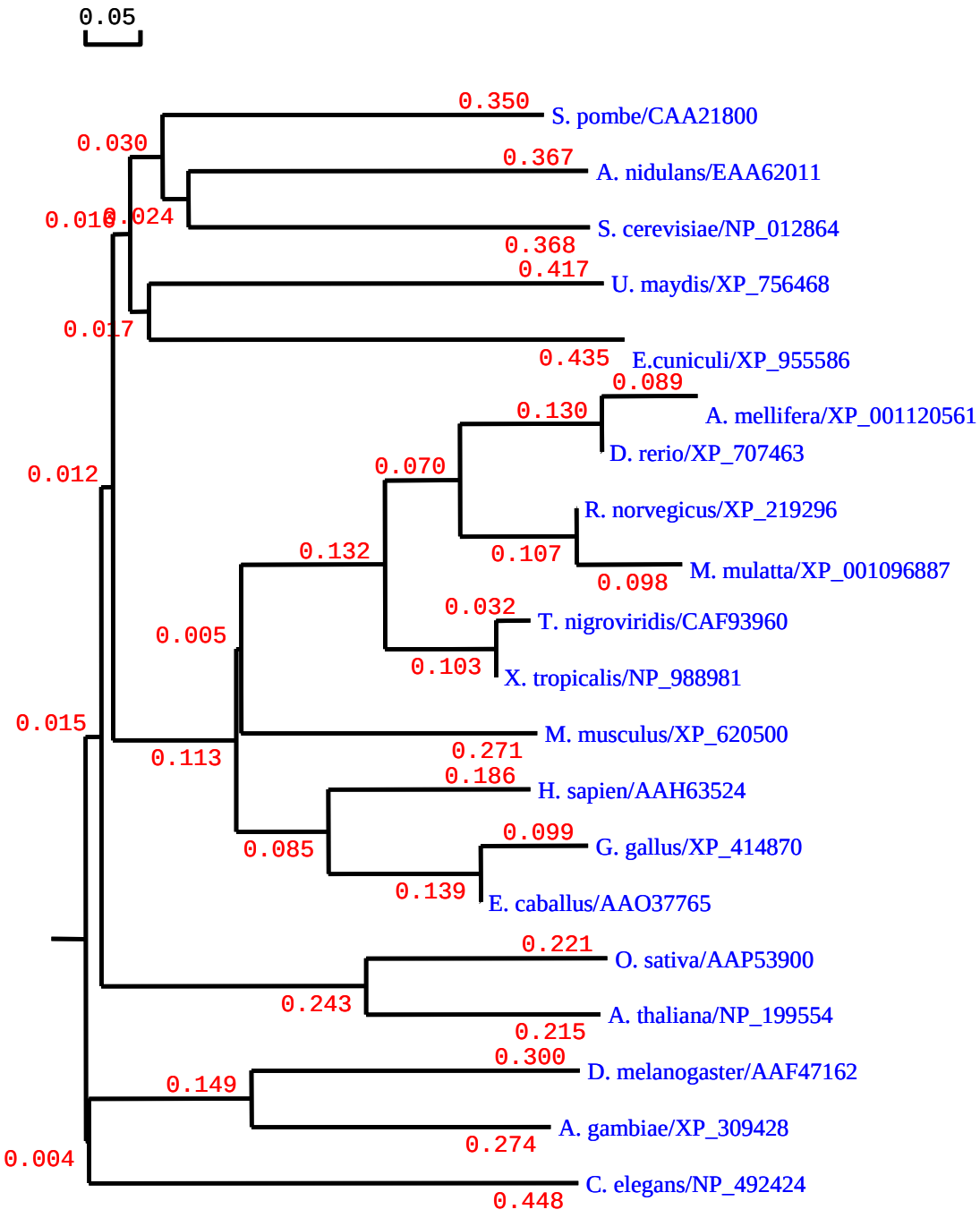


Figure 9: **Phylogenetic analysis of SNAMA and related proteins.** The tree was generated using DNAMAN (Lynnon Biosoft) multiple sequence alignment program. The whole protein sequences were used in the alignment. The numbers on the tree signify branch distances between different organisms.

1.4 Apoptosis

Apoptosis or programmed cell death is a major form of cell suicide, by which animals eliminate unwanted, damaged or harmful cells (Garcia-Domingo *et al*., 1999; Song *et al*., 2000). The process is highly conserved and is triggered in a well defined pattern of cellular and biochemical events independent of the origin of the death stimulus (Claveria *et al*., 2002). It commonly occurs during animal development and metamorphosis (Colussi *et al*., 2000; Dorstyn *et al*., 2002). It is mostly a gene-directed process, orchestrated by a handful of genes in *C. elegans* and *D. melanogaster* (Green, 2000; Melino, 2001).

Apoptosis is characterised by a series of biochemical events, which are divided into three functional components: caspases, caspase activators and a family of apoptotic regulators and inhibitors (Fraser *et al*., 1999; White *et al*., 1994). Several inputs involving signal transduction pathways are integrated into the apoptotic pathway, which controls the response of cells to external stimuli (Widmann *et al*., 1998). The process is defined by morphological characteristics such as cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation (Yue *et al*., 1999). In metazoans it usually occurs during development, immune selection, maintenance of cellular integrity and tissue homeostasis (Sah *et al*., 1999). Apoptosis can be triggered by many different stimuli, like death factors, steroid hormones, DNA damage, cytotoxic cells, removal of extracellular survival signals, anticancer drugs and viral infection (Song *et al*., 2000; Yokoyama *et al*., 2000). Although most proapoptotic signals are internal, some external factors, such as ultraviolet (UV) irradiation, may also

trigger this process. Regardless of the stimuli, most of the downstream cell elimination is carried out by the activation of conserved sequence specific proteases known as caspases (cysteine aspartases).

1.5 Apoptosis in *Drosophila melanogaster*

The apoptotic machinery is highly conserved in metazoans, whereby components that mediate apoptosis in higher eukaryotes, have homologoues in simple eukaryotes like *Drosophila melanogaster* and *Caenorhbditis elegans*. The death receptor mediated pathway in flies consists of transmembrane proteins similar to the Fas/CD95 Tumor Necrosis Receptor Family. For instance Eiger (*Drosophila* tumor necrosis factor) is a type II transmembrane protein (Igaki, 2002), whereas Wegen (member of the *Drosophila* tumor necrosis factor receptor (TNFR) superfamily) is a type III transmembrane protein with a TNFR homology domain (Kanda, 2002). Transmembrane proteins like Fas/CD95 and Tumor necrosis factor receptor family are some of the best-characterized pathways involved in the initiation of apoptosis (Yue *et al*., 1999). Fas is a type I membrane receptor, whereas its ligand is synthesized as a type II membrane protein. They are members of the TNF receptor and TNF family of proteins respectively and they have their conserved C-terminal region in the extracellular region and the variable N-terminal in the cytoplasmic region (Nagata, 1997). The intracellular regions of these death receptors are composed of components like death effector domain (DED) or death domain (DD). The receptors interact via the DD and DED to bind and cleave initiator caspases (caspase 8 and 10). The activated initiator caspases then cleave and activate downstream effector caspases; caspase

3, 6 or 7, which in turn cleave and activate endonucleases such as DNA fragmentation factor (DFF) and cell death-inducing DFF45-like effector (CIDE). This mechanism of cell death is widely known as an extrinsic pathway. This mechanism of cell death begins in the environment outside the cell, the stimuli triggers the cascade of events that leads to cell death, whereas the intrinsic pathway is triggered by an internal stimuli. Through sequence homology searches proapoptotic genes in the nematode *Caenorhbditis elegans* were observed to be highly conserved in which some genes (*ced*-9 and *ced-3*) were found to have a high sequence homology with the mammalian (*bcl-2*) family of genes (Fraser and Evan, 1997).

In *Drosophila* much of the programmed cell death occurs during development mediated mostly by activation of the killer genes. Studies have uncovered these cell death genes in the fruit fly *Drosophila melanogaster* (Garcia-Domingo *et al*., 1999). The four closely linked genes *Reaper, Hid, Grim* and *Sickle* are located at region H99 of the third chromosome (Haining *et al*., 1999; Igaki *et al*., 2000; Claveria *et al*., 2002). They act as death switches regulated at the level of transcription. Ectopic expression of these genes leads to apoptosis in viable cells and their inactivation prevents apoptosis in the cells that were fated to die (Bergmann *et al*., 1998). The N-terminal region of these proteins is similar to a proapoptotic mitochondrial protein homologous to the proapototic protein second mitochondria-derived activator of caspases/Direct IAP Binding protein with Low pI (Smac/DIABLO) (Verhagen and Vaux, 2002). Smac/DIABLO is released during stress related apoptosis and inactive inhibitor of apoptosis (IAP) (Hu and Yang, 2003).

Even though these genes are closely linked in the chromosome, their regulation and function appear to be different. Reaper and Grim are only expressed in the cells that are going to die, whereas Hid has been detected even in the cells that were going to live (Bergmann *et al*., 1998). Claveria *et al*. (1998) also reported that Grim and Reaper triggered the apoptosis programme via the *Drosophila* caspase 1 (DCP1) and Hid activated apoptosis via a caspase that is still to be identified, thus suggesting that Hid might be playing a distinct role in developmentally regulated apoptosis (Kurada and White, 1998). Hid, grim and reaper were found to have different expression patterns, their N-terminal sequences bind to inhibitors of apoptosis and are conserved (Claveria *et al*., 2002). Although apoptosis is a highly regulated and specific programme, some defects in the machinery are linked to a number of pathological conditions. Various disorders such as cancer and autoimmune diseases have been attributed to the failure of programmed cell death (Seshagiri *et al*., 1999), whereas, diseases like AIDS, neurodegenerative disorders and cell loss are associated with increased apoptosis (Gil-Gomez *et al*., 1998).

As many proapoptotic enzymes like caspases are almost always present in the cell, the cell must have the means of preventing the triggering of that pathway (Jones *et al*., 2000). As mentioned earlier, the apoptotic machinery consists of the proapoptotic and the anti-apoptotic elements, which the latter elements are also important in the cell cycle. The anti-apoptotic activity is provided by a battery of cell death inhibitors, which are present in various steps of the apoptotic machinery. The inhibitors of apoptosis (IAP) were first discovered in the

baculoviruses, which are arthropod-specific double stranded DNA viruses (Seshagiri *et al*., 1999). These IAP antagonized the host defense mechanisms by attenuating the apoptotic pathway and thus assisting in the propagation and the spreading of the pathogen (Liston *et al*., 1996).

In *Drosophila* IAP orthologs namely DIAP1 and DIAP2, have similar structural domains like RING finger motifs and baculovirus inhibitor of apoptosis type repeats BIR (Salvesen and Duckett, 2002). Jones *et al*. (2000) reported a new type of IAP in the fly, namely deterin, which has some deviations from traditional structures of DIAP. The protein has only one BIR and no ring finger motifs.

1.6 Aim of this study

The aim of this study was to characterise SNAMA by identifying the proteins which interact with it using the yeast two hybrid system and immunoprecipitations assays and also by determining its role in cell cycle regulation. The primary aim of this project was to identify genes coding for proteins, which interact with SNAMA. The yeast two-hybrid system was chosen, as it would enable the isolation of the genes coding for the interacting proteins. The coding region of the DWNN domain of *SNAMA* was inserted into phyblex/zeo plasmid and the protein produced was used as bait to trap any protein that might interact with the domain in yeast two-hybrid assays. The prey vector used was pJG4-5, and contained the *Drosophila* 0-12 hours embryo cDNA library. The yeast two hybrid system is an in-vivo expression system which can mimic interaction likely to happen in the organism from which the genes coding for the proteins being investigated were cloned from. Since several proteins require posttranslational modification the some interactions might not be detected using the yeast two hybrid system. The immunoprecipitations were carried out to probe for interactions likely to occur in organism of interest. The region encoding DWNN was also inserted into a prokaryote expression vector pGEX6p. Antibodies against the fusion protein were immobilized on a protein A matrix and were used to immunoprecipitate the fusion protein. The immunoprecipitated fusion protein was the further exposed to the crude extract from adult flies to probe for any proteins which interact with DWNN. Since SNAMA was identified in the screen for gene involved in cell cycle regulation the secondary aim was to determine the exact role of the protein in that pathway.

SECTION 2

2. MATERIALS AND METHOD

2.1 Yeast strains

Strain	Genotype	Phenotype
Saccharomyces cerevisiae L40 (Invitrogen)	*MATα his3Δ200 trp1-901 leu2-3112 ade2 LYS2:: (4lexAop-His3) URA3::(8lexAop-lacZ) GAL4*	His-, Trp-, Leu-, Ade-
S. cerevisiae EGY48 (Gyuris *et al*., 1993)	*MATα ura3 trp1 his3 6lexAop-LEU2*	Ura-, Trp-, His-, Leu-
S. cerevisiae Rfy206 (Finley and Brent, 1994)	*MATa trp1∗::hisG his3∗200 ura3-52 lys2∗201 leu2-3*	Ura-, Trp-, His-, Leu-

2.2 Bacterial strains

Strain	Genotype
Escherichia coli XL-1 blue (stratagene)	*recA*1 *end A*1 *gyrA*96 thi-1 *hsd*R17 *supE*44 *relA*1 *lac* [F'*proAB lacI*^{q}ZΔM15 *Tn 10 (Tet*^{r}*)]*
E. coli BL 21 (stratagene)	*F-ompT [lon] hsdSB(rB-mB-BL 21(DE3)* is an *E. coli* B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene

2.3 Cell Cultures

Strain	Source
Drosophila melanogaster Schneider 2 cells	Invitrogen

2.4 Plasmids

Yeast plasmids	Genotype	Purpose
pHyblex/Zeo (Invitrogen)	*Zeo^R, 2 μ ori, ADH prom. and ter. LexA ORF, TEF1 and EM-7 prom. ColE1 ori, CYC1 ter*.	Vector "for bait" protein
pJG 4-5 (Gyuris *et. al*, 1993)	*TRP1, 2μ ori, Amp^R, GAL 1* prom. *B42-HA tag, NLS, ADH1* ter.	Vector for prey protein)
pYESTrp2 (Invitrogen)	*TRP1*, 2μ ori, *Amp^R, GAL 1* prom. *B42-V5 epitope tag, NLS, ADH1* ter. *f1 ori, ColE1 ori, CYC1* ter.	Vector for prey protein
pSH18-34 (Gyuris *et. al*, 1993)	*URA3*, *2μ ori, Amp^R, 8 lexA* operators l*acZ ORF, ColE1* (pBR322 derived)	Reporter

Bacterial plasmids	Genotype	Purpose
pGEM-T Easy (Promega)	*Amp*^{R}	Cloning vector, for PCR products.
pGEX-6P-2 (Pharmacia Biotech)	*Amp*^{R}	Overexpression of recombinant proteins

2.5 Bacterial and yeast growth conditions

2.5.1 Bacteria

Escherichia coli XL-1 blue and *E. coli* BL 21(DE3) were used throughout the project. *E. coli* XL-1 blue cells were used as host cells and for subsequent amplification of recombinant plasmid DNA molecules, whereas *E. coli* BL 21(DE3) cells were used for heterologous expression of recombinant protein.

2.5.2 Selection of transformed cells and positive clones

E. coli XL-1 blue bacterial cells that were transformed with recombinant pGEM-T Easy plasmid were selected on LB agar plates supplemented with 100 μg/ml ampicillin, 12 μg/ml tetracycline, 25 μg/ml X-gal and 100 mM IPTG. Cells containing recombinant clones were white in colour, due to the disruption of the β-galctosidase coding region. The β-galactosidase gene produces an enzyme that catalyzes x-gal and cells carrying the non-disrupted gene turn blue when grown on a plate containing x-gal. In instances where other plasmids (pGEX 6P, pHyb lex/zeo and pJG4-5) were used in transformations, cells were selected on

plate containing ampicillin (*E. coli* BL 21(DE3)) or together with tetracycline (*E. coli* XL-1 Blue), whereas cells transformed with pHyblex/zeo were selected on media containing 100 μg/ml zeocin.

2.6 Yeast

Untransformed yeast cells were maintained and grown on Yeast peptone dextrose (YPD) growth media (Appendix 1). Transformed cells were grown under selective pressure on an appropriate yeast nitrogen base (YNB) dropout media (Appendix 1).

2.6.1 Selection of transformed cells and screening for interactors

The 'bait' constructs was transformed into an *Saccharomyces cerevisiae* EGY 48 (Matά *ura 3 his3 leu2::3lexop-LEU2 trp lys2*) yeast strain using the Lithium acetate yeast transformation method (Golemis, 1995). This yeast strain contains the pSH18-38 ß-gal reporter plasmid and an internal leucine reporter gene. The transformed organisms were then plated on (YNB) minimal plates lacking uracil supplemented with zeocin and glucose (YNBglu/zeo/ura) to select for pSH18-34 and phyblex/zeo.

2.6.2 Interaction mating

The "prey" plasmid containing the *Drosophila* library was transformed into the *S. cerevisiae* RFY 206 (Mata *ura3-52 his3 AE200 leu2-3 lys2 AE201*

trp1::HisG) mating yeast strain using the same method stated above. Transformants were selected by plating the organisms on YPD/glu/ trp- plates.

2.7 Polymerase Chain Reaction (PCR)

PCR was performed in the Perkin Elmer Geneamp 2400 thermal cycler. The reaction consisted of 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl; pH 8.3), 1.5 mM MgCl, 3 μg template DNA, 200 μM dNTPs, 0.2 μM of each primer, and 5 units *Taq* DNA polymerase. The final volume was made up to 50 μl with sterile distilled water. The run was through 40 cycles: 94°C for 30 seconds (denaturation), 55°C for 1 minute (annealing) and 72°C for 45 sec (extension). The amplification cycles were preceded by 95°C pre PCR denaturation for 5 minutes and followed by 10 minutes final extension step at 72°C. Following the reaction, the PCR product was then qualitatively analysed on a 1% agarose gel.

2.8 Extraction of DNA from agarose gels

The gel containing PCR products was subjected to long wavelength ultraviolet light to visualize DNA. The DNA band of interest was excised from the gel and transferred into a clean eppendorf tube. Extraction was performed using the concert matrix gel extraction system from Gibco Life Technologies (11457-017). The system uses silica resin to capture and purify DNA molecules.

2.9 Restriction endonuclease digestion of plasmid DNA

Restriction analysis reaction was done according to the manufactures instructions. The corresponding 10x buffer constituted 10% of the total reaction with one unit of enzyme for each μg of DNA. The reaction was carried out at 37°C for 16 hours, whereupon the restricted DNA was analysed on agarose gel. *Eco*RI and *Xho*I were used throughout this project and the restrictions were carried out in buffer H (50 mM Tris-HCL, 10 mM $MgCl_2$, 100 mM NaCl, 1mM 1-4 Dithioerythritol [DTE]) from Roche.

2.10 Removal of 5' end phosphate overhangs

Calf intestinal phosphatase was used to treat linear plasmid molecules at a ratio of 0.5 units per μg DNA in a suitable buffer. The reaction was incubated at 37°C for 30 minutes. Heating the tube at 65°C for 15 minutes followed by phenol chloroform isoamyl alcohol (25:24:1) extraction stopped the reaction as outlined in section 2.11.

2.11 Recovery of DNA from liquid mixture

The volume of the reaction was adjusted to starting volume of 300 μl. The sample was extracted twice, first with phenol:chloroform:isoamyl (25:24:1) followed by chloroform. The pure aqueous layer was transferred into a fresh eppendorf tube, followed by DNA precipitation with ethanol acetate for 30 minutes at –70°C. The DNA was recovered by centrifugation at 12, 000 xg for 10 minutes, washed with 70% ethanol and air dried at room temperature for 10 minutes. DNA was reconstituted with appropriate volume of sterile distilled

water.

2.12 Ligation of DNA molecules

Ligation into pGEM-T easy vector (Promega) was done according to the manufacturers instructions (A1360). The ligation cocktail consisted of 200 ng of the PCR amplified DNA, 50 ng plasmid and 2.5 units of T4 DNA ligase. Ligations into pHyb/lex zeo and pGEX-6P-2 were conducted in such a way that the ratio of vector to insert was 2:1 where dephosphorylated vector DNA was used. The T4 DNA ligase was at ratio of 1unit/μg of DNA. The reactions were incubated overnight at 4°C.

2.13 Preparation of competent cells and transformation

2.13.1 Bacterial competent cells

A single colony of the desired bacterial strain was used to inoculate into 5ml of LB broth (Appendix 1), and the culture was incubated for 16 hours on a shaker platform at 37°C with shaking at 150 xg rpm. The culture was then used to sub-inoculate 100 ml of fresh LB broth medium and incubated further until the cells were at OD_{550} of between 0.4 and 0.6. The culture was cooled on ice, and was then centrifuged at 1100 xg for 10 minutes at 4°C. The supernatant was removed and the cells resuspended on ice with 35 ml of ice cold transformation buffer (TFB) (Appendix 1). The cell suspension was incubated on ice for 15 minutes after which the cells were harvested by centrifugation (4428 xg) at 4°C. The

pellet was gently resuspended in 2 ml of cold TFB. To the cell suspension 150 µl of 1M dimethyl formamide (DMF) was added followed by 5 minutes incubation after which 150 µl of 1M Dithiothritol (DTT) was added and the suspension was incubated for further 10 minutes on ice. After incubation 70 µl of 1 M DMF was added and the cells were kept on ice for 30 minutes followed by centrifugation (13,250 xg) at 4°C. The pellet was resuspended in 0.1 M Calcium chloride/20% glycerol solution and then snap frozen in 0,2ml aliquots using liquid nitrogen. The aliquots were stored at –70°C.

2.13.2 Yeast competent cells

Yeast cells were made competent using modified version of the lithium acetate method by Ito *et al*., (1983). A single colony of the desired yeast strain was grown in 10 ml YPD broth overnight at 30°C. The overnight culture was used to sub-inoculate 50 ml YPD and grown further for 4 hours after which cells were harvested by centrifugation at 1100 xg. The pellet was resuspended in 40 ml 1x tris-EDTA (TE) buffer, spun again followed by resuspension in 2 ml Lithium acetate-tris-EDTA buffer and the cell suspension was incubated at room temperature for 10 minutes.

2.14 Transformations

2.14.1 Transformation of bacterial cells

Transformation was done according to Lederberg and Cohen (1974), where

200 ml of competent cells was thawed on ice and 20 µl of ligation reaction added followed by incubation on ice for 15 minutes. The cells were then heat shocked for 2 minutes at 42°C, after which 900 µl of cold LB was added and the mixture was incubated at 37°C for 60 minutes. The transformed cells were then plated on LB agar plates containing ampicillin.

2.14.2 Transformation of yeast cells

For each transformation 100 µl yeast suspension aliquot was mixed with 1 µg plasmid DNA and 100 µg salmon sperm DNA followed by addition of 700 µl Lithium acetate polyethyleneglycol 330 buffer. The cells were then incubated at 30°C for 30 minutes followed by addition of 88 µl dimethylsulfoxide, whereupon they were heat shocked at 42°C for 15 minutes. The cells were centrifuged briefly and the pellet was resuspended in 1x TE and plated on appropriate YNB selective plates. Plates were incubated at 30°C for 3 days.

The method was scaled up to 1 liter for transformation with library plasmid and 20 ml aliquots of yeast suspension were mixed with 1 ml (10 mg/ml) of denatured salmon sperm DNA and 500 µg library DNA.

2.15 Isolation of plasmid DNA from *E. coli*

Plasmid DNA isolation was done according to Birnboim (1983), based on alkaline lysis method.

2.15.1 Small-scale plasmid preparation

A colony of transformed *E. coli* was used to inoculate 2 ml of LB containing 100 μg/ml of ampicillin, and was grown overnight with shaking at 37°C. A 1.5 ml aliquot of this culture was centrifuged at high speed for 1 minute in a micro-centifuge and the supernatant was carefully removed. The cells were resuspended by vortexing in 100 μl of cold solution 1 (Appendix 1), followed by 200 μl of solution 2 (Appendix 1). The cells were mixed gently by inversion, incubated for 3 minutes at room temperature followed by addition of 150 μl of solution 3 (Appendix 1). The suspension was then mixed gently by inversion and left on ice for 20 minutes. Cell debris was removed by centrifugation at 13250 xg for 10 minutes and the plasmid containing supernatant was transferred to a fresh eppendorf tube. The plasmid was precipitated with 2 volumes of 95% ethanol for 20 minutes at –20°C prior to centrifugation for 15 minutes. The resulting pellet was washed once with 70% ethanol. The plasmid DNA was recovered by centrifugation for 10 minutes and the DNA was reconstituted with 200 μl of sterile distilled water.

2.15.2 Large-scale plasmid preparation

Plasmid DNA was isolated using Qiagen maxiprep kit (12163). A 10 ml overnight culture of transformed *E. coli* was used to inoculate 500 ml of LB broth containing 100 μg/ml ampicillin and the mixture was shaken overnight at 37°C.

The cells were harvested at 5000 rpm for 10 minutes at 4°C in a Beckman JA 10 rotor. Further on cells were treated according to the manufactures instructions. Isolated DNA was resuspended in 1 ml of sterile distilled water.

2.16 Protein extraction and purification

2.16.1 Extraction of soluble protein

A single colony of *E. coli* BL 21 cells transformed with pGEX-6P-2 recombinant plasmid was inoculated into 10 ml LB broth containing ampicillin and grown overnight with vigorous shaking at 37°C. 5 ml of the overnight culture was used to inoculate 200 ml of fresh LB ampicillin broth and the culture was incubated further until the cells were at OD_{600} of 0.6. The culture was induced with 0.5 mM IPTG for 4 hours. After induction cells were harvested in pre-weighed centrifuge tubes at 5000 rpm for 10 minutes at 4°C in a Beckman JA 10 rotor. The pellet was resuspended with Bugbuster reagent from Novagen (Cat no:70584) at a ratio of 5 ml per gram of wet cell paste. Sodium benzonase (25 units/ml of bugbuster) and 4 mM Pefabloc were added to the cell suspension followed by incubation on a shaker platform at room temperature for 15 minutes. The insoluble cell debris was removed by centrifugation at 12, 000 xg for 15 minutes and the supernatant was stored for further purification and SDS PAGE analysis.

2.16.2 Extraction from embryos

Wild type and l(3)rQ13 mutant fly embryos were collected at different stages of development. Embryos were then washed with distilled water and

homogenized by pestle in cell lysis buffer (Appendix1). The homogenate was centrifuged for 10 minutes at 4°C and the supernatant was transferred into a fresh eppendorf tube and stored at -70 ^{o}C.

2.16.3 Extraction from adult flies

Adult flies were collected into 1.5 ml eppendorf tubes and homogenized with pestle in cell lysis buffer (Appendix1). The supenatant was collected after centrifugation at 12, 000 xg for 5 minutes and stored at -70 ^{o}C.

2.17 Polyacylamide gel electrophoresis

Denaturing SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) under reducing conditions was used to separate the proteins. Gels were prepared from 30% pre-made stock of 29:1 acrylamide: bisacrylamide (Sigma). The resolving gel consists of 7% acrylamide mix, 0.375 M Tris HCl pH8.8, 0.1% SDS, 0.1% ammonium persulphate and 0.1% Tetramethylethylenediamine (TEMED). The stacking gel added on top of the resolving gel, consisted of 4% acrylamide mix, 0.125 M Tris HCl pH6.8, 0.1% SDS, 0.1% ammonium persulphate and 0.1% TEMED. Samples were prepared for electrophoresis by mixing protein with 2 x SDS PAGE loading buffer (Appendix 1) and then boiling for 5 minutes. The samples loaded onto the gel were electrophoresed in SDS PAGE running buffer (Appendix 1) at 120 V for 2 hours.

2.18 Staining SDS PAGE gels

Immediately after electrophoresis, the gel was immersed in 5 volumes of the Coomassie blue staining solution (Appendix 1) for 3 hours at room temperature. The gels were then destained until the bands were visible or overnight with gentle agitation.

For sensitive applications silver stain plus kit from Bio-Rad (cat no: 161-0449) and Gelcode blue stain reagent from Pierce biotechnology (cat no: 24592) were used. These reagents were more sensitive and less time consuming as staining was generally completed in less than 2 hours.

2.19 Immunoprecipitations

Protein was extracted from adult flies and embryos in RIPA buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5mM PMSF, 1μg/ml aprotinin, 4.0mM Pefabloc). The lysate was then incubated with 1.0 μg of antibody (anti-*Drosophila* p53, anti-Human DWNN and anti-SNAMA) overnight at 4°C. Following incubation 20 μl of Protein A agarose from Santa Cruz (sc-2001) was added to the crude protein antibody mixture followed by incubation on a rotating device for 2 hours at 4°C. The protein matrix complex was then washed twice with 1.0 ml RIPA buffer. After final wash the immunoprecipitated proteins were recovered by boiling in SDS sample buffer and analyzed on an SDS PAGE.

2.20 Western blotting

Denaturing SDS-polyacrylamide gel electrophoresis under reducing conditions was used to separate proteins. Following electrophoresis, the gel was equilibrated in an electroblotting buffer (Appendix 1) for 5 minutes. The gel was then positioned on top of the Whatman filter paper. Wet Hybond PVDF membrane soaked in electroblotting buffer was then placed on top of the gel, taking care to remove air bubbles and another Whatmann paper placed on top of the membrane and the sandwich was placed inside a blotting cassette. Proteins were electroblotted onto the membrane at 300 mA for 3 hours. After blotting, the PVDF membrane was blocked for 1 hour at room temperature in superblock solution, followed by incubation for 1 hour at room temperature or overnight at 4°C with a 1:5000 dilution of rabbit anti SNAMA, anti Human DWNN (provided by Prof J. Rees) or goat anti *Drosophila* p53 (Santa Cruz: sc-17577) in 1x phosphate buffered saline (PBS). The membrane was washed twice with PBS, 0.1% Tween 20 and then incubated with 1:10 000, dilution of secondary antibody Immunoglobulin G (sc-2768) conjugated to horseradish peroxidase for 1 hour at room temperature. The membrane was finally washed 6 x 10 minutes with PBS, 0.1% Tween 20 and detection of the secondary antibody performed using enhanced chemiluminescence system from Pierce (34080) according to the manufactures instructions. The membrane was then exposed to X-ray film.

SECTION 3

3. RESULTS

Macromolecular interactions occur in all living organisms thus enabling biological systems to carry out essential functions such as cell differentiation, metabolism and maintenance of cellular homeostasis. These interactions involve essential components such as RNA, DNA and proteins. Execution of processes such as apoptosis relies heavily on these interactions. The main focus of this study was to characterise SNAMA, by isolating proteins which interact with it. The SNAMA homologue was isolated in the process of identifying proteins which play a role in apoptosis. The protein was initially referred to as DWNN (Rees *et al*., personal communication). Bioinformatics analysis revealed that the human homologue of SNAMA is RBBP6. SNAMA was identified through homology searches using the RBBP6 (Pugh *et al*., 2006). The hypothesis of this study was that it plays a role in apoptosis. Since apoptosis involves multiple interactions, my hypothesis thus suggests that SNAMA interacts with other proteins such as transcription factors and stress related proteins. SNAMA orthologues such as P2P-R and PACT were previously shown to interact with cell cycle regulators such as p53. In order to test this hypothesis the yeast two-hybrid assay and immunopecipitations were carried out.

3.1 SNAMA interacts with other proteins

The yeast two hybrid system is a very powerful tool for identifying protein-protein interactions. The system exploits the fact that eukaryotic transcription

factors have multiple domains. A transcription factor can be separated into DNA binding and activation domains, whereby neither of the two domains is capable of activating transcription on its own. The DNA binding (DB) domain is contained in the bait plasmid (phyblex/zeo) whereas the activation domain is in the prey plasmid pJG4-5 (Appendix 3). Interaction of the proteins expressed from both prey and bait plasmids create the complete transcription system. The lexA DB domain in the bait vector and a B42 transcription activation domain in the prey plasmid can only activate reporter genes when brought together by interacting proteins, thus eliminating chances of nonspecific reporter activation. An advantage of this system is immediate isolation of the interacting proteins DNA coding region as compared to the classical biochemical methods, which involve first identification, and later sequencing of the interaction peptides (Van Criekinge and Bayaert, 1999). The *Drosophila* 0-12 hours embryonic cDNA library in pJG4-5 (prey) plasmid was kindly provided by Professor R. Finley (Finley *et al*., 1994).

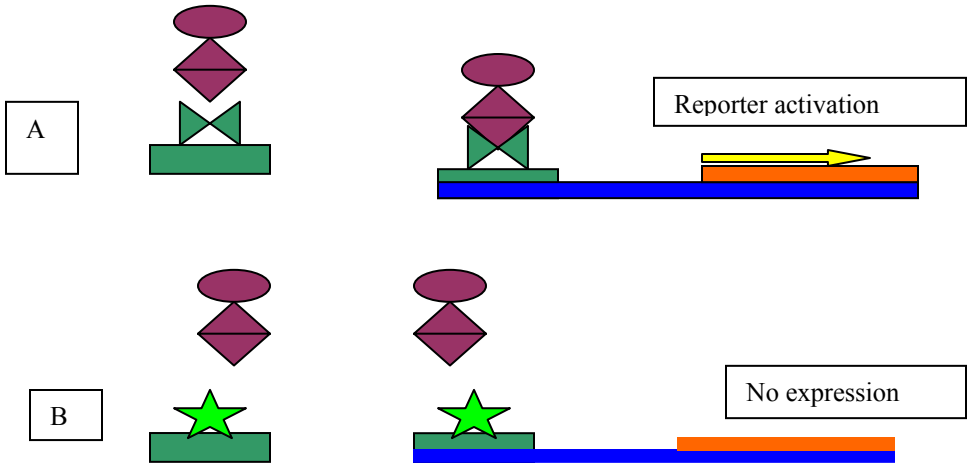


Figure 12: Schematic representation of the yeast two hybrid system. Panel A show interaction between the bait and prey and panel B depicts non interacting proteins. Where  is the bait protein,  stands for the activation domain,  DNA binding domain and  prey protein.

3.1.1 Yeast two hybrid assay

3.1.1a Cloning into pGEM T-easy vector

A *SNAMA* clone in pOT2 was obtained from the Berkely *Drosophila* Genome Project (AF132177) and used as a PCR template for DWNN amplification. The domain specific primers DWNN1a (5'-GAATTCATGTCGGTACACTAT-3') and DWNN2a (5'-CTCGAGGGCGATGGGGATGCG-3') were designed in such a way that they contained *Eco*RI and *Xho*I restriction sites respectively (underlined sequences). Following PCR, the amplified DNA product was analysed qualitatively by electrophoresis on 1% agarose gel and the fragment size of approximately 200 bp corresponding with the expected size of 213 bp was obtained Figure 13.

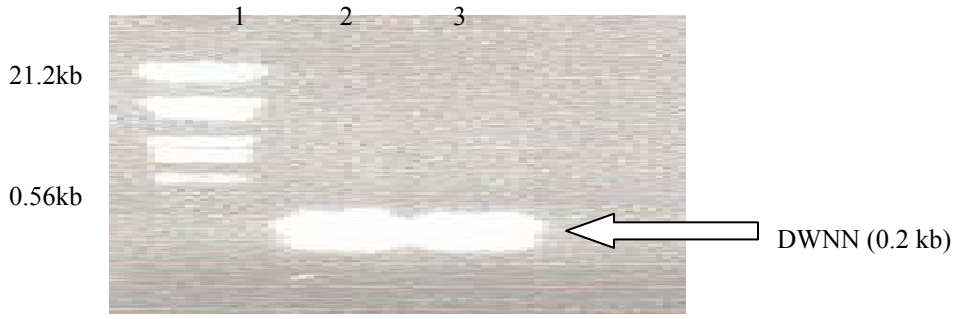


Figure 13: **PCR amplification of DWNN using domain specific primers DWNN1a [forward] and DWNN2a [reverse].** Lane1, λ DNA molecular weight marker III sizes of the bands is in kilo base pairs (kb); lane 2 and 3 PCR amplification products.

The PCR product was then extracted and ligated into the pGEM-T Easy vector followed by transformation of competent *E. coli* XL-1 blue cells. White colonies were screened by colony PCR to verify the presence of DWNN in the recombinant plasmids. Furthermore plasmid DNA was isolated the positive (white) colonies and subjected to restriction endonuclease analysis.

Figure 14 depicts the DNA bands obtained from the screening process. The unrestricted pGEM T-easy recombinant vector in lane 2 and the restricted vector is shown in lane 3 where the DWNN fragment can be seen at the 200 bp region. The colony PCR product in lane 4 was used to further verify that the cloned fragment was indeed DWNN.

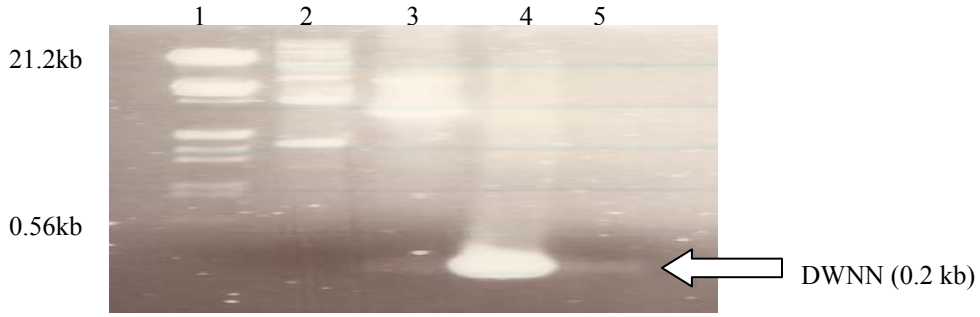


Figure 14: **Screening of pGEM-T easy-DWNN recombinant plasmid using colony PCR and restriction endonucleases.** The fragments were resolved on a 1% agarose gel with ethidium bromide. Lane 1 contains DNA molecular weight marker III; lane 2 unrestricted pGEM T Easy recombinant plasmid; lane 3 recombinant plasmid restricted with *Eco*RI and *Xho*I, lane 4 Colony PCR amplification product; lane 5 Restriction digest product recovered from the gel.

3.1.1b Construction of the bait plasmid

To prepare a bait vector for yeast two hybrid assays, the DWNN fragment was excised from the pGEM-T easy with *Eco*RI and *Xho*I. The resulting fragment was extracted from the gel as outlined in "Materials and Methods". The fragment was sub-cloned into dephoshorylated phyblex/zeo plasmid, which was treated with the same restriction endonuclases used to release the DWNN fragment from the primary host vector. The construct was propagated in *E. coli* XL-1 blue cells and the recombinant plasmid was subjected to restriction

analysis to confirm the presence of the insert (figure15).

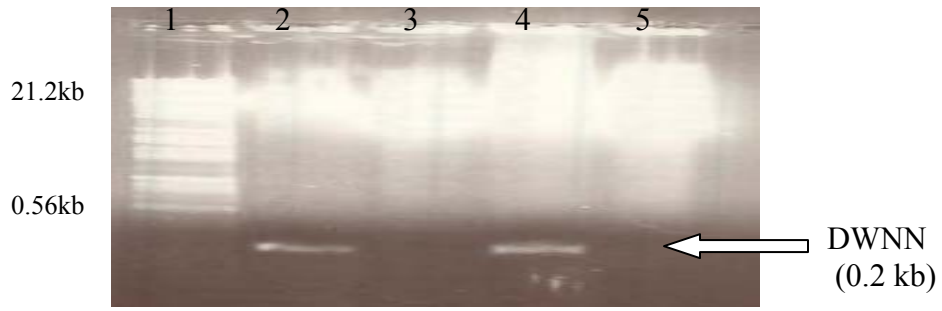


Figure 15: **Screening of phyblex/zeo recombinant plasmids with restriction endonucleases.** Gel showing electrophoresis of pHybLex/Zeo DWNN clones**.** Lane 1, DNA molecular weight marker III; lane 2 and 4 Recombinant plasmids restricted with *Eco*RI and *Xho*I; lane 3 and 5 Unrestricted recombinant plasmids

```
GTCACGTTCGCATCTCTAGTAAATTGGAAAAAAATAAAATCGTCGGAGTTTTTATGTGCT
||       |    | ||     |  || |  |  |                        | | |
GTTTAAACCAATTGTCGTAGATCTTCGTCAGCAGAGCTTCACCATTGAAGGGCTGGCGGT

TGCAGAGTAGTATTTCTTTCATATGCAACT....ATGTCGGTACACTATAAATTTAAGAG
||   |           |     |   ||         ||||||||||||||||||||||||
TGGGGTTATTCGCAACGGCGACTGGCTGGAATTCATGTCGGTACACTATAAATTTAAGAG

TACACTCAACTTTGATACAATTACTTTTGATGGACTTCACATTTCTGTCGGGGACTTAAA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TACACTCAACTTTGATACAATTACTTTTGATGGACTTCACATTTCTGTCGGGGACTTAAA

AAGGGAGATTGTGCAGCAGAAGCGACTGGGCAAAATCATCGACTTTGATCTCCAAATAAC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AAGGGAGATTGTGCAGCAGAAGCGACTGGGCAAAATCATCGACTTTGATCTCCAAATAAC

AAATGCGCAGAGTAAAGAAGAATACAAGGACGATGGGTTCCTTATTCCCAAAAACACAAC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AAATGCGCAGAGTAAAGAAGAATACAAGGACGATGGGTTCCTTATTCCCAAAAACACAAC

GCTGATCATATCGCGCATCCCCATCGCCCATCCCACAAAAAAGGGCTGGGAGCCACCAGC
|||||||||||||||||||||||||||||||       | |       ||
GCTGATCATATCGCGCATCCCCATCGCCCTCGAGTCGACCTGCAGCCA
```

Figure 16: **Optimal alignment of the cloned DWNN gene fragment (lower sequence) with the SNAMA cDNA sequence from NCBI and BDGP (upper sequence) see appendix 2.** Purple coloured nucleotides are restriction sites used for in frame directional ligation of the coding region of the domain domain with lexA DNA binding module. These sequences were aligned using DNAMAN and were found to be 100% complimentary to the original published sequence.

Since PCR introduces mutations, plasmid DNA obtained from the positive clones was sequenced and analysed. The cloned DWNN fragment was identical to the one available on the NCBI and BDGP databases and it contained no mutations (figure16). The results also confirmed that the inserted DWNN-encoding fragment was in frame with the coding region for the lexA DB domain (figure17).



Figure 17: **Sequence analysis of the pHybLex/Zeo DWNN plasmid construct**. The sequence was generated by the automated DNA sequencer ABI prism using pHybLex/Zeo reverse primer 5'-GAG TCA CTT TAA AAT TTG TAT ACA C-3' and the sequence was analysed using DNAMAN. Purple coloured nucleotides are restriction sites used to insert the DWNN encoding region into the vector, green is the stop codon and the underlined sequence is the vector. Blue letters represent the amino acid sequence of DWNN whereas the black letters represent the phyblex/zeo sequence.

3.1.1c Preparation of the bait strain

S. cerevisiae EGY 48 competent yeast cells containing pSH18-34 reporter plasmid were transformed with the pHybLex/Zeo-DWNN construct. The transformants were selected on YPD rich media supplemented with zeocin.

3.1.1d Transformation of bait strain with cDNA library recombinant plasmids

The 0-12 hour *Drosophila* embryonic cDNA library in pJG 4-5 was used to transform competent *S. cerevisiae* EGY 48/pSH18-34/phyblex/zeo-DWNN-yeast strain and plated on YNB/glu/zeo/ura-/trp- selective media. Transformants were then frozen and replated to select for all the plasmids. For interaction mating assays the library plasmid was transformed into *S. cerevisiae* RFY 206 strain and selected on YNB/glu/trp- plates. *S. cerevisiae* RFY 206 is a MAT *a* haploid strain of *Saccharomyces* which can mate with any MAT *ά* to form a diploid strain. In this case the mating strain was *S. cerevisiae* EGY48.

3.1.1e Interaction hunt

Interaction hunt was performed by plating transformed yeast cells which were selected and shown to carry all the required recombinant plasmids. Transformed yeast cells were replica plated onto YNB selective media lacking uracil, tryptophan and leucine supplemented with galactose (for activation of the GAL1 promoter), raffinose (to encourage yeast growth) and zeocin. Figure 18 shows assays carried out to test for β-gal reporter activation. Colonies were lifted off the plate onto a filter paper and tested for β-gal activation by incubating them in an X-gal containing solution. Figure 18a is a positive control picture showing reporter activation caused by interaction between Fos

and Jun and figure 18b is a negative control with unaltered phylex/zeo and library plasmid. Transformed cells carrying plasmid which express proteins that interact with DWNN were expected to turn blue when treated with X-gal plates and also to grow on plates lacking leucine. This assay produced no interactors Figure 18 c-f.

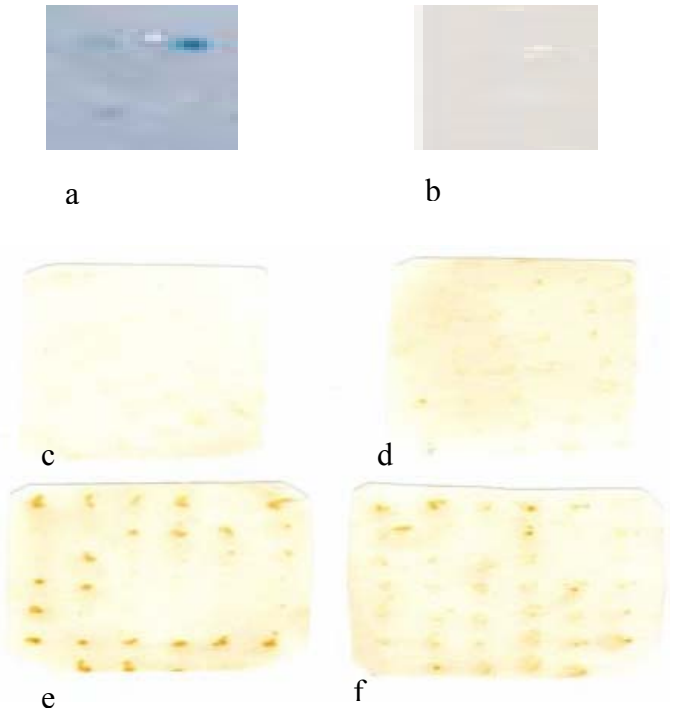


Figure 18: Yeast two hybrid assays. The filter lift assay was carried out on two days old colonies. A, Positive controls with bait (phyblex/Zeo-Fos) and prey (pYestTrp-Jun) plasmids containing genes of proteins which are known to interact. B, Negative control to test for reporter autoactivation pJG4-5 library plasmid with phyblexzeo. c-f, Experimental results showing assay carried out with pHyblex/Zeo-DWNN (Bait) and pJG4-5 library (prey plasmids).

3.1.2 Immunoprecipitation assays

3.1.2a Heterologous expression of DWNN in *E. coli* cells

The recombinant pGEX 6P2 DWNN plasmid construct was obtained from Zungu, (2003) and the construct was used to transform *E. coli* BL-21 (DE3) competent cells. The protein was overexpressed in *E. coli* BL 21 (DE3) with 0.1 M IPTG for 4 hours and the GST-DWNN fusion was isolated in soluble

fraction using the bugbuster reagent (Novagen). Figure 19 shows the SDS-PAGE profile of proteins extracted with the bugbuster reagent. The majority of the fusion protein was obtained in the soluble fraction.

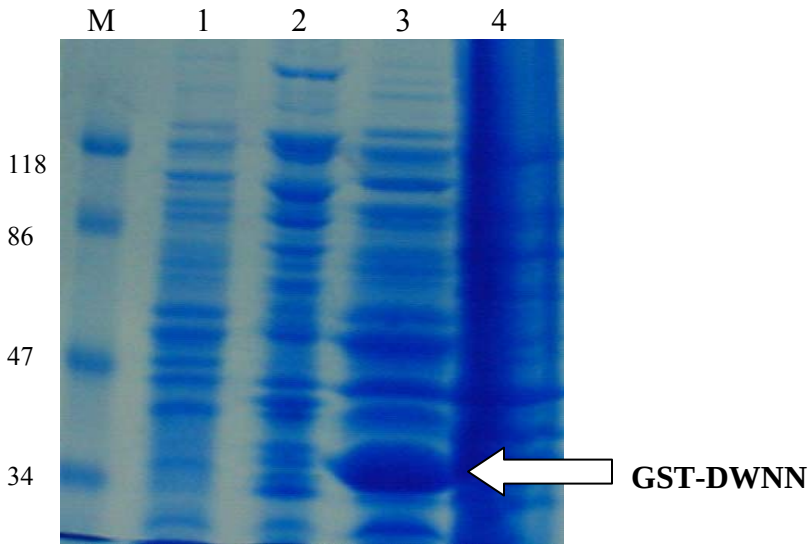


Figure 19: **Large-scale expression of GST-DWNN fusion protein**. Proteins were resolved on a 12% SDS PAGE gel. Lane marked M is Prestained protein molecular weight markers (Fermentas). Lane 1, Soluble extract from cells before induction, lane 2, insoluble extract from cells before induction of expression; lane 3 soluble crude extracts after induction with 0.1M IPTG at 37°C; lane 4, Insoluble extract after induction.

3.1.2b Immunoprecipitations with the GST-DWNN recombinant protein

The crude protein extract from *E. coli* BL-21(DE3) cells was incubated with anti-GST or anti-SNAMA antibodies for 2 hours at 4°C. Protein A sepharose matrix was then added to the antibody fusion protein reaction and incubated for further 2 hours followed by stringent washes using the RIPA buffer. The eluted protein was analysed on a 12% SDS-PAGE. Figure 20 lanes 1 and 5 depict samples which were immunoprecipitated with anti-GST and anti-SNAMA respectively. To test if DWNN is involved in any protein interactions the adult fly crude protein extract was added to the antibody fusion protein reaction

immobilized on a protein A matrix and the samples were also subjected to stringent washes with RIPA buffer. Lane 2 and 3 of figure 20 shows the crude extract from wild type flies and heterozygous SNAMA mutant fly line *l*(2)rQ313w;GFP;Cyo respectively after immunoprecipitating with the anti-GST and GST-DWNN fusion, whereas lanes 6 and 7 shows products immunoprecipitated with anti-SNAMA.

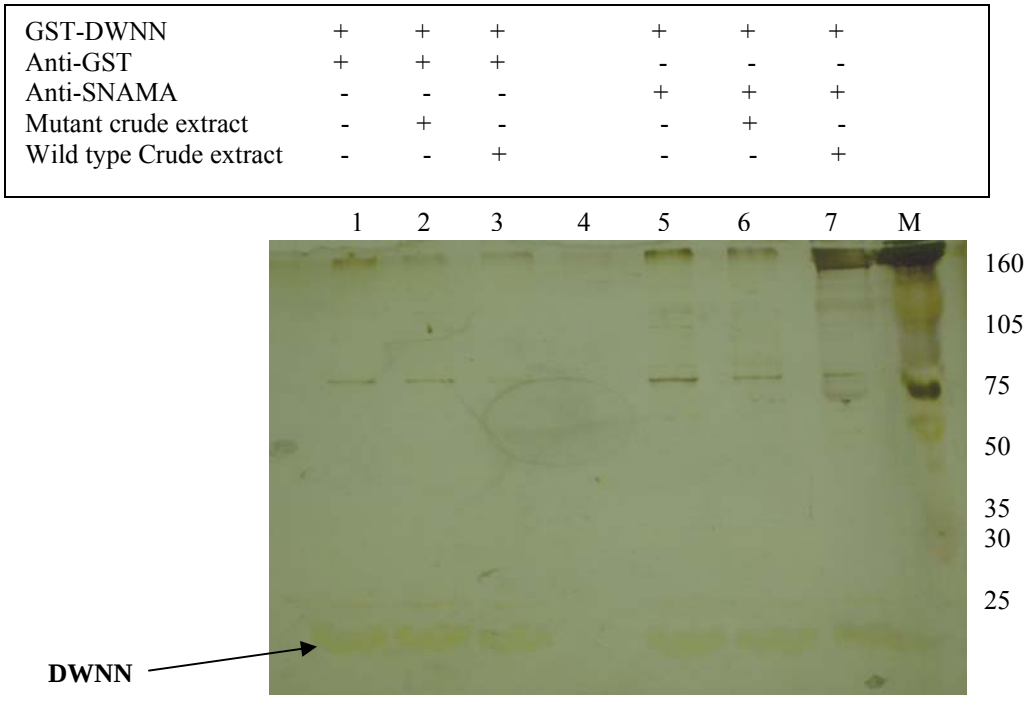


Figure 20: **The GST-DWNN fusion protein was immobilized on a protein A sepharose matrix using anti-GST or anti-SNAMA antibodies.** *In vitro* interaction assay was carried with crude protein extract from adult mutant and wild type flies out using fusion protein.

The SDS-PAGE profiles obtained from this assay revealed that the GST-DWNN fusion is unstable, especially when using buffers that have detergents. The DWNN band could be seen at the bottom of the gel, thus implying that the fragment might have been cleaved from the GST during the washing steps

(Figure 20). Zungu (2003) also reported that the DWNN-GST fusion tends to be unstable which results in the auto-cleavage of DWNN from GST.

To counteract the pre-cleavage of the fusion protein the GST-fusion antibody complex immobilised on the protein A matrix was washed with 1 x PBS followed by the addition of the crude protein extract from flies. The proteins eluted from the matrix are shown below (figure 21). Lane 5 (*l*(2)rQ313w;GFP;Cyo crude protein and GST-DWNN fusion) and lane 7 (Canton S wild type crude protein and GST-DWNN) show proteins immunoprecipitated with anti-SNAMA, whereas lane 6 (*l*(2)rQ313w;GFP;Cyo crude protein and GST-DWNN fusion) and 8 (Canton S wild type crude protein and GST-DWNN) are proteins immunoprecipitated with anti-GST.

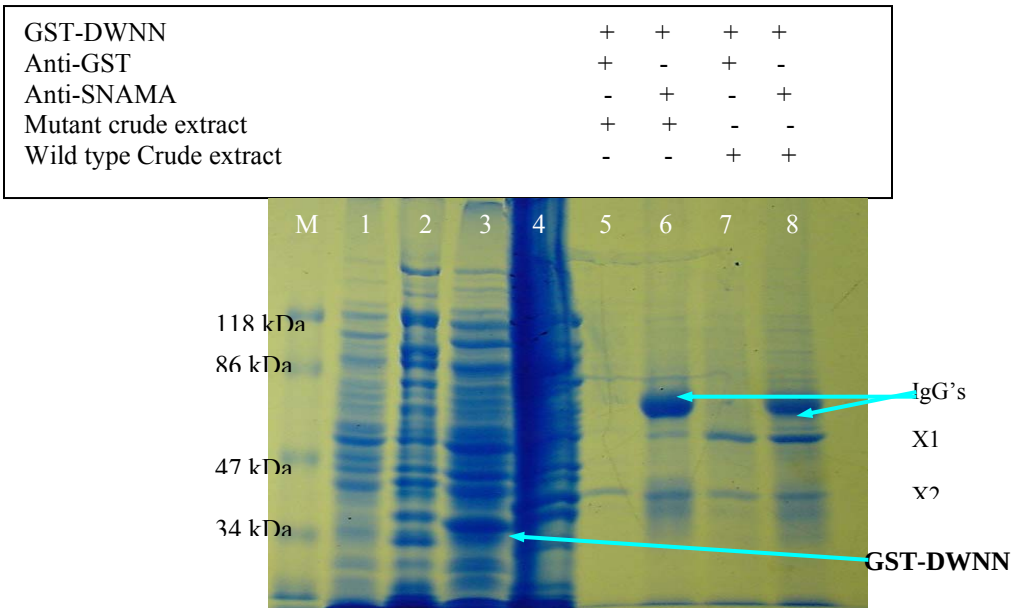


Figure 21: **Immunoprecipitations with GST-DWNN.** M=Prestained molecular weight marker (Fermentas). Lanes 1 preinduction soluble extract and lane 2 preinduction insoluble extract. Lane 3 soluble extract after 4 hours induction with 100mM IPTG and lane 4 is the insoluble extract. Lanes 5 and 7 immunoprecipitations using fusion protein immobilized on the protein A matrix with anti-GST. Lane 6 and 8; Immunoprecipitation assay using fusion protein immobilized on the protein A matrix with anti-SNAMA.

Washing the column with PBS buffer did not affect the integrity of the fusion protein. Figure 21 shows some of the proteins which were isolated from the assay. Interestingly two bands at 45 kDa (X2) and 50 kDa (X1) appear reproducibly in three reactions (lanes 6-8). The protein marked X2 might be Dmp53. More experiments are underway to check if the 50 kDa band is Dmp53.

3.1.2c Interactions with Dmp53

Immunoprecipitations were carried out to further verify that SNAMA interact with other proteins as suggested by the primary structure analysis. Homologues of the zinc finger motifs of SNAMA are known to promote protein-protein interactions. The CCHC belongs to a family of zinc finger motifs, which interact with a transcription factor GATA (Fox *et al.*, 1999). The RING finger-like motif also suggests that the protein might be covalently attached to other proteins.

Crude protein extracts from adult wild type Cantons S and *l*(2)rQ313w;GFP;Cyo mutant fly lines was used in the immunoprecipitations with anti-Dmp53, anti-SNAMA and anti–human DWNN. The *l*(2)rQ313w;GFP;Cyo fly line is a heterozygous mutant where *SNAMA* has been disrupted. Figure 22 depicts the immunoprecipitation profiles with anti-Dmp53, anti-SNAMA or anti–human DWNN (RBBP6). There was a noticeable difference between the proteins in the mutant and the wild type flies.

Anti-humanDWNN-13	-	-	-	+	+	+	-	-	-
Anti-Dmp53	+	+	+	-	-	-	-	-	-
Anti-SNAMA	-	-	-	-	-	-	+	+	+
Mutant crude extract	-	+	-	-	+	-	-	+	-
Wild type Crude extract	-	-	+	-	-	+	-	-	+
Protein A	+	+	+	+	+	+	+	+	+

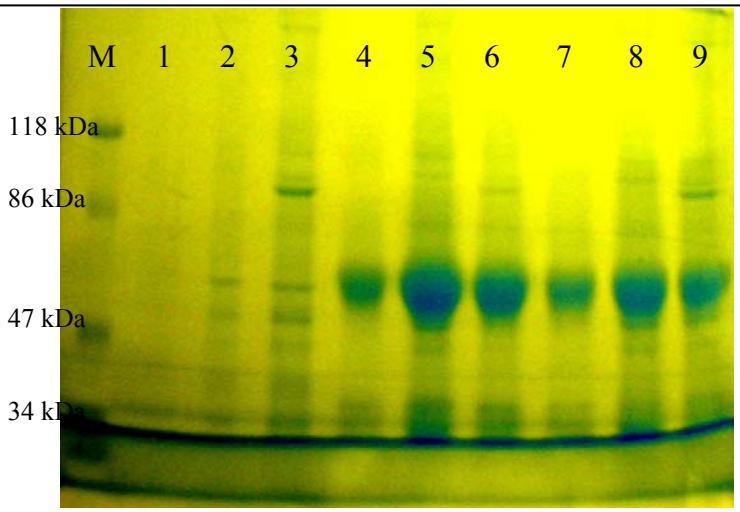


Figure 22: **Immunoprecipitation assays of crude protein extract from l(2)rQ313/w; GFP; cyo and Canton S wild type adult fly extract with anti-Dmp53 anti-SNAMA and anti-HumanDWNN-13.** M is Prestained molecular weight marker (fermentas). Lane 1-3, immunoprecipitations with anti-Dmp53, lanes 4-6 and 7-9 are products immunoprecipitated with anti-SNAMA and anti-HumanDWNN-13 antibodies respectively.

Immunoprecipitations with protein from wild type flies gave a distinct band of approximately 86 kDa with all the three antibodies (Figure 22 lanes 3, 6 and 9). The size of the band did not correspond with the predicted molecular weight of either SNAMA or Dmp53. Even though the size of the product immunoprecipitated by the three antibodies did not correspond to the expected size of either protein (SNAMA or Dmp53) or the predicted molecular weight of the complex between the two; these results suggest that Dmp53 and SNAMA might be interacting. Immunoprecipitations with the mutant flies protein did give two distinct bands in the sample lanes were anti-SNAMA and anti–human

DWNN antibodies were used lane 5 and 8 respectively. However, no bands could be seen where Dmp53 antibody was used (lane 2).

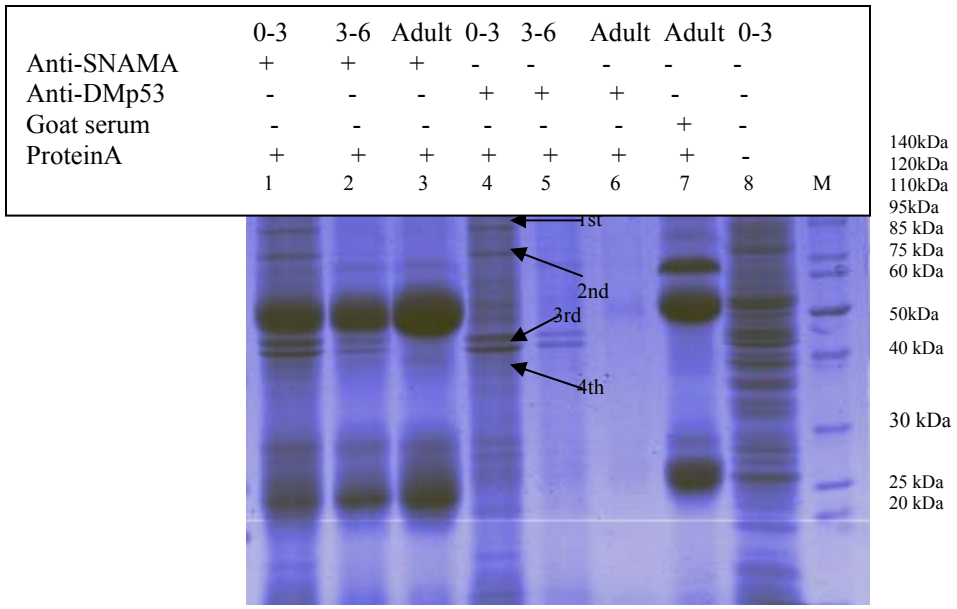


Figure 23: **Immunoprecipitation assay of crude protein extracts from CantonS wild type flies with anti-SNAMA and anti-Dmp53**. Lanes 1-3 crude protein extracts from 0-3, 3-6 and adult flies immunoprecipitated with anti-SNAMA. Lanes 4-6 crude protein extracts immunoprecipitated with anti-Dmp53. lane 7 goat serum with adult flies crude, whereas lane 8 is the 0-3 hour embryos crude extract.

Based on the results obtained using adult flies the search was broadened further by probing the early developmental stages of wild type flies for any interesting interactions. Crude extracts from wild type 0-3 hours and 3-6 hours old embryos were used in immunoprecipitations with anti-SNAMA and anti-Dmp53 antibodies and compared with the results from adult flies. It must be noted that the adult only control was used as the immunoprecipitations with 0-3 and 3-6 hours old embryos were included for comparison with results obtained previously with adult flies (See figure 22) where an 86 kDa band was observed. Interestingly, the 0-3 hour's crude protein immunoprecipitations with anti-SNAMA and anti-Dmp53 antibodies did show additional protein bands of approximately 75 kDa, 50 kDa

and 45 kDa (figure 23 lanes 1 and 4). Proteins that were immunoprecipitated were identified using MALDI-TOF mass spectrophotometer at the Institut de Biologie Moleculaire et Cellulaire at Strasbourg Cedex in France. They were identified as heat shock protein 82 (hsp82), LP19893p (HSP70) and CG2985-PA (Pancreatic lipase like) marked 1st, 2nd and 3rd respectively on the gel (figure 23). Identification of these proteins in early embryonic stages suggests that they are crucial in development. Results obtained with 0-3 hour's embryos thus suggest that SNAMA plays a different in adult flies compared to early developmental stages. Previous studies have already shown that Dmp53 and SNAMA play a role in apoptosis, thus these results further suggest that SNAMA and Dmp53 are involved in similar processes. Even though the controls used were from adult flies the identification of heat shock proteins immunoprecipitated by both anti-SNAMA and anti-Dmp53 suggest that the SNAMA and Dmp53 might be regulating the activity those proteins. Heat shock proteins are known to be involved in protein turnover by stabilizing the 26s proteasome (Kiss *et al* 2005). Heat shock protein 70 is expressed at high levels in stressed cells and it tends to reduce cell proliferation whenever it is overexpressed (Krebs and Feder, 1997).

3.1.2d Western analysis of immunoprecipitated proteins

Immunoprecipitations with protein from flies using anti-Dmp53 suggest that Dmp53 might be modified by SNAMA. Crude protein extracts were collected from 0-3, 3-6 hours old embryos and adult flies. Immunoprecipitations with anti-Dmp53 and anti-SNAMA were carried out and the products were resolved and blotted onto a PVDF membrane. Immunoblot analysis of the immunoprecipitated proteins with anti-SNAMA and anti-Dmp53 further

confirmed the specificity of the immunoprecipitation. Figure 24 is a blot probed with anti-Dmp53, the 53 kDa band observed on the gel corresponds to the molecular masses of one Dmp53 isoform and the heavy chain of the antibody. Interestingly the band was also detected in the lanes where anti-SNAMA antibodies were used in the immunoprecipitations, thus suggesting that it might be one of the Dmp53 isoforms.

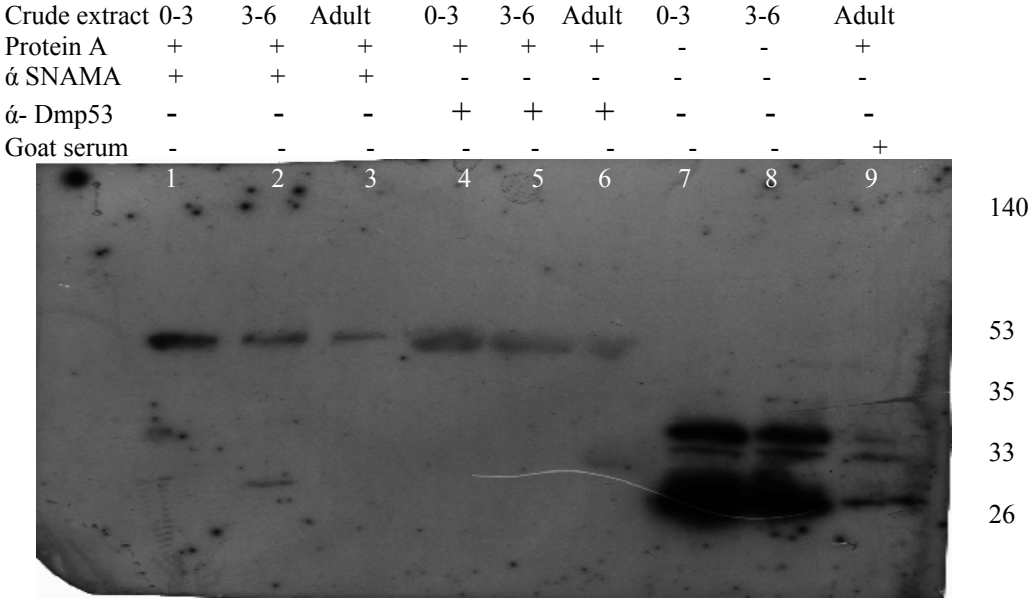


Figure 24: **Western blot analysis of proteinns immunoprecipitated with anti-SNAMA and anti-Dmp53**. Protein extracts from wild type Canton S flies used in the immunoprecipitations with anti SNAMA(lanes 1-3), anti Dmp53(lanes 4-6). Lanes 7-8 crude protein extracts and lane 9 Crude proteins extract from adult flies immunoprecipitated with goat serum. The blot was probed with anti-Dmp53

Figure 25 below shows blot probed with anti-SNAMA. The 53 kDa and the 25 kDa bands detected on the blot correspond to the expected molecular weight of the heavy and light chain SNAMA immunoglobulins. The only interesting band is the ≈140 kDa at the top. Even though SNAMA has a predicted molecular weight of 148 kDa, all previous western blots with anti-SNAMA

antibodies failed to elicit the band of that molecular weight, thus the high molecular weight band in the blot might be SNAMA.

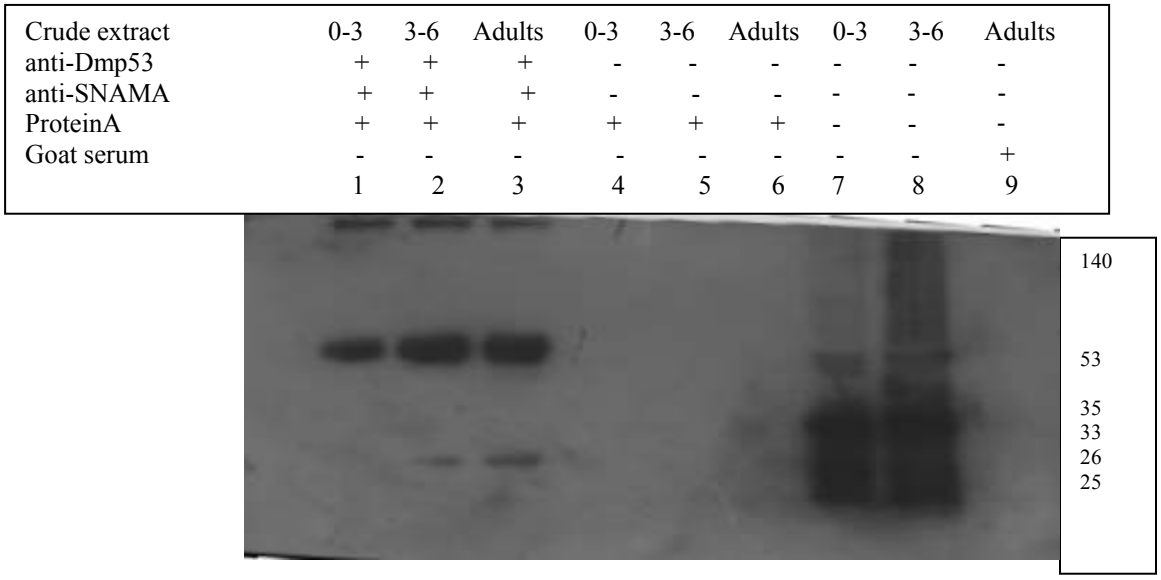


Figure 25: **Detection of SNAMA by immunobloting**. Crude protein extracts from wild type Canton S flies used in the immunoprecipitations with anti SNAMA (lanes 1-3) and anti Dmp53 (lanes 4-6). Lanes 7-8 crude protein extract from 0-3 and 3-6 hours old embryos, lane 9 Crude proteins extract from adult flies immunoprecipitated with goat serum. The blot was probed with anti-SNAMA.

SECTION 4

4. DISCUSSION

Previous studies have revealed that SNAMA plays a role in apoptosis (Mather *et al*., 2005). The protein is expressed at very high levels at early developmental stages and is crucial in embryonic development since deletion results in recessive lethality. SNAMA ortholgues such as PACT, P2P-R and Mpe1 play diverse roles in cell cycle regulation. PACT is known to interact with p53 and Rb (Simons *et al*., 1997), whereas the Mpe1 in yeast is involved in mRNA processing. Sakai *et al*., (1995) also found that RBBP6 interacts with Rb. The homology tree below depicts how closely SNAMA is related to its orthologues (figure 26).

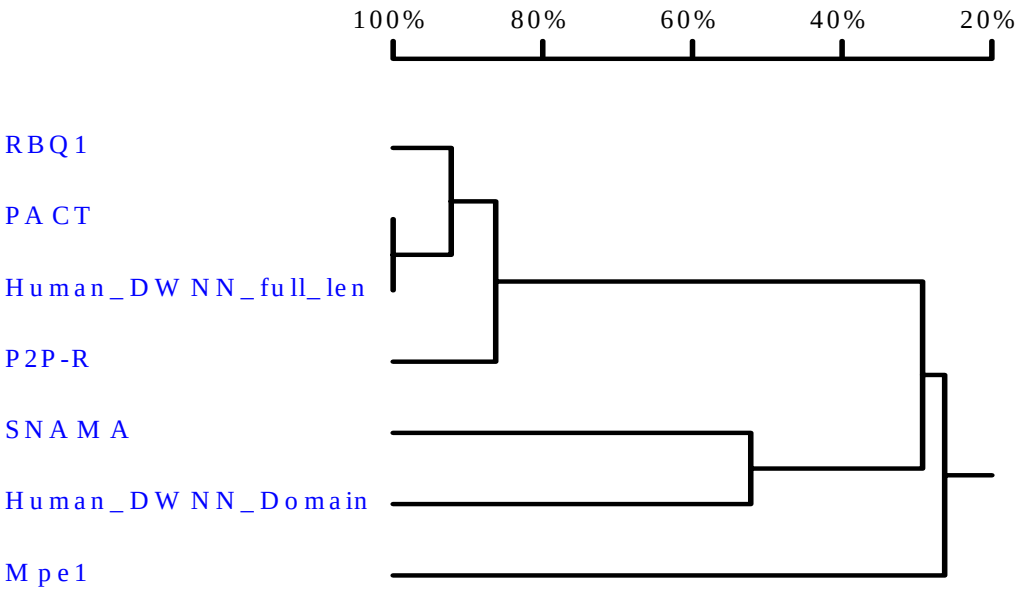


Figure 26: Sequence comparison between SNAMA and related proteins. The scale bar above signifies the percentage homology between the proteins

Even though the overall sequence homology between SNAMA and its orthologues from higher eukaryotes appears to be low, they share a similar primary structure. The RING finger-like motif is present in all the orthologues, whereas Mpe1, SNAMA and the human homologue contain the N-terminal extension which includes the unique DWNN domain and the zinc finger.

The focus of this study was to characterise the unique DWNN domain and SNAMA by isolating proteins which interact with them. It was mentioned in section 1.1 that structural prediction studies revealed that the domain has an ubiqutin like fold. This prediction implies that the protein might interact with other proteins. Bioinformatic analysis of SNAMA orthologues revealed that some have ubiquitin-like features.

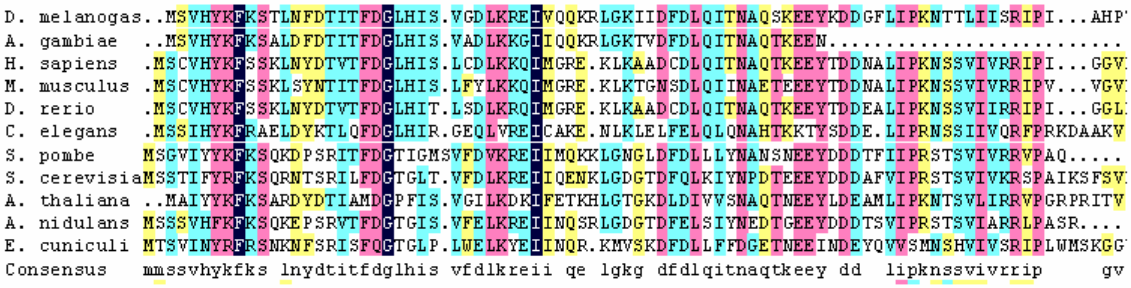


Figure 27: **Alignment of insects, mammals, yeasts, worms and plants DWNN domains**. The alignment was done using DNAMAN version 4.03 (Lynnon Biosoft).

Ubiquitin related proteins are divided into two different categories termed ubiquitin-like modifiers (ubl) and ubiquitin-domain proteins (ubp) (Jentsch and

Pyrowolakis, 2000). Ubp are proteins such as NIRF, scythe and parkin which possess the ubiquitin like domain (Mori, 2002; Jentsch and Pyrowolakis 2000). Proteins such as SUMO1 and NEDD8 are the best known ubiquitin-like proteins. Figure 27 shows the alignment of the N-terminal domain of SNAMA and its orthologues. The human (*H. sapien*), zebrafish (*D. rerio*) and a protozoan (*E. cuniculi*) orthologues might be classified as ubiquitin-like proteins. These proteins have the di-glycine residues, which are characteristic of ubiquitin and most ubl. One of the most common features with these proteins is the proline at position 76 of SNAMA's N-terminal region; the amino acid is highly conserved and might be important in the biological processing of the protein. Gilchrist *et al*., (1997) reported that some ubiqiutin-specific proteases cleave ubiquitin fusion proteins at the ubiquitin proline bond. Proteins such as HUB1 covalently modify other proteins even though it lacks the C-terminal glycine residues (Yoshiroda and Tanaka, 2004).

4.1 Analysis of protein-protein interaction using the yeast two-hybrid system

DWNN was inserted into a yeast expression plasmid pHyblex/zeo as a lex A fusion construct. The recombinant plasmid molecule was confirmed by sequencing to be in frame with the lex A coding region. The construct was used to transfect *S. cerevisiae* EGY 48 yeast cells and characterised using standard two hybrid assays.

Yeast cells containing the bait plasmid were further transformed with the library. Approximately $4x10^3$ transformants were obtained per transformation. The minimum amount of plasmid DNA had to be used in these transformations so as to make sure that only one plasmid molecule is incorporated into the yeast cells. The galactose inducible system in the pJG4-5 library plasmid offers an added advantage, as expression of the library proteins is repressed by the presence of glucose. Plating the transformants on the galactose containing media induces expression of the library proteins. All the transformants that were screened showed no noticeable activation and expression of reporter proteins.

The presence of the 20 amino acid at the c-terminal end however, may have caused structural change affecting interaction with target proteins. Heterelogous expression of proteins in yeast has always been problematic because yeast cells tend to be highly codon biased (Akashi, 2001), whereas a strain such as *Saccharomyces cerevisiae* tends to hyperglycosylate expressed proteins.

SNAMA homologue Mpe1 in yeasts is involved in mRNA splicing. It forms part of the CPF complex which processes single stranded RNA. The domain might also be acting as an inert scaffold, thus aiding the motif associated with it in interactions.

4.2 Analysis of protein-protein interaction

DWNN was expressed successfully as a GST tagged protein in *E. coli* BL 21(DE3) cells. Initially, when the protein was isolated from these cells using the

sonication method, this protein was expressed in inclusion bodies. The sonication method was replaced by the bugbuster reagent based method that proved to be more efficient. High quantities of this fusion protein were soluble with none expressed in the insoluble extract. In vitro studies with the heterologously expressed DWNN revealed that the protein does interact with other proteins. Figure 21 shows immunoprecipitations with the GST-DWNN fusion protein. Proteins of approximately 45 and 50 kDa where isolated in this assay. It may be significant that the 45 kDa band corresponds to the estimated size of one isoform of Dmp53.

Immunoprecipitations also revealed that SNAMA interacts with other proteins. Crude protein extracts from adult wild type and SNAMA knockout mutants used were found to co-elute with other proteins. The 90 kDa band (figure 22) was obtained with the crude protein extract from wild type flies. The proteins were immunoprecipitated with anti-SNAMA, anti-Dmp53 or anti-humanDWNN. The band size did not correspond with either SNAMA or any of the Dmp53 isoforms. Even though the size of the band did not correspond to the expected sizes of the both proteins, the fact that it was immunoprectipitated by both anti-SNAMA and anti-Dmp53 implies that Dmp53 and SNAMA might be involved in similar pathways. Proteins from mutant flies gave a band slightly bigger than the one obtained with the wild type flies.

Further immunoprecipitation with crude extracts from embryos showed that SNAMA and p53 might be involved with the 26S proteasome, figure 23 shows

that the antibodies against SNAMA and Dmp53 immunoprecipitate similar proteins. The proteins identified were hsp82, Hsp70 and CG2985-PA (Pancreatic lipase like). Hsp82 has been shown to associate with the 26S proteasome (Kiss *et al* 2005). Krebs and Feder (1997) reported that overexpression of Hsp70 causes reduction in cell proliferation. Figure 24 and 25 show a Western blot analysis of the proteins obtained with the immunoprecipitations. Immunoblot probed with anti-SNAMA and anti-Dmp53 was used to confirm the specificity of the immunoprecipitations. The immunoblot probed with anti-SNAMA antibody (figure 25) shows a high molecular weight band which corresponds with a predicted molecular weight of SNAMA (135 kDa). The presence of this high molecular weight band in the immunoblots is highly interesting as it is not apparent in the immunoprecipitation gel (figure 23). Thus the presence of the protein might have led to the precipitations of the proteins identified above. These results thus suggest that SNAMA might be playing a role in stress related cell cycle regulation.

SECTION 5

5. CONCLUSION AND FUTURE PROSPECTS

This study was undertaken to investigate the association between SNAMA and other proteins. The major hypothesis was derived from previous studies with SNAMA orthologues such as P2P-R, PACT and RBBP6 which were shown to interact with other proteins. Immunoprecipitation assays with antibodies against SNAMA have shown that the protein does associate with other proteins. Interestingly similar experiment with anti-Dmp53 gave a similar profile to that obtained with anti-SNAMA. The fact that SNAMA and Dmp53 might interact with similar proteins makes this study very interesting and might contribute further to the growing body of scientific knowledge about p53. Even though p53 has been widely studied its exact role in carcinogenesis is not clearly known. This study thus presents another dimension in the study of p53 and SNAMA as some of the proteins which were identified are known to be involved in cell cycle. Hsp82 plays a role in the stabilization of the 26S proteasome, whereas Hsp70 tend to be involved in cell cycle regulation by slowing down proliferation of the affected cells. The association of the Hsp70 with Dmp53 appears to be highly significant as the Dmp53 in flies does not play a role in cell cycle regulation. The protein bands which were identified with the immunoprecipitations were observed in embryos only and not adult fly extract. Even though the controls shown in the manuscript were done with crude extract from adult flies the results obtained are still significant. The proteins which were identified participate in the processes

such as cell cycle regulation and protein turnover. Dmp53 and SNAMA are involved in apoptosis, whereas the predicted structure of SNAMA's N-terminal domain suggests that it is ubiquitin like.

Assays with the yeast two hybrid system using the conserved DWNN did not produce any interacting proteins. The assay is prone to limitations as it relies heavily on physical interactions between two proteins, SNAMA could interact with other proteins through covalent interactions involving the DWNN domain these could be observed on the immunoprecipitation experiments. Further experiments with the full length SNAMA might help in elucidating the characteristics of the protein even though results obtained with the immunoprecipitations suggest that SNAMA might be part of a regulatory complex.

SECTION 6

6. APPENDIX 1

Media and Supplements

LB Agar:	Tryptone 10 g/l, NaCl 10 g/l, yeast extract 5 g/l, agar 28 g/l
LB Broth:	Components of this media were the same as the LBagar above with the exception of agar.
Yeast peptone dextrose:	10 g/l yeast extract, 20 g/l of peptone, 0.1 g/ladenine, 2% glucose.
Yeast nitrogen base media:	6.7 g/l yeast nitrogen base w/o amino acids, 0.6 g/l amino acid dropout mix, 20g/l glucose or (20 g/lgalactose + 10 g/l raffinose)
IPTG:	the stock was made at 0.1 M in water and the solution was filter sterilized and stored at 4°C
X-gal:	the stock was made at 50 mg/ml in N, N, Dimethyl formamide. The working concentration was 25 μg/ml
Ampicillin:	the stock solution was made at 100 mg/ml in water and sterilized by filtration.
Tetracycline:	the stock was made at 12 mg/ml in 70% ethanol.

General stock buffers solutions and kits components

TE buffer (10x):	100 mM Tris, 1 mM EDTA adjusted to pH 7.5 with concentrated HCI. This stock solution was diluted 10 fold with sterile water to make the working solution.
TAE (50x):	2 M Tris, 5.7% glacial acetic acid, 0.5 M EDTA pH 8.0
Transformation buffer (TFB):	10 mM K-Mes (pH 6.2), 100 mM RbCl, 45 mM $MnCl_2.4H_2O$, 10 mM $CaCl.2H_2O$, 3 mM Hexamine cobalt chloride
Ethanol acetate:	96% (v/v) absolute ethanol and 4% (v/v) 3M Sodium acetate
LiPEG:	100 mM Lithium acetate pH 7.5, 40% PEG-3350, 10 mM Tris-HCI pH 7.5.
LiTE :	1x Lithium acetate pH 7.5, 0.5x TE
Solution 1:	50 mM glucose, 25 mM Tris, 10mM EDTA pH 8.0
Solution 2:	0.2 M NaOH, 1% SDS (w/v)
Solution 3:	3 M sodium acetate
Cell lysis buffer:	25 mM Tris, 150 mM NaCl, 0.2% SDS, 0.5 mM PMSF (sigma), 1 μg/ml aprotinin, 4.0 mM Pefabloc (Roche)
TBS:	10 mM Tris pH 8.0, 150 mM NaCl

PBS:	150 mM NaCl, 9.1 mM K_2HPO_4, 1.7 mM KH_2PO_4, adjusted to pH 7.4 with NaOH
SDS-PAGE sample buffer:	0.225 M Tris-Cl (pH 6.8), 50% glycerol, 5% SDS, 0.05% bromophenol blue, 0.25 mM DTT
SDS PAGE Running buffer:	0.5 M Tris base, 1.92 M glycine, 0.5% SDS
Electroblotting buffer:	25 mM Tris, 192 mM Glycine, 3.5 mM SDS, 20% (v/v) methanol
Coomassie blue stain:	0.1% Coomasie blue R-250 (w/v), 10% acetic acid, 40% methanol
Destain solution:	40% methanol, 10% acetic acid
RIPA buffer:	1x PBS, 1% Nonidet P-40, 0.5% sodium dioxycholate, 0.1% SDS
Z Buffer:	60 mM $Na_2 HPO_4$, 40 mM NaH_2PO_4, 10 mM KCI, 1 mM $MgSO_4$-$7H_2O$ pH 7.0
Glycerol solution:	65% glycerol, 0.1 M $MgSO_4$, 25 mM Tris-HCl, pH 8.0
GST purification kit (Cat no:70794-3) :	BugBuster protein extraction reagent (cat no: 70584-3), and 10, 000 units Benzonase nuclease (cat no: 70746-3), GST Bind resin (cat no: 70541-3)
GST bind wash buffer (10x):	43 mM Na_2HPO_4, 14.7 mM KH_2PO_4, 1.37 M NaCl, 27 mM KCl pH 7.3

GST elution buffer:	500 mM Tris-HCl pH 8.0, 100 mM glutathione
GST mag agarose beads:	50% slurry, 0.05 M phosphate buffer pH 7.5, 0.15 M NaCl, 0.1 M NaN_3
Membrane striping buffer:	2% (w/v) SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM β-mecaptoethanol
Protease inhibitor stocks:	10 mg/ml PMSF (Sigma, cat no: P 7626), 20 mg/ml Pefabloc (Roche, cat no: 1873601), 1mg/ml Aprotinin (Roche, cat no: 981532)

APPENDIX 2

```
1 GCATTTCACATCTCTGGGGCTTTGGCGTCACGTTCGCATCTCTAGTAAATTGGAAAAAAA
1
61 TAAAATCGTCGGAGTTTTTATGTGCTTGCAGAGTAGTATTTCTTTCATATGCAACTATGT
21                                                          M  S
121 CGGTACACTATAAATTTAAGAGTACACTCAACTTTGATACAATTACTTTTGATGGACTTC
41     V  H  Y  K  F  K  S  T  L  N  F  D  T  I  T  F  D  G  L  H
181 ACATTTCTGTCGGGGACTTAAAAAGGGAGATTGTGCAGCAGAAGCGACTGGGCAAAATCA
61     I  S  V  G  D  L  K  R  E  I  V  Q  Q  K  R  L  G  K  I  I
241 TCGACTTTGATCTCCAAATAACAAATGCGCAGAGTAAAGAAGAATACAAGGACGATGGGT
81     D  F  D  L  Q  I  T  N  A  Q  S  K  E  E  Y  K  D  D  G  F
301 TCCTTATTCCCAAAAACACAACGCTGATCATATCGCGCATCCCCATCGCCCATCCCACAA
101    L  I  P  K  N  T  T  L  I  I  S  R  I  P  I  A  H  P  T  K
361 AAAAGGGCTGGGAGCCACCAGCAGCAGAAAATGCCTTTTCGGCGGCGCCTGCCAAGCAGG
121    K  G  W  E  P  P  A  A  E  N  A  F  S  A  A  P  A  K  Q  D
421 ACAACTTCAACATGGACCTGTCCAAAATGCAAGGCACGGAGGAGGACAAAATCCAGGCCA
141    N  F  N  M  D  L  S  K  M  Q  G  T  E  E  D  K  I  Q  A  M
481 TGATGATGCAGAGCACAGTCGACTATGATCCTAAGACGTACCATCGTATTAAAGGACAAT
161    M  M  Q  S  T  V  D  Y  D  P  K  T  Y  H  R  I  K  G  Q  S
541 CGCAAGTGGGAGAAGTTCCCGCATCCTACCGATGCAACAAATGCAAGAAAAGCGGACACT
181    Q  V  G  E  V  P  A  S  Y  R  C  N  K  C  K  K  S  G  H  W
601 GGATCAAAAACTGTCCCTTTGTGGGGGGAAAGGACCAGCAAGAGGTCAAACGGAATACTG
201    I  K  N  C  P  F  V  G  G  K  D  Q  Q  E  V  K  R  N  T  G
661 GTATTCCGCGGTCTTTCCGCGACAAGCCAGATGCGGCTGAGAACGAATCAGCCGATTTTG
221    I  P  R  S  F  R  D  K  P  D  A  A  E  N  E  S  A  D  F  V
721 TGCTGCCTGCTGTACAAAACCAAGAGATACCGGAGGATCTGATATGCGGCATATGCCGAG
241    L  P  A  V  Q  N  Q  E  I  P  E  D  L  I  C  G  I  C  R  D
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261    I  F  V  D  A  V  M  I  P  C  C  G  S  S  F  C  D  D  C  V
841 TGCGAACCTCCTTATTGGAGTCAGAGGATAGTGAGTGCCCCGATTGCAAGGAGAAGAACT
281    R  T  S  L  L  E  S  E  D  S  E  C  P  D  C  K  E  K  N  C
901 GTTCGCCTGGCTCCCTGATACCTAATCGGTTCTTGAGGAATTCGGTGAACGCCTTTAAAA
301    S  P  G  S  L  I  P  N  R  F  L  R  N  S  V  N  A  F  K  N
961 ATGAGACTGGGTATAACAAAAGCGCGGCTAAGCCAGCTGCAGTAAAAAATGAGGAAAAAC
321    E  T  G  Y  N  K  S  A  A  K  P  A  A  V  K  N  E  E  K  P
1021 CTCCTGTTGAAAAAGAAGTGGAGAAAAAGCCAGTCGCGGAGGTGGAACCCGAAGAGACTG
341     P  V  E  K  E  V  E  K  K  P  V  A  E  V  E  P  E  E  T  E
1081 AGGTGAAACCTGAAAAGCAAAAAGAATCCGAAACCAATGGCAGTAATCCGCCAAAATCGG
361     V  K  P  E  K  Q  K  E  S  E  T  N  G  S  N  P  P  K  S  E
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1201 CAGACTACGAGGATAACATTACCATAAAAATGCCCCAGCCTGCAGCTGATTCTACAACAG
401     D  Y  E  D  N  I  T  I  K  M  P  Q  P  A  A  D  S  T  T  V
1261 TGCCCAGCAAAAGATCCCCCAGTTATTCCCACAGAAGTGAATCCTCTCATCGACGGGACA
421     P  S  K  R  S  P  S  Y  S  H  R  S  E  S  S  H  R  R  D  R
1321 GGTCGGATTATGTTTCCGATCACGATCACAAGCACCAACGTCCATCAAAATCGGAGTCTG
441     S  D  Y  V  S  D  H  D  H  K  H  Q  R  P  S  K  S  E  S  V
1381 TTAACAAGGATCGCAGTCTCCTGCCCTTGCCCATTGGCACCCTGCCTAGCTACCAGGGCC
461     N  K  D  R  S  L  L  P  L  P  I  G  T  L  P  S  Y  Q  G  H
1441 ACATGATGGCCGAATCAGAAGAAGCTCGTCGATCGAGTGCCTATAAGCCCCCTTATATGC
481     M  M  A  E  S  E  E  A  R  R  S  S  A  Y  K  P  P  Y  M  Q
1501 AAATGCAGCGGGGCCCACCTCCTATGCACATGATGAGTCACCACATGCCAGCCTACAACA
501     M  Q  R  G  P  P  P  M  H  M  M  S  H  H  M  P  A  Y  N  N
1561 ACGGGTTTAACAACATGGGACAGAGGCCTCCCCTCAGCTATGTGCCGTATCAAAACCAAT
521     G  F  N  N  M  G  Q  R  P  P  L  S  Y  V  P  Y  Q  N  Q  S
1621 CCGTACACCCAATGCGTGCGCCGTACGGATCTGCAGGCGGAGGTATGAATATGAATATGT
541     V  H  P  M  R  A  P  Y  G  S  A  G  G  G  M  N  M  N  M  S
1681 CACAACCATTTCAGTCCCCAAATTTAGCCTCGATATACCAAGGGGTGGCAGCGAAGGTCG
561     Q  P  F  Q  S  P  N  L  A  S  I  Y  Q  G  V  A  A  K  V  G
```

```
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581     S  G  P  I  D  D  P  L  E  A  F  N  R  I  M  K  E  K  E  R
1801 GGAAGAAGGTGGACCGCTTTCGAAGCTCTGACCGCCACAGGTCAAGGTCCCCGGATAGAC
601     K  K  V  D  R  F  R  S  S  D  R  H  R  S  R  S  P  D  R  Q
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621     R  H  R  F  K  S  P  M  Y  E  K  D  N  S  R  D  N  L  K  D
1921 ACAAAAGACCGCGATCCCGGGAAAGGAAGCGAGAACATAGCTACGAACGGCATATACGCC
641     K  R  P  R  S  R  E  R  K  R  E  H  S  Y  E  R  H  I  R  H
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661     P  R  S  S  R  Q  P  N  D  G  S  K  S  P  G  G  R  I  K  R
2041 GATCTGGACATCGTCGCTCTGCATCTCCAAAGCCGGGCTACAAGAGTGATTACAGAGACA
681     S  G  H  R  R  S  A  S  P  K  P  G  Y  K  S  D  Y  R  D  K
2101 AGCCGTACAACAAGCCTAGTGCTCCCAAAACGGAGGCAGTTGAGCCTCCTCCCCCCGGAT
701     P  Y  N  K  P  S  A  P  K  T  E  A  V  E  P  P  P  P  G  F
2161 TCGAGCCGTTGCAGCTGACGGATGAAGACGGCTACAGGAACAAGCACCCGACCAGTTCGG
721     E  P  L  Q  L  T  D  E  D  G  Y  R  N  K  H  P  T  S  S  E
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741     A  S  Q  S  S  K  G  D  S  S  K  K  R  G  E  N  R  H  E  E
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761     A  P  R  K  R  H  R  S  R  S  I  S  K  E  P  K  P  N  D  S
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781     N  Y  R  S  L  T  P  P  A  K  I  T  T  P  K  M  T  A  A  Q
2401 AGTTGAGGCAACGCGAAAGTTCACCGAAGACGCCGGAAAAGAGTCACGACGATTATCTGA
801     L  R  Q  R  E  S  S  P  K  T  P  E  K  S  H  D  D  Y  L  T
2461 CCGCGAAGGCCAGAATTATGGCCTCCCAGCCCGTCATCAACGACACGGAAATGGAGACCA
821     A  K  A  R  I  M  A  S  Q  P  V  I  N  D  T  E  M  E  T  N
2521 ATGTGGGCAAGGAGAACAAGGCCAAGAGTCCGTTGTCAAAAGATCGCAAGAAGAAGAAGA
841     V  G  K  E  N  K  A  K  S  P  L  S  K  D  R  K  K  K  K  K
2581 AGGACAAGGACAAGGCTGAGCGCAAGAAAAACAAGAAGGACAAGCGCGCTAAGAAGGAGA
861     D  K  D  K  A  E  R  K  K  N  K  K  D  K  R  A  K  K  E  K
2641 AAGGGGATCGCCAGAAGAAGAGCTCCTCAGTTAATCGATCTGACTCGGATATTAACAACA
881     G  D  R  Q  K  K  S  S  S  V  N  R  S  D  S  D  I  N  N  S
2701 GCTCACTAATGAACGAGTCAAATTATAAAGTATTGTCTCCCAGGGCTCAAAGTCCCAGCA
901     S  L  M  N  E  S  N  Y  K  V  L  S  P  R  A  Q  S  P  S  I
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921     E  I  N  A  A  Q  L  S  P  T  H  N  A  T  E  N  V  N  P  K
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941     S  H  S  I  L  T  V  G  A  A  S  D  D  N  L  G  P  R  S  K
2881 AACTCAGCGAGGCTAATTCTGTCAATCTATCCAAATGGGAAATCGACGAGAATATCTTAG
961     L  S  E  A  N  S  V  N  L  S  K  W  E  I  D  E  N  I  L  G
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981     L  E  D  S  S  K  K  A  A  G  A  S  D  D  P  S  E  I  T  S
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1001    D  V  L  R  K  A  E  N  A  I  F  A  K  A  I  N  A  I  R  P
3061 CTATGGAGTTTCAAGTTATTATCAATTCCAAGGACAACAGCAAGGACCGCTCCGTAGTTC
1021    M  E  F  Q  V  I  I  N  S  K  D  N  S  K  D  R  S  V  V  R
3121 GAAGTGACAAGGATCGCTCCTCCTCACCCAGGCGTAACAACAGCAGCAGGTCGGTAAAGG
1041    S  D  K  D  R  S  S  S  P  R  R  N  N  S  S  R  S  V  K  D
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1061    R  L  G  T  K  I  S  N  D  R  S  R  S  R  D  K  S  K  G  R
3241 GGCGCCGGGCCGCAAGGAGCTCCGACGACGATGCGAACCGCGGCAGGTCGGATCGTCATG
1081    R  R  A  A  R  S  S  D  D  D  A  N  R  G  R  S  D  R  H  G
3301 GCAGCCGGAAGAGGGACAACAGATCCCGCGACAGGGCGGCGCCTTCAGAGAAGAGGCAGG
1101    S  R  K  R  D  N  R  S  R  D  R  A  A  P  S  E  K  R  Q  E
3361 AGCGTTCGTACAAGCGAAGCTCGCCGGAGGACGACAAGCTGAGGCGCCAGAACAAGGAAC
1121    R  S  Y  K  R  S  S  P  E  D  D  K  L  R  R  Q  N  K  E  Q
3421 AGTCCGAATCCAAGCACGGAAAGCATGATCAAAACAATAGCGACGACTCGGATCGGAGGG
1141    S  E  S  K  H  G  K  H  D  Q  N  N  S  D  D  S  D  R  R  A
3481 CGGCCAAAAACACCAAGTCCAGCGACAGCCGAGTGGTCTCCTCTGTAACAGCCGTGGTTG
1161    A  K  N  T  K  S  S  D  S  R  V  V  S  S  V  T  A  V  V  A
3541 CTCCTCCCAAACCCTGTCGTCCAGACAACCCGTTCCGCAAGTTCGTCGACACCAGTTCGT
1181    P  P  K  P  C  R  P  D  N  P  F  R  K  F  V  D  T  S  S  S
3601 CGAGCAGCTTAGTTGTAAAATATGATAACACGATACAGAAGGAGGGCGCGTCCTCGGACA
1201    S  S  L  V  V  K  Y  D  N  T  I  Q  K  E  G  A  S  S  D  N
```
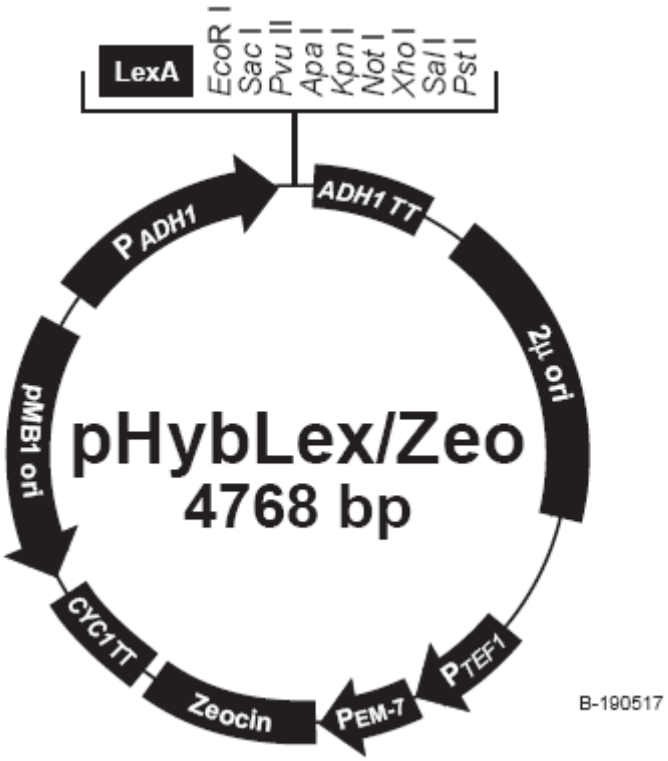
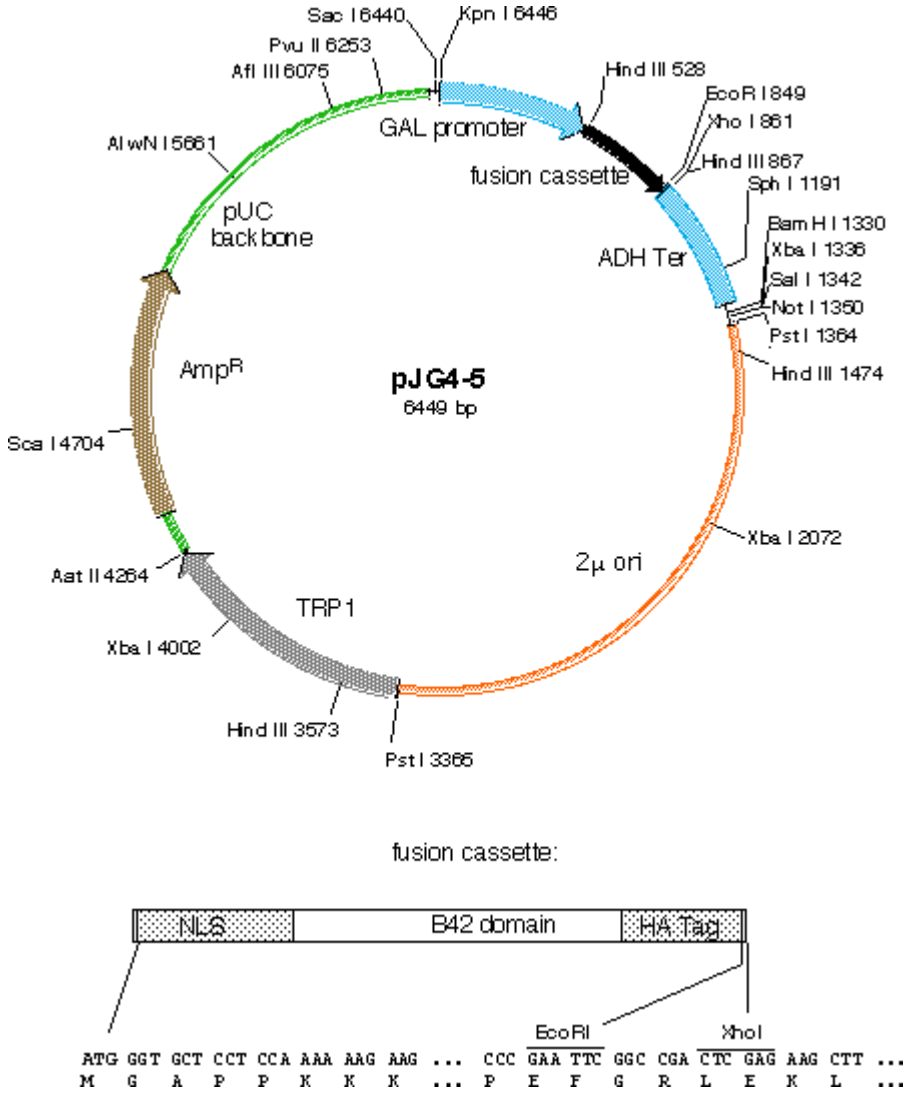

```
3661 ACGGCATGGAGCACAGGAAGCAGAGGGATAAGAAGCTGAAGAAACATTCAAAATATTCGT
1221    G  M  E  H  R  K  Q  R  D  K  K  L  K  K  H  S  K  Y  S  S
3721 CAACCGATTCGTTGAAGAGCGAGAAGCGCAAGGATCCGAAGAGCAAAAAGAAGAGCAAGA
1241    T  D  S  L  K  S  E  K  R  K  D  P  K  S  K  K  K  S  K  I
3781 TTTTGAAGAAGAAGAAAAAATCAAAGAAGTAGGTTACGGTAGGCTACGAGATAAGAATGA
1261    L  K  K  K  K  K  S  K  K *
3841 TATAAATATTGAAGATTAATGTGTACAAATCAAAGATTTTTAATGTATGTATTTATCATG
1281
3901 CAACTATAAGTATACAAATAAAACAGAACTACTCAAGGA
1301
```

Nucleic acid and corresponding amino acid sequence of SNAMA. The sequence in bold letters is DWNN, whereas the zinc and RING finger like motif are shaded in red and green respectively.

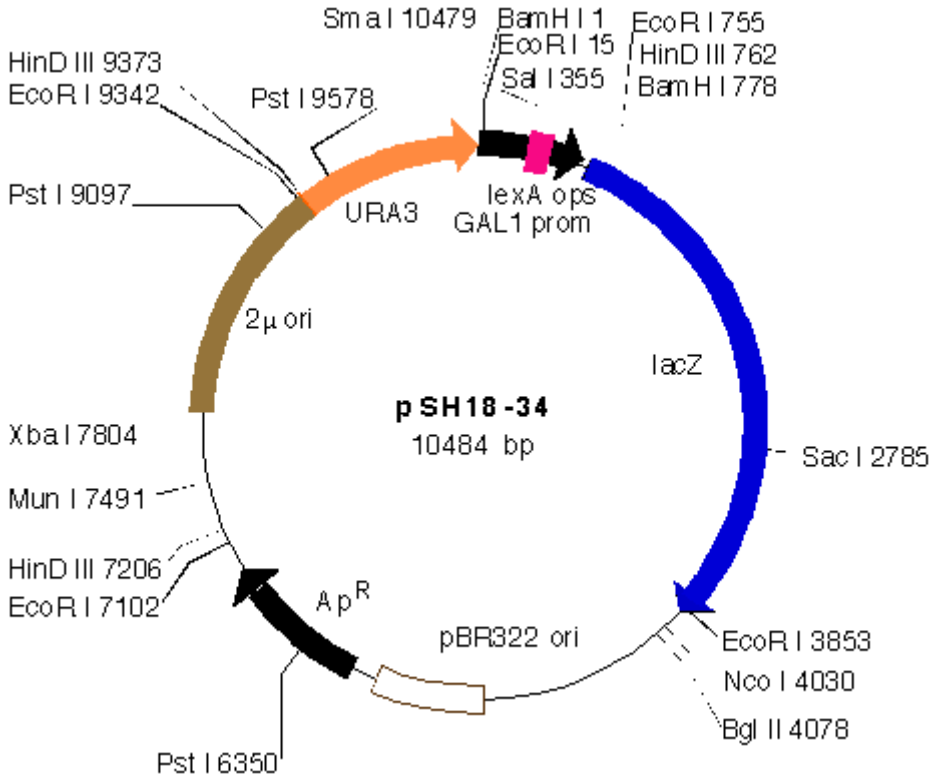
APPENDIX 3



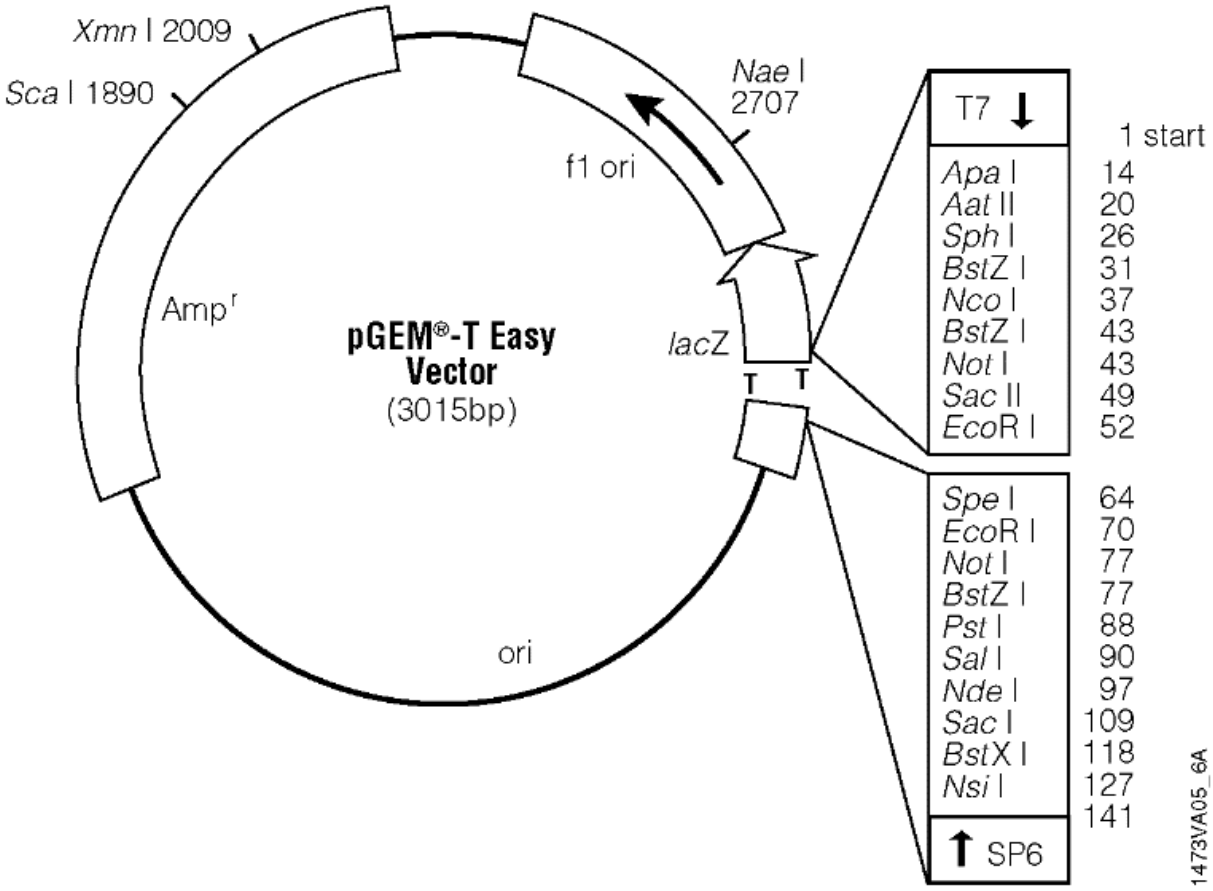
Plasmid map of pHybLex/Zeo (Invitrogen)



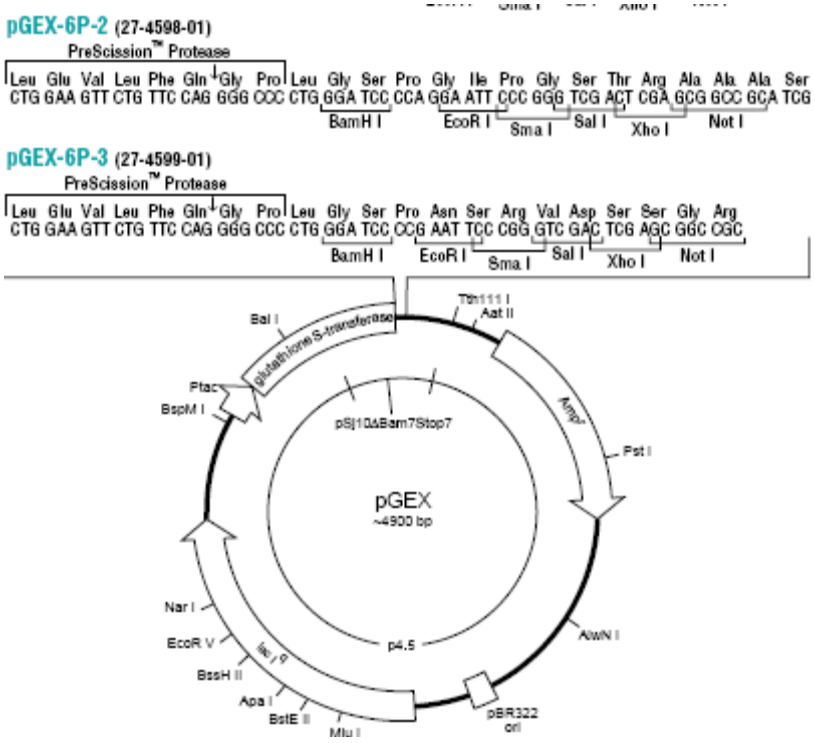
Map of pJG4-5 library plasmid. Showing the fusion cassette and the restriction sites which were used to insert the library cDNA. (Gyuris *et al*., 1993)



Plasmid map of pSH18-34 reporter plasmid (Gyuris *et al*., 1993)



Map of pGEM-T easy cloning vector (Promega)



Map of pGEX easy cloning vector (Amersham)

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