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Temporal expression of Dmp53 and SNAMA isoforms and their relation to genotoxic stress.

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Temporal Expression of Dmp53 and SNAMA Isoforms and their relation to genotoxic stress.

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science.

Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



_____

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University of Witwatersrand, Johannesburg.

22nd April 2015.

Abstract

RBBP6 is an E3 Ubiquitin ligase protein with a U-box motif. It interacts with p53 and Rb and is linked to several cellular functions. SNAMA is the *Drosophila* RBBP6 homolog, but is less characterized than its vertebrate counterparts. Gene expression studies on *Drosophila* have a potential to advance the knowledge on molecular mechanism underlying genotoxic stress. Previous studies have shown that SNAMA plays a critical role as an apoptosis suppressor and possibly in responses to genotoxic stress. The molecular basis for this is, however, unknown. Initially, two isoforms were identified by bioinformatics and one (*Snama A*) experimentally as well. Here, we confirm experimentally the existence of the second isoform (*Snama B*). We also show that these are differentially expressed during development and when the organism undergoes genotoxic stress. Total RNA samples were used to demonstrate gene expression by using Reverse Transcriptase Polymerase Chain Reaction. Using samples collected at different stages of development and from adult flies treated with the DNA damaging agent, irinotecan, it is shown that these isoforms are differentially expressed throughout development and upon genotoxic stress. This knowledge may help to understand the functional role SNAMA plays in normal physiology and in response to genotoxic stress. Furthermore, the results show that SNAMA is involved in a potentially beneficial intervention whereby the glycolytic pathway is bypassed by the addition of methyl pyruvate.

Quotation

“The will to excel and the will to win, they endure. They are more important than any events that occasion them”

- *Vince Lombardi*

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To the Almighty God; His grace is always sufficient.

To my family; they are my pillar of strength.

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Research paper- (Accepted)

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Awards-

First time inventor award presented by WITS enterprise

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List of Abbreviations

ADP- Adenosine diphosphate

ATP- Adenosine triphosphate

ATM- Ataxia telangiectesia mutated

ATR- Ataxia telangiectesia and Rad 3-related protein

BIR- Baculovirus inhibitory repeat

cDNA- Complimentary DNA

CK1- Casein kinase 1

CPT- Camptothecin

DEPC- Diethylpyrocarbonate

DNA- Deoxyribonucleic acid

Dmp53- *Drosophila* p53

DMSO- Dimethylsulfoxide

DREF- DNA replication-related element

DWNN- Domain with no name

EDTA- Ethylenediaminetetraacetic acid

Fwd- Forward

HIF-1- Hypoxia-inducible transcriptional factor

IPTG- Isopropyl β-D-1-thiogalactopyranoside

JNK- Jun NH2- terminal kinase

LB- Lysogeny broth

M- Molar

MDM2-Mouse double minute 2

mm- Millimetres

ml- Millilitres

Mins- Minutes

MOPS- 3-(N-morpholino)propansulfonic acid

mRNA- Messenger RNA

NADH- Nicotinamide adenine dinucleotide hydrogenase

PACT- p53 activated cell testes derivative

PBS- Phosphate buffer solution

PCR- Polymerase chain reaction

PKR- Protein kinase R

RBBP6- Retinoblastoma binding protein

RING- Really Interesting New Gene

Rev-Reverse

RNA- Ribonucleic acid

Rpr- Reaper

TAE- Tris-Acetate-EDTA

TNF-R- Tumour necrosis factor receptor

Topo1- Topoisomerase

UTR- Untranslated region

XGAL- 5-bromo-4chloro-3-indolyl-beta-D-galacto-pyranoside

β-cell- Beta cell

µl- microlitres

1. Introduction

1.1 The *Drosophila melanogaster*

The life cycle of the *Drosophila melanogaster* involves four major stages; Embryo, Larvae, Pupae and Adult. At an optimal temperature of about 25°C, it takes about 12 days for the embryo to develop into an adult.

1.1.1 The Life cycle of the *Drosophila melanogaster*

At 25°C, it takes the *Drosophila* about 12 days to complete its life cycle, while at 20°C it takes about 15 days as seen in figure 1.1. This indicates that the lower the temperature, the longer it takes for development to proceed. However, it is important to note that an increase in temperature may result in the death of the fly. After fertilization has occurred, the female fly produces embryos which later, are transformed into larvae, and then into pupae after three instar larval stages. Finally, the adult fly emerges from the pupal case. This stage is important because it allows the isolation of virgins.

The Embryo

The *Drosophila melanogaster* embryo is about 0.4 mm in length. It has a pair of filaments called micropyles that protrude from its dorsal surface. This pair of filaments functions to transfer the sperm into the ovum during fertilization. The female requires just one mating process from which it can store many sperm cells. After the sperm enters the female and makes contact with the egg via a tiny opening located at the anterior ending, meiosis occurs and this result in the formation of the egg nucleus. The egg and sperm nuclei join side by side to form the zygotic nucleus in the embryo.

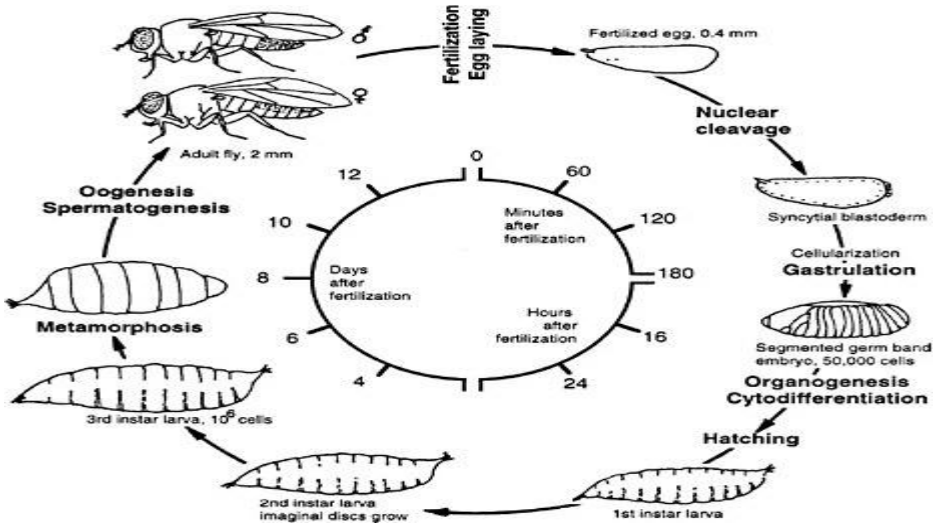


Figure 1.1: Life cycle of *Drosophila melanogaster*. 1 day after the egg is fertilized, the larva hatches. First, second, and third instar are the different larval stages after which the pupae emerges. During the pupae stage most of the larval tissues are removed, shortly after adults emerge. Duration of time is given at 25°C.

Source: Adapted from (Wolpert *et al* 1998)

The Larva

The larvae develop in three stages (called instars); the 1st instar, 2nd instar and 3rd instar. They can grow up to 4.5 mm in length by the 3rd instar. A male larva can be differentiated from its female counterpart by the size of its gonad. The testis of a male is much larger than the ovary. This is readily distinguishable by the naked eyes without dissecting the larva.

The Pupa

After the 3rd instar, the larva begins to slowly crawl to a dry surface (normally the side of the vial or bottle). During the process of pupation the white larva turns into a dark brown case and becomes harden. The entire process takes about 2 days.

Adults

Four (4) days after pupation the adults emerge. An adult fly can either be male or female and they can be distinguished in several ways (Table 1.1).

Table 1.1: Distinguishable characteristics of the male and female *Drosophila melanogaster*

FEATURES	ADULT MALE	ADULT FEMALE
Size	Much smaller than the female.	Bigger in size than the male.
Tip of the abdomen	Not elongated.	Elongated.
Abdominal markings	Darker Markings.	Lighter markings.
Abdominal segments	5 segments	7 segments

1.1.2 *Drosophila* embryonic development and axis formation

During early development, several genes are expressed by the female and deposited at specific positions in the embryo. These genes are called "maternal effect" genes as the phenotype of the offspring is dependent on the genotype of the female (mother) regardless of the male genotype. At the cellular blastoderm stage the embryo expresses zygotic genes, to continue embryonic development.

Maternal effect genes are critical in forming the two axes (the antero-posterior axis and the dorso-lateral axis) within the embryos. The products of these maternal effect genes define the correct establishment of axes by spatially distributed RNA and proteins (figure 1.2). Zygotic genes in the nuclei are then

activated by these proteins in preparation for the next round of patterning; segment formation.

Maternal effect genes that specify the antero-posterior axis

Prior to fertilization of the egg, differences in the egg are created along the antero-posterior axis by the expression of maternal effect genes. The future anterior head and posterior ends of the fly are distinguished by these differences. However, these mutations strongly effect the development of the offspring. Four maternal effect genes (*bicoid*, *hunchback*, *nanos* and *caudal*) are dispersed along the antero-posterior axis and are critical in endowing it (St Johnston and Nusslein-Volhard, 1992).

Four classes of these maternal effect genes exist;

1. **Those affecting the anterior region**: For example, loss of function mutations in maternal *bicoid*, lead to loss of structures in the head and thoracic regions. In some cases, these structures can be substituted with structures from the posterior regions.
2. **Those affecting the posterior regions**: Mutations in *nanos* , lead to an absence of abdominal region structures resulting in smaller abnormally sized embryos.
3. **Those affecting both terminal regions:** Mutations in *torso* has serious effects to the acron and telson located at the head and tail sections of the embryo resulting to interference in the developmental process of the fly.
4. **Those affecting the dorso-ventral regions**: Mutations in *toll*, results in defects during embryogenesis.

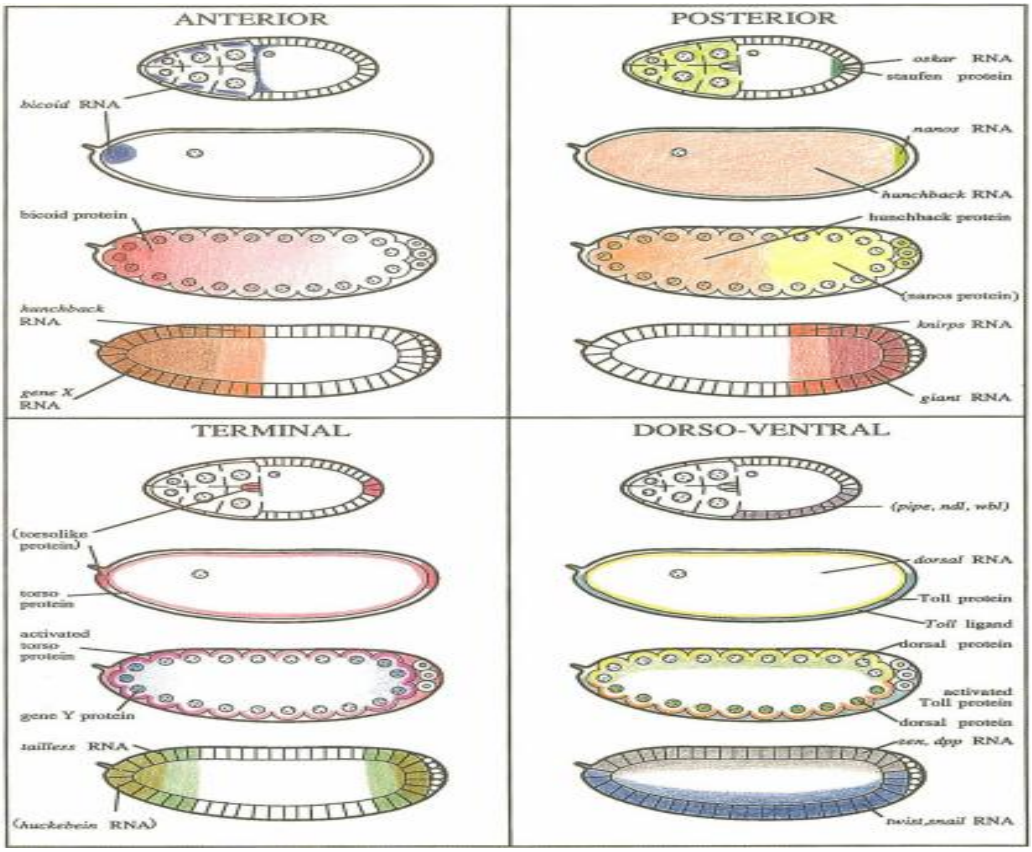


Figure 1.2: Summary of the critical steps of all maternal pathways (Anterior, posterior, terminal and dorso- ventral pathways). The location of Gene X, Gene Y and genes showed in parenthesis are speculative.

Source: Adapted from (St Johnston and Nusslein-Volhard, 1992).

The bicoid gene establishes the antero-posterior axis

Before fertilization of the egg, the *bicoid* mRNA can be found at the anterior end. After fertilization, translation occurs and the protein moves from the anterior end to the posterior end forming an antero-posterior concentration gradient. Based on the formation of this gradient, positional information necessary for future patterning is obtained. The bicoid protein functions as a morphogen by turning on specific zygotic genes at differing concentrations thereby begin a fresh pattern of

expressed genes along the antero-posterior axis. Thus, the *bicoid* is a crucial maternal effect gene involved in the early stages of development.

Nanos and Caudal proteins control posterior patterning.

The posterior end is controlled by nine maternal effect genes. These genes are responsible for the localization of distinct maternal factors within the oocyte. Amongst them is *oskar,* which functions in localizing the *nanos* mRNA at the posterior end of the unfertilized egg. A deficiency in nanos results in the absence of abdominal regions. The nanos protein is responsible for the suppression of another maternal effect gene called *hunchback.* This suppression is crucial because the *hunchback* is also zygotically expressed and controls the establishment of the bicoid gradient. During early embryogenesis, *hunchback* becomes activated by elevated concentrations of the bicoid protein at the anterior of the embryo. This results in the formation of an antero-posterior gradient of the hunchback protein. But since *hunchback* is also maternally expressed at the posterior end, a very high concentration of the hunchback protein is observed at that end. Therefore, for a distinct antero-posterior gradient to be observed *nanos* has to inhibit its own expression at the posterior end by binding to the *hunchback* mRNA (Rivera-Pomar et al., 1995).

Another important maternal product, *caudal*, is expressed ubiquitously within the embryo. The bicoid protein inhibits the caudal protein leading to the formation of a posterior to anterior gradient. At the posterior end, the concentration of the caudal protein is highest because of the low concentration of its inhibitor; the bicoid protein. When the *caudal* gene is mutated it results in some abdominal segments being abnormally developed (Struhl, 1989).

1.2 The p53 pathway during DNA damage

Structural and Functional Characteristics of p53

p53 plays a very important role in, protecting genome integrity, cell cycle regulation and apoptosis. Mutation in p53 occurs in many human cancers (Levine, 1997). Tp53 (Human p53) is made up of 393 amino acids and is divided into four domains (Zhu, 2000). These domains each have their own unique functions. At the N-terminus is the transactivation domain which is composed of 42 amino acids. Within this domain is a region (amino acids 13-29) that interacts with Mdm2 protein (Kussie et al., 1996). The DNA binding domain is found between amino acids 94-292 and it possesses the ability to fold into a β-sheet sandwich thereby forming a scaffold for a large loop and a helix motif which has a direct interaction with DNA. The majority of the missense mutations that occur in p53 leading to cancers occur within this region (Levine, 1997). These missense mutations alter the amino acids that interact and bind with DNA or which are responsible for maintaining the three-dimensional structure of p53. Mutated p53 is defective in binding DNA therefore cannot undergo transactivation. At the C-terminus of p53 is the tetramerization domain composed of amino acid 324-355 and is responsible for the oligomerization of p53 subunits (Jeffrey et al., 1995). p53 is functional as a tetramer (Jin et al., 2000).

1.2.1 p53 induces apoptosis.

Under normal physiological conditions, the p53 levels are kept very low by its short half-life and by downregulation induced by proteins such as Mdm2, cop1 or pirh2. These proteins induce p53 degradation via the ubiquitin/proteasome

pathway. When the cell becomes stressed due to hypoxia, damage to the DNA, or when oncogenes are activated abnormally, signals are sent to p53 to stabilize it. The level of p53 increases rapidly and becomes active to function as a transcriptional factor. This activation of target genes located downstream like cyclin G, Bax and p21 appears to be responsible for the mechanism through which cell cycle progression and apoptosis are regulated by p53 (Jin et al., 2000).

1.2.2 Regulation of p53

In normal healthy cells, p53 is tightly regulated causing it to be present in very low amounts due to the fact that the protein is very quickly degraded after its been synthesized (Kubbutat et al., 1997). Regulation of p53 stability ensures that its levels are well under control. One of the major mechanisms by which low p53 levels are maintained is by ubiquitination and subsequent degradation in the proteasome. This mechanism involves a group of enzymes that join several ubiquitin chains to lysine residues in the protein of interest (Varshavsky, 1997). The joining of ubiquitin to proteins for degradation requires the activities of; Ubiquitin-activating enzyme (e1), Ubiquitin-conjugating enzyme (e2) and the Ubiquitin-ligase (e3).

Degradation of p53 and Mdm2

Mdm2 is a critical component in the pathway leading to the degradation of p53. Its expression is upregulated when a p53 response is activated , although unlike other p53 targets there is no proof that it controls any effects of p53 downstream such as apoptosis or cell cycle arrest (Marston et al., 1994). The major role of Mdm2 is to directly bind p53 thereby inhibiting its activity. Mdm2 binds to the transactivation domain of p53 at the N-terminus. This interaction alone can inhibit

the function of p53 by decreasing its ability to stimulate gene expression. *In vitro* studies have shown that Mdm2 can also function in the degradation of p53 by functioning as an E3 ubiquitin-ligase (Honda et al., 1997). E3 ubiquitin-ligases possess the RING (Really Interesting New Gene) finger domain and function by attracting ubiquitin to the protein that is to be degraded; this then leads to the degradation of the protein in the proteasome. Significantly, Mdm2 not only targets p53 for degradation but also auto-ubiquitinates, therefore it likely regulates itself (Honda and Yasuda, 1999). Regulation of p53 by Mdm2 is very crucial in normal development, since deficiency in Mdm2 results in early embryonic lethality (Jones et al., 1995). This lethality is rescued by the deletion of p53 and this supports the theory that a loss of Mdm2 activity leads to an increase in the activity of p53. The importance of the Mdm2/p53 pathway can be further illustrated by cancerous cells expressing the inactive form of p53. Mutant p53 lose their tumour suppressing abilities and become unable to activate the expression of Mdm2. Consequently mutant p53 are stable and accumulate to large amounts within the cell (Kubbutat et al., 1997).

In *Drosophila*, Mdm2 is absent but it is hypothesized that SNAMA might have similar functions. This hypothesis was drawn from the fact that SNAMA possesses the RING finger domain, which has the E3 ubiquitin-ligase activity. Also, like Mdm2, SNAMA possesses potential binding sites for p53 and Rb. (Mather et al., 2005).

Stabilization of p53

The degradation of p53 in normal cells is essential to reduce p53 activity. Since it has been observed that the degradation of p53 is mostly regulated by Mdm2, the

stabilization of p53 will be largely dependent on mechanisms that inhibit the binding of Mdm2 to p53. The stabilization of p53 is a critical response to different forms of stress within the cell. When p53 is activated and cell growth inhibited in response to stress, it prevents the progression and development of tumour cells by causing an inhibition of the proliferation of abnormal cells (Levine, 1997).

One of the most common ways to inhibit interaction between Mdm2 and p53 is by the modification of either of the two proteins. p53 undergoes modification by phosphorylation at various sites after DNA damage (Meek, 1998) and various kinases are involved in this process. *In vitro* experiments have shown that several kinases (ATM,JNK,DNA-PK,ATR and CK1) are involved in the phosphorylation of residues at the N terminus of p53 (Jayaraman and Prives, 1999). Evidence has shown that ATR and ATM phosphorylates serine15 *in vivo* (Khanna et al., 1998) and PKR phosphorylates serine18 in mouse p53 (Cuddihy et al., 1999). Phosphorylation at the N-terminus of p53 correlates with its stabilization, although it has been observed that different patterns of phosphorylation occur in response to varying stress signals indicating that no single site is liable for the stabilization of p53 (Ashcroft and Rorsman, 1989). Mdm2 can also undergo phosphorylation (Mayo et al., 1997).

1.3 Impact of p53 in energy metabolism: Glycolysis and oxidative phosphorylation

In the regulation of energy metabolism, p53 interposes at different points in the glycolytic and oxidative phosphorylation pathways (Figure 1.3). One major way in which p53 functions is by slowing down glycolysis and enhancing oxidative phosphorylation. Through transcriptionally regulating target genes, p53 balances

the use of both pathways and this may provide a way in which tumouregenesis can be blocked (Puzio-Kuter, 2011).

Metabolic pathways are influenced by p53 because of the ability of p53 to regulate the expression levels of genes that affect metabolic products. For example, p53 elevates the expression levels of SCO2 (cytochrome c oxidase 2), PUMA (the p53 modulator of apoptosis), GLS2 (glutaminase 2), GLUT1 (Glucose transporter 1), GLUT4 (Glucose transporter 4), TIGAR (Tp53-induced glycolysis and apoptosis regulator) and p53 reduces the expression of PGM (Phosphoglycerate mutase) (Schwartzenberg-Bar-Yoseph et al., 2004). The induction of TIGAR by p53, leads to the reduction of fructose-2,6-bisphosphate lowering glycolysis (Bensaad and Vousden, 2007). When glycolysis is decreased, the pentose phosphate pathway is activated and resulting in limiting of cell death induced by oxidative stress. Glycolysis is also reduced when PGM expression is reduced by p53. Increasing PGM results in an increase in glycolytic flux and excessive proliferation, this occurs in certain cancers possessing mutant p53 (Kondoh et al., 2005). p53 also functions in regulating the expression of GLUT1 and GLUT4 by repressing these genes (Schwartzenberg-Bar-Yoseph et al., 2004). Mutant p53 cannot repress the expression of GLUT1 and GLUT4, allowing for the supply of energy to the cell and an increase in glucose metabolism.

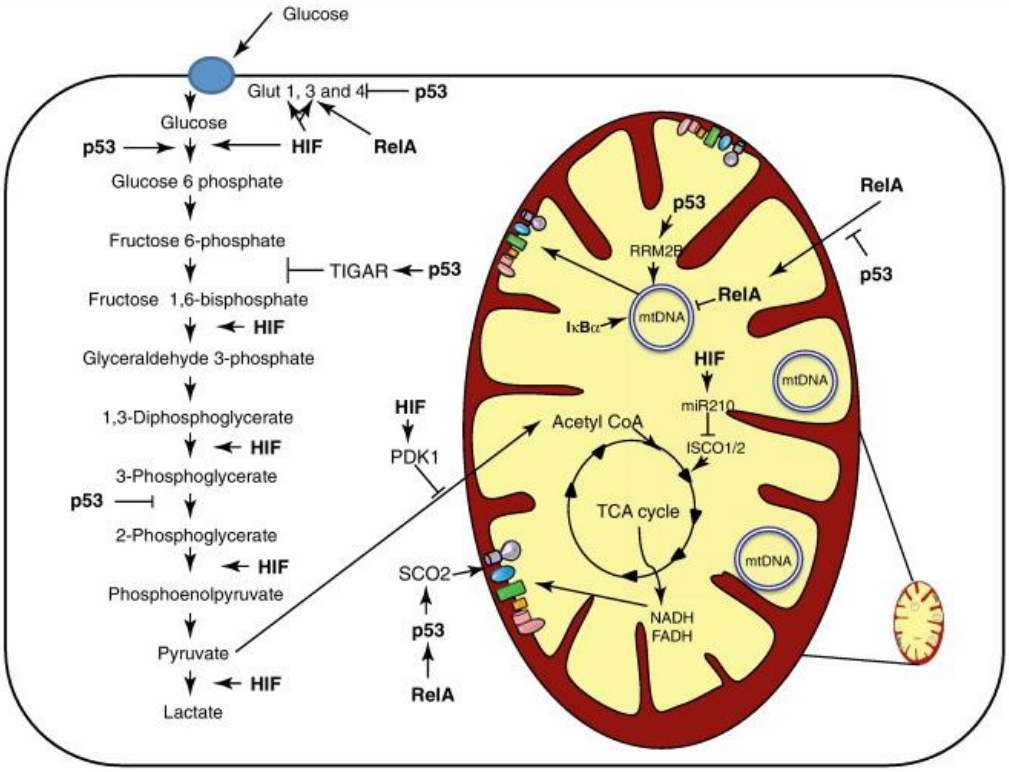


Figure 1.3: Role of P53 and HIF(Hypoxia-inducible transcriptional factor) in the regulation of metabolic pathways. p53 regulates the expression of critical components of the glycolytic pathway (in the cytosol) and TCA pathway (in the mitochondria).

Source: Adapted from (Johnson and Perkins, 2012).

1.4 Methyl pyruvate, is an essential component of glycolysis

Production of ATP in the mitochondria is a very crucial step in stimulus-secretion coupling. This coupling is glucose-induced and it occurs in the beta cells of the pancreas (Duchen, Smith et al. 1993). When ATP/ADP levels are elevated, it results in K_{ATP} channels closure, depolarization and the opening of Ca^{2+} channels, the influx of Ca^{2+} and finally secretion of insulin (Ashcroft and Rorsman, 1989). Pyruvate metabolism in the citrate cycle produce reduction equivalents for the production of ATP in the mitochondria (Maechler and Wollheim, 1999). Pyruvate is the major intermediate of glycolysis in the metabolism of glucose but does not in fact play a role as a primary stimulus for the secretion of insulin in β-cell of the

pancreas. However, various studies show that methyl pyruvate is a cogent stimulator of the secretion of insulin (Dukes et al., 1998). The lipophillic properties of methyl pyruvate enable it to accumulate within the mitochondria. Studies with *Drosophila* have tested the effects of methyl pyruvate in food fed to flies. Adult flies were treated with a combined treatment with camptothecin and 10% methyl pyruvate for three days and then left to recover for another nine days and it was observed that although this treatment was observed to be toxic to the flies, there was also an increase in the number of eggs that developed into larvae compared to eggs produced by camptothecin treated flies (Hull and Ntwasa, 2010). This observation indicates that methyl pyruvate might have a positive effect in development of flies but is toxic to the adult fly at 10% concentration. It is therefore necessary to investigate the effects of methyl pyruvate at varying concentrations as this might produce interesting results.

1.5 *Drosophila* p53

The *Drosophila* homologue is encoded by a lone gene that is structurally similar to the human family members including *Tp53, Tp63* and *Tp73*. Structurally, both *Tp53* and *Dmp53* contain 2 promoters; the first located upstream in the regulatory region and the second in the first intron.

Transcriptional activity of Dmp53

In most cases, Tp53 functions as a transcriptional factor by binding to DNA with the specific DNA binding domain. When p53 binds DNA, it represses or induces target genes to control particular cell responses. Transcriptional activity of Tp53 determines the process by which the cell responds to genotoxic stress (Beckerman and Prives, 2010). Dmp53 possesses a DNA binding domain identical to that

found in Tp53 and the transcriptional activity of Dmp53 has been shown (Brodsky et al., 2000).

Dmp53 is expressed during development.

Maternal RNA Dmp53 is transferred to eggs in abundant quantities and its levels in the embryo remain elevated until cellularization of the blastoderm. In the oocyte, Dmp53 is expressed ubiquitously, however, during the mid- stage of embryogenesis RNA distribution becomes more prominent in certain positions such as the mesoderm but low in the neural and epidermal layers (Ollmann et al., 2000).

Regulation of Dmp53

In the regulation of Dmp53, dRad6 plays a very critical role. dRad6 is the *Drosophila* homolog of Rad6 (Chen et al., 2011). Rad6 is an E2 enzyme (ubiquitin-conjugating enzyme) involved in the repair of DNA damage and in the regulation of gene transcription by catalyzing of the ubiquitination of specific target proteins in mammalian cells and yeast (Kim et al., 2009; Koken et al., 1991). Reduction of dRad6 increases the amounts of Dmp53 which in turn causes the degradation of dRad6. dRad6 interacts with Dmp53 by binding to its transcriptional domain thereby regulating the ubiquitination of Dmp53. A loss of function in dRad6 results in lethal consequences and morphological changes in adult flies (Chen et al., 2011).

Role of Dmp53 in apoptosis

During DNA damage, for example one caused by radiation, Dmp53 is activated resulting in the activation of ATM kinase located in the mei-41 locus. Dmp53 then interacts and binds to enhancer element (which is radiation specific) and

activates its transcription. Consequently, the Reaper protein binds DIAP1 (*Drosophila* inhibitor of apoptosis protein) and blocks it from inhibiting caspase cascade activation. ICAD (inhibitor of caspase deoxyribonuclease) is then cleaved to give free CAD by caspase 3, resulting in apoptosis (Steller, 2000).

Role of reaper-Family proteins in the induction of apoptosis

The Reaper-family proteins namely grim, hid (head involution defective) and reaper play a critical role in inducing apoptosis (Kornbluth and White, 2005). The absence of all these genes results in the complete blocking of apoptosis (White et al., 1994) while their expression leads to the induction of apoptosis (Grether et al., 1995). Proteins encoded by these genes contain a short motif located at the N-terminus called the IAP-Binding-Motif (IBM), which is essential for IAP-binding and apoptosis (Shi, 2002). Reaper-family proteins use their IBM to bind a unique domain present in IAPs (inhibitor of apoptosis proteins) called BIR (Baculovirus inhibitory repeat) (Kornbluth and White, 2005), and this interaction prevents IAPs from inhibiting caspases (cysteine-aspartate proteases that play a huge role in apoptosis) (Salvesen and Duckett, 2002). Reaper can also promotes the auto-ubiquitination and subsequent degradation of IAPs (Ryoo et al., 2002).

1.6 SNAMA, the *Drosophila* homologue of RBBP6

SNAMA (Something that sticks like glue) is a protein with 1231 amino acids. It has a conserved DWNN (Domain With No Name) made up of 76 amino acid residues, a Zinc finger domain of a CCHC kind, a RING finger domain, a lysine rich domain and a NLS (Nuclear localisation signal) (Mather et al., 2005). Its domain structures indicate that it might play a crucial role within the cell and may act in apoptosis and RNA processing. Previous studies have also indicated that

there might be an interaction between SNAMA and Dmp53, in which SNAMA might act as a suppressor of apoptosis or in negatively regulating activators of apoptosis. SNAMA possesses the DWNN domain (which is ubiquitin-like) and a RING finger domain and this suggests that it could be an UDP (Ubiquitin domain protein) and have an E3 ligase activity (Mather, 2006). Two Isoforms of *Snama* exists; *Snama A* and *Snama B. Snama A* possess 10 exons (3 more than *Snama B*).

DWNN domain

The 3-dimensional structure of this domain is identical to that of ubiquitin. The primary structure of the DWNN domain is, however, 22% similar to ubiquitin (Mather et al., 2005). The resemblance of the DWNN domain to ubiquitin might stipulate that RBBP6 acts via ubiquitin modification. DWNN is unique to the RBBP6 family only and is located at the N-terminus of the orthologues (Pugh et al., 2006).

ZINC finger domain

They make up an important group of factors that play a role in gene expression and transcription. The Zinc finger motifs are the most occurring motifs found in eukaryotic proteins (Jantz et al., 2004). They are rich in cysteine and histidine and found in a variety of proteins with one of their most important characteristic being their binding with zinc ions. In the *Drosophila,* almost all zinc fingers play critical roles in some developmental processes and also in protein-protein synergy (Hart et al., 1996). They also orchestrate the way in which DNA, RNA and proteins interact with each other. The most frequently occurring zinc finger motif is the CCHH (Miller et al., 1985).

Cysteine-rich domain

SNAMA possesses a cysteine-rich domain that is very similar to a RING finger. A RING finger can be defined as a zinc finger that has been altered (Freemont et al., 1991). The amino acid histidine, located at position 4 is unique to RING finger motifs but in the cysteine-rich domain, it is replaced by the amino acid, serine. However, this domain is often considered as "RING finger-like" because the RING finger domains usually possesses ubiquitin-ligase activity that allows the SNAMA protein to function in adequately altering other proteins. In SNAMA, ubiquitin-ligase activity has not been shown but it undoubtedly contains a cysteine-rich region (Mather et al., 2005).

Snama-p53 interaction; a role in Apoptosis

SNAMA has been shown to play a role during apoptosis and in the development of embryos (Mather et al., 2005). The DWNN domain in SNAMA includes a region that has striking structural resemblance to PACT. PACT (the mouse homologue of RBBP6) is a murine polypeptide that has the ability to bind non-mutated p53 subsequently disrupting p53-DNA specific binding (Simons et al., 1997). The PACT protein has been identified to be involved during cell damage or stress by its binding to p53 (Sakai et al., 1995) which implicates its role in apoptosis. Apoptosis is programmed cell death and occurs in all organisms that are multicellular. Apoptosis controls the proliferation of normal and damaged cells. SNAMA appears to function with p53 during apoptosis (Mather et al., 2005). p53 suppresses tumours by inducing apoptosis and it is well known that mutations in p53 occurs in more than half of all human cancers.

SNAMA is expressed during development

SNAMA is expressed as a 4.6kb throughout development and it is expressed (in varying amounts) at every stage of the development of the *Drosophila* by being expressed in high amounts at the early stages of development and in lower amounts in adults (Mather et al., 2005).

1.7 Camptothecin, a DNA damaging agent

Camptothecin (CPT) and its analogues are extensively used to treat several cancers including ovarian and colorectal cancers. These drugs target cells that are rapidly dividing causing strand breaks during replication resulting in apoptosis (Liu et al., 2000). CPT analogues like Irinotecan have improved pharmacokinetic properties and are preferred to CPT, for instance irinotecan is water-soluble and is a better CPT- derivative (Schulz et al., 2009). These structural analogues had to be developed because of the high toxicity and poor solubility of CPT. CPT and its structural analogues are topoisomerase poisons that function by inhibiting DNA topoisomerase 1 (Topo1). Topo1 is an enzyme located within the nucleus and it introduces temporary breaks that are single-stranded on the DNA, thereby relieving positive supercoils during the process of replication. Topo1 re-joins nicked strands to form unaltered DNA. A crucial step in this process is forming a complex (which is capable of being split) between the DNA and the Topo1 via a covalent bond between the 3'end of the strand of DNA and an amino acid (tyrosine) at the active site of the enzyme. Topo1 is a critical enzyme during *Drosophila* embryonic development and is found mainly within the ovaries (Lee et al., 1993). CPT and its analogues transform Topo1 into poison by ensuring the cleavable complex is stable for preventing re-ligation of the nicked strands. This

stabilization results in double-strand breaks during DNA replication compromising the genome. Topo1 is the only target of CPT (Hsiang et al., 1985). This allows us to target specific pathways. However, since replication occurs in all cells albeit at higher rate in dividing cells, this specificity is somewhat reduced. Consequently, while CPT drugs act against cancer cells, they also have an adverse effect on normal cells. Irinotecan, which is a CPT derivative, can induce programmed cell death in a manner that is p53 dependent (Takeba et al., 2007).

1.8 Aim.

The main aim of this study is to define temporal expression patterns of Dmp53 and SNAMA during development and during response to genotoxic stress in order to understand their biological functions.

1.9 Objectives.

1. Total RNA Extraction.
2. Temporal expression patterns of *snama* isoforms using RT-PCR.
3. Differential expression patterns of *snama* isoforms and Dmp53 when treated with a camptothecin derivative; irinotecan.
4. The effects of bypassing glycolysis during and after treatment with irinotecan.

2. Materials and Methods

2.1 Materials.

2.1.1 Media

Table 2.1: Media used in the duration of this study

MEDIA	COMPOSITION
Apple juice agar	2% agar; 1.25% sucrose; 0.025% streptomycin; 0.15% methylparaben and 24-25% apple juice(v/v)
LB medium	1% NaCl; 1% tryptone; and 0.5% yeast extract
LB agar	LB medium and 1.5% agar
LB/XGAL/IPTG plates	LB medium; 1.5% agar; 0.2 mM IPTG; and 40 µg/ml X-gal
LB/Ampicillin medium	LB medium and 50 µg Ampicillin
LB/Ampicillin plates	LB medium; 1.5% agar; and 50 µg Ampicillin

2.1.2 Solutions and buffers.

Table 2.2: Molecular biology solutions and buffers

SOLUTIONS/BUFFERS	COMPOSITION
10X MOPS buffer	50 mM Sodium Acetate; 10 mM EDTA (pH 7); and 0.25 MOPS. To be stored in the dark
0.5M Na_2EDTA (pH 8.0)	186.12 g Na_2 EDTA-2H_2O
0.1M IPTG- 50 ml	1.19 g 1PTG
Xgal (20 mg/ml)- 20 ml	400 mg X-gal; and 20 ml N, N'-dimethyl formamide.
Ethanol Acetate (1:25)	1 ml Ethanol; 25 ml Acetate.
10X PBS- 1 litre	80 g NaCl; 2 g KCl; 26.8 g Na_2HPO_4-7H_2O; and KH_2PO_4.
50X TAE- 1 litre	2 M Tris-base; 5.7% Glacial acetic acid; and 0.5 M EDTA.
RNA Loading Buffer	DEPC-treated water; 50% glycerol; 1 mM Na_2EDTA; and 0.4% Bromophenol Blue.

RNA Sample buffer	10 ml deionized formamide; 2.5 ml 37% formaldehyde and 2 ml 5X MOPS.
DEPC- treated water	0.1- 0.2 ml DEPC; and Water.

2.1.3 Oligonucleotides (primers)

All the Oligonucleotides used were purchased from Inqaba-Biotec, produced by Thermo-Scientific.

Table 2.3: Oligonucleotides and their Sequences used in this study

PRIMER	SEQUENCE
Dmp53 Fwd	5′ CAATCCGTGCTGCGCGAAAT 3′
Dmp53 Rev	5′ CCCACAGATGCAACAGATTTC 3′
Snama A Fwd	5′ ATCTGGACATCGTCGCTCTG 3′
Snama A Rev	5′ CTTCTTCTGGCGATCCCCT 3′
Snama B Fwd	5′ GATGCCTTGCAATCCTCAGC 3′
Snama B Rev	5′ CAAAGTATGCCGAATATAGATTC 3′
RP49 Fwd	5′ TGTTGTGTCCTTCCAGCTTCAA 3′
RP49 Rev	5′ ACTGATATCCATCCATCCAGATAATG 3′
AB fwdexon3 *Snama*	5′ TGAGGAATTCGGTGAACG 3′
Brevutr *Snama*	5′ ATCCACATGGTAGCGGGAT 3′
Arevexon7 *Snama*	5′ CAACGGATCGTCAATGG 3′
DREF Fwd	5′ TGAGAGTGCATTTGAAGCAT 3′
DREF Rev	5′ TTGGTCAGCACCTTGTGA 3′

Reaper Fwd	5′AAACCAGAATTCATGGCAGTGGCATTCTAC3′
Reaper Rev	5′TTACTCGAGCTCTCATTGCGATGGCTTGCG3′

2.1.4 Chemicals and kits

Table 2.4: Chemicals /Kits used and their manufacturers

CHEMICALS/KITS	MANUFACTURER with catalogue Number
Chloroform (99+ %)	Sigma- Aldrich C-2432-500 ml
EDTA	United States Biochemical CAT 15698
Nuclease free water	Qiagen CAT 129117
2-Propanol	Merck 1036573
Dimethyl Sulfoxide	Sigma-Aldrich D8418- 100 ml
Glycerol	Saarchem 1025192
Acetic acid	Saarchem 10274367
MOPS	Sigma M1254- 250 G
Methyl Paraben	Sigma H5501- 100G

Agarose powder	Seakem 5004- 500 g
Plasmid plus maxi kit (25)	Qiagen 12963
RevertAid first strand cDNA synthesis kit	Thermoscientific #K1622
PCR mastermix(2X)	ThermoScientific #K0171
PGEM®-T Easy vector system	Promega A3610
E cloni 10G and 10GF chemically competent cells	Lucigen 60106- 1
Irinotecan hydrochloride	Sigma L1406- 50 mg
Ampicillin Sodium salt	Sigma A29518 - 25 G
Streptomycin sulfate salt	Sigma 56501-50 G
Tri Reagent	Sigma BCBF12310

2.1.5 Laboratory equipments used

Table 2.5: Laboratory Equipment/Machinery used in this study

EQUIPMENT	MANUFACTURER with Model Number
Microwave Oven	KIC MWS- 900M
Hot plate/Magnetic stirrer	Freed Electric MH4-9517
Digital dry bath	Labnet D1100-2300
GeneAmp PCR System	Perkin Elmer 2400
Biofuge	Heraeus Instruments D-31520
Microscope	Olympus SZ40
Weighing balance	Precisa XT220A
Gel doc System	Bio-Rad XR^+

2.1.6 Molecular Biology Enzymes

Table 2.6: Molecular biology enzymes used and Manufaturers

ENZYME	MANUFACTURER
NdeI	New England Biolabs (NEB)
NcoI	New England Biolabs (NEB)
SalI	New England Biolabs (NEB)
AatII	Fermentas
PstI	Fermentas
PvuII	Promega
HinfI	Promega
EcoRI	New England Biolabs (NEB)

2.2 Methods

2.2.1 Fly maintenance, treatment and stocks

Wild type Canton S flies were placed in vials containing cornmeal agar plus yeast and reared at 25°C. Flies were transferred to new vials on a weekly basis to maintain healthy fly stocks. Flies were exposed to different dozes of combination of methyl pyruvate and irinotecan by making a mixture of these chemicals in yeast blobs. Duration of exposure was 3 days after which they were transferred to fresh vials containing untreated yeast blobs and left to recover for a period of 3- 9 days depending on the experiment to be performed.

Drosophila stocks

The *Snama* and *p53* genes were both simultaneously knocked out in wild type Canton S flies producing $p53^{-/-}$/$Snama^{-/-}$ mutant flies. The mutant flies were a kind

gift from Professor Jean-Marc Reichhart in Strasbourg niversity, France. The mutant phenotype observed possessed curly wings and red eyes.

A B C



Figure 2.1: An example of a vial (A), bottle (B) and a cage (C) respectively, used in rearing flies. The cage is used to rear large amounts of flies and is effectively used in the collection of embryos.

2.2.2 Collection of developmental stages of the flies

Embryos were collected with apple juice agar plates attached to carefully labelled cages. Adult flies were put in these cages and embryos collected within the specific time periods. Larvae and Pupae were collected by setting up larval boxes. Adult males and females of the same age were collected directly from either vials or bottles.

2.2.3 RNA Extraction

RNA extraction was done based on the guanidinium method of (Chomczynski, 1993) using the TRIzol®LS reagent. Embryos/Larvae/Pupae/Adult flies were

collected and transferred to 1.5 ml eppendorf tubes. 500 µl of TRIzol was then added and a plastic homogeniser used to homogenise the samples. The sample was vortexed and 200 µl of chloroform was added. Sample was then centrifuged at 13,000 g at 4°C. Supernatant was collected and 200 µl of isopropanol and centrifuged at 13,000 g at 4°C. Pellet was collected and washed with very cold ethanol (75 µl). Ethanol was then removed and pellet left to air dry. The pellet was dissolved in 50 µl of RNase free water. The samples were then heated in a dry bath at 65°C for about 10 mins and then placed on ice immediately. Concentration of the RNA samples was determined by use of the Nanodrop®.

2.2.4 Reverse Transcriptase Polymerase Chain Reaction

CDNA Synthesis

A reaction tube was placed on ice and about 5 µg of RNA was placed into the tube. 1 µl of Oligo (dT) primer was added and Nuclease free water was also added to make up 12 µl. 1 µl each of Rnase inhibitor (Ribolock) and Revertaid reverse transcriptase was placed in the tube followed by 4 µl of the reaction buffer and 2µl of the 10 mM DNTP mix. Samples were then incubated for 60 mins at 42°C and the reaction terminated by heating at 70°C for 5 mins.

Polymerase Chain Reaction

RT-PCR was performed based on the protocol provided on the PCR Mastermix booklet #K1071. All reagents were added onto a PCR tube standing on ice. After all the reagents have been properly added, the tubes were placed onto the PCR machine. Below is an example of a PCR program.

Table 2.7: An example of a PCR program

	Temperature	Time	Number of cycles
Initial denaturation	94°C	2 mins	1 cycle
Denaturation	94°C	20 secs	30-40 cycles
Annealing	50-65°C	30 secs	
Extension	72°C	60 secs	
Final Extension	72°C	5mins	1 cycle

Annealing temperatures are determined by the Tm of primers used.

Agarose gel Electrophoresis

1% Agarose gels were prepared by mixing the exact adequate amount of agarose powder in the electrophoresis buffer (1X TAE). The mixture is then heated until solution is clear. When solution was cooled to about 60°C, ethidium bromide (0.5 mg/ml) was then added and the solution poured onto a casting tray containing the comb and left to solidify at room temperature. After solidifying, the comb was gently removed and the gel placed in the chamber. The 1X TAE buffer was poured into the chamber and samples loaded into the gel wells. The electrophoresis tank was connected to a power supply and left to run. After a successful run the gel was then visualized using a Bio-Rad Gel doc System.

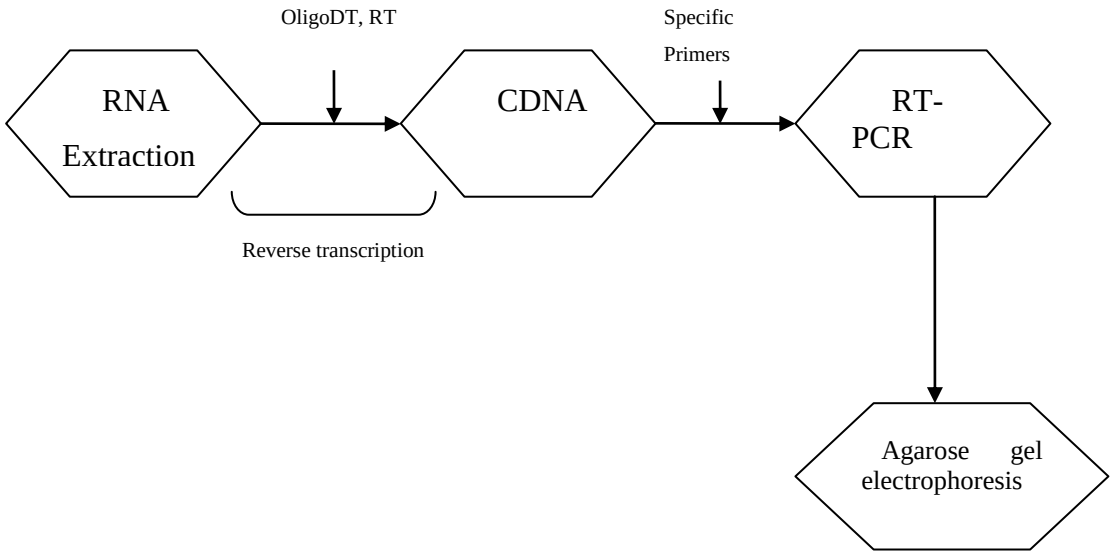


Figure 2.2: Schematic representation of RT-PCR showing binding of primer (oligoDT) and enzyme (Reverse transcriptase) to synthesize cDNA from mRNA and binding of primers to cDNA to produce multiple copies of the gene.

2.2.5 Cloning

Ligation

Ligation was performed according to the protocol in the pGEM®-T Easy vector system II Catalogue #A1360 including Positive and Negative controls. After all reagents were added into the tubes, the reactions were mixed properly and incubated overnight at 4°C. Overnight incubation was performed to get the maximum number of transformants.

Transformation of cells

LB/Ampicillin/IPTG/X-Gal plates were made in preparation for cell transformation. Ligation reactions were centrifuged briefly and 2 µl of the reaction transferred to sterile 1.5 ml eppendorf tubes on ice. The JM109 High-Efficiency cells were placed on ice for about 5 mins to thaw. Then 50 µl of the cells was added to each of the tubes containing the ligation reaction. After flicking

the tubes gently, they were left to incubate in ice for 20 mins. They were then removed and heat shocked in a water bath for about 45-50 seconds at 42°C and then returned on ice immediately for 2 mins. 950 µl of room temperature LB was added and the mixture was left to incubate for 90 mins at 37°C. 100 µl of mixture was plated in the LB/ampicillin/IPTG/X-Gal plates previously prepared and incubated overnight at 37°C. Colonies were observed and counted manually. White colonies were then selected for plasmid DNA purification and also plated in fresh new plates to generate and store more clones.

Plasmid Purification using Qiagen mini purification kit

A single white colony was cultured overnight 1.5 ml eppendorf tubes and centrifuged at 13,000 g for 10 mins. The pellet was then re-suspended in 150 µl of buffer 1. 150 µl of buffer 2 was then added and mixture is left to stand at 25°C for 5 mins. 150 µl of buffer 3 was added and mixture was again gently mixed and left to stand at room temperature. The mixture was then centrifuged at 13,000 g for 10 mins and the supernatant collected. 2.5 volumes of Ethanol Acetate (1:25) was then added and mixture is kept at -70°C for 40 mins. After spinning at 13,000 g for 10 mins, the pellet was washed with 70% ethanol. Ethanol was then discarded and pellet allowed to air dry. 50 µl of Nuclease free water was then added and mixture left on ice to dissolve.

Restriction digestion

Restriction enzymes were used to digest the plasmid DNA. 0.5- 1 µg of DNA was added into a tube on ice followed by 3 µl of the appropriate buffer. 1 µl of the enzyme is then added. Nuclease free water was then used to make the volume up to 30 µl.

Nucleic acid sequencing

Samples of DNA in the form of PCR products or plasmid clones were sent to Inqaba-biotec for sequencing.

2.2.6 Bioinformatics analysis

Sequence alignments were done using the DNAMAN programme version 4.03 (Lynsoft Biosoft). Using the National centre for biotechnology information (NCBI) database several blasts were performed to compare the sequences with other sequences in the database.

2.2.7 *In vitro* Transcription

In vitro transcription was performed based on the protocol described in the Riboprobe® *in vitro* transcription system catalogue. After adding the required reagents into a tube standing at room temperature, the mixture was then incubated at 37°C for 1hr. To test the integrity of the sample afterwards, the sample was then run on a gel and viewed using the Gel Doc system. After the integrity of the sample had been ascertained, the template DNA was then removed based on the protocol also described in the Riboprobe® *in vitro* transcription system catalogue.

2.2.8 Survival experiment

Fly treatment and counting

800 flies (400 males and 400 females) were placed in different vials of 20's. Five independent experiments was set up. The flies were then exposed to various forms of treatments for 3 days and then were transferred to fresh vials containing normal yeast for them to recover.

The treatment categories include;

a. 1mM irinotecan
b. 2.5% methyl pyruvate only
c. 5% methyl pyruvate only
d. 10% methyl pyruvate only
e. Irinotecan + 2.5% methyl pyruvate
f. Irinotecan + 5% methyl pyruvate
g. Irinotecan + 10 % methyl pyruvate

The recovery period lasted for nine days and flies were counted every three days in order to ascertain the number of dead and alive flies.

Kaplan-Meier Survivorship analysis

The number of dead and living flies was observed at an interval of three days over a 12 day period. This raw data was then analysed using the Kaplan- Meier survivorship plots. The Kaplan-Meier survival plot is defined as the probability of an organism to survive in a given time period, while taking into consideration time in short intervals (Goel et al., 2010). Along the x-axis of the Kaplan-Meier plot are horizontal lines that represent the duration of survival at that particular interval. This interval is aborted by the occurrence of an event of interest, which in our case is death. The vertical lines however, are just to make the plot more appealing, although, the distances between the horizontal lines are critical because the show the changes in cumulative probability as the plot progresses.

The formula for calculating the survival probability at a given time is;

$$S_t = \frac{\text{Number of subjects living at the start} - \text{Number of subjects dead}}{\text{Number of subjects living at the start}}$$

The plot is a series of declining “staircase-like” horizontal steps. It is widely used in the field of medical sciences to estimate the number of patients alive after a particular period of time. This estimation is somewhat complicated by the fact that not all patients survive through the duration of the experiment as some patients drop out or die. The group of patients that die or drop out are called “censored observations”. Kaplan-Meier plot is unique in that it is able estimate a survival curve that includes censored observations.

3. Results

3.1 Introduction

The aim of this study was to define temporal expression patterns of Dmp53 and SNAMA during development and during response to genotoxic stress. We also investigated the effects of bypassing the glycolytic pathway by the treatment of adult flies with methyl pyruvate. Genetic studies using *Drosophila* have resulted in elucidation of the expression of numerous genes that are observed during development and during various kinds of stresses (Arbeitman, 1995). The recent discovery of a second smaller *Snama* isoform (*Snama B*) has prompted the study of their functional importance, particularly their role in DNA damage response.

3.2 Temporal expression of *Snama* isoforms.

In order to show the expression of *Snama* isoforms during development of the *Drosophila* adult males and females were placed in several fly cages and at varying time intervals correlating to various stages of development of *Drosophila*, agar plates were used to collect samples. Temporal expression patterns were demonstrated by RT-PCR; Total RNA from adult males and females, embryos, larvae and pupae was used. RT-PCR performed in three independent experiments showed that both isoforms are differentially expressed during development (Figure 3.1). Higher amounts of these isoforms are present in the embryos but there is an observed decrease during the adult stages. *Snama B* seems to be decreased at the late embryonic stage (E3), pupal and adult stages. *Snama A* is highly expressed in all stages except in the adult males where it is highly reduced and in the E2 embryonic stage where it is undetectable by RT-PCR.

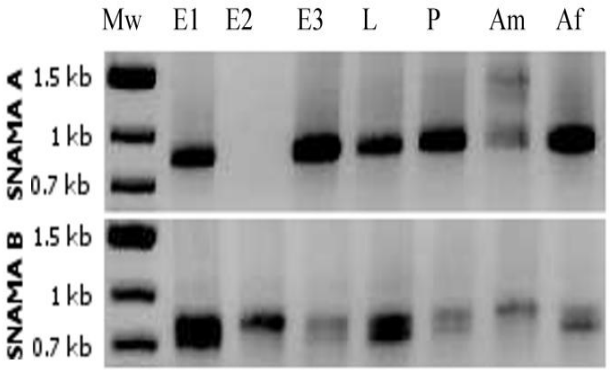


Figure 3.1: Expression of *Snama* isoforms during development. Agarose gel showing RT-PCR products on RNA obtained from embryonic stages E1 (0-3hrs), E2 (3-6hrs), E3 (6-9hrs), Larvae (L), Pupae (P), adult males (Am) and from adult females (Af). *Snama* A and *Snama B* are both differentially expressed during development, although *Snama A* is undetectable during E2.

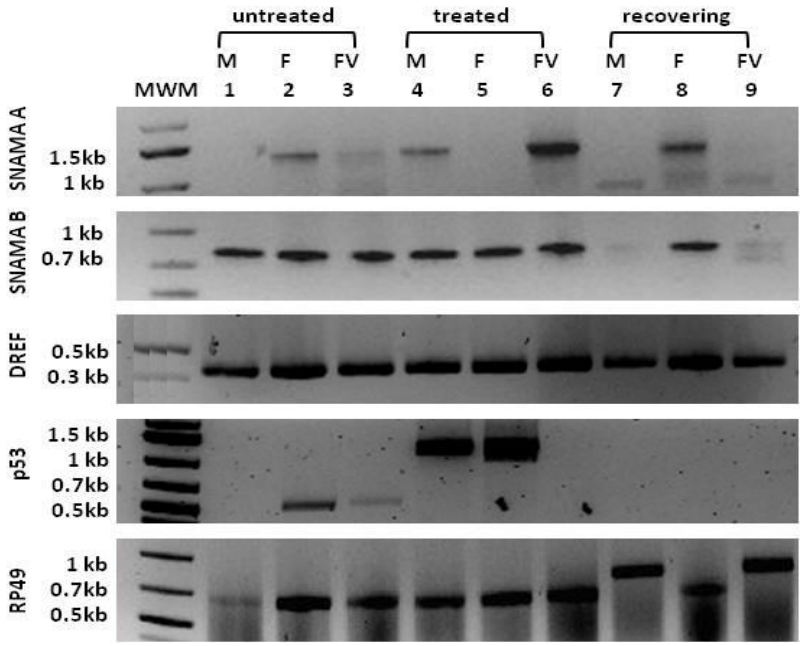
3.3 Expression of *Snama* isoforms, *DREF* and *p53* in untreated and in flies undergoing genotoxic stress.

To induce genotoxic stress, flies were exposed to 1mM irinotecan. A group of these flies were left to recover for another three days by transferring them into fresh vials. RT-PCR performed in three independent experiments *Snama* isoforms are expressed differentially during treatment (Figure 3.2). *Snama B* appears to be expressed in the same amounts in untreated and treated flies; however slight upregulation is observed in treated female flies. Differential expression of *Snama A* is observed in treated and untreated flies. It is upregulated in treated males and in virgins and returns to "normal" levels upon recovery.

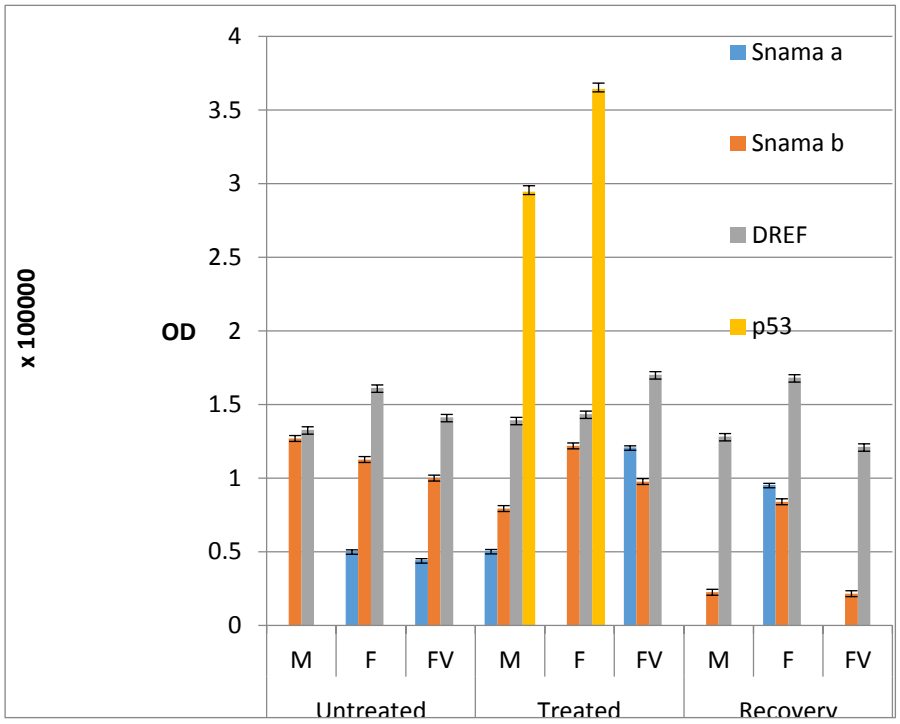
Three independent experiments show that p53 is expressed probably as a shorter isoform in untreated females whereas during treatment a larger isoform is expressed in males and females (Figure 3.2). An upregulation of the level of p53 is observed during irinotecan treatment.

The master cell proliferation gene, *DREF* (Matsukage et al., 2008), is observed to be present in relatively same amounts in the untreated, treated and recovering flies.

RP49 (Ribosomal protein 49), a house keeping gene, is shown to be expressed in all categories, however, the gene expression observed during recovery in males and female virgins seem to be slightly different in size (Figure 3.2). After several repetitions, consistent results were observed and we suggest that a larger isoform of *RP49* is being expressed under those conditions. However, we are still unsuccessful in sequencing after several trials.



a



b

Figure 3.2: Expression patterns of *Snama A*, *Snama B*, *DREF*, *p53* and *RP49* in untreated, treated and flies recovering from irinotecan treatment.(a) Agarose gel depicting expression of the various genes at various conditions. (b) Densitometry reading of 3 independent experiments normalized by RP49 expression. (M=male, F=female, FV= virgins).

3.4 Survival Analysis of Adult male and female flies when exposed to Methyl Pyruvate alone and in combination with Irinotecan.

Irinotecan, a derivative of camptothecin, targets and inhibits the Topo1 enzyme which plays a crucial role in replication of DNA (Hsiang et al., 1985). Irinotecan is often used in cancer treatment. In the current study, irinotecan is used to induce genotoxic stress.

Methyl pyruvate provides the end-product of the glycolytic pathway. By treating flies with methyl pyruvate, the glycolytic pathway is bypassed. Previous experiments conducted on flies show that methyl pyruvate resulted in toxicity at the concentration of 10% (Hull and Ntwasa, 2010). In the current study, 800 adult flies (400 males and 400 females) were collected, placed in carefully labelled vials flies and treated for 3 days with 1mM irinotecan, 10% methyl pyruvate and a combined treatment of 1mM irinotecan and 10% methyl pyruvate by administration in yeast blobs. Flies were then transferred to fresh vials for 9 days to allow for recovery. In adult males and females, the combined treatment (methyl pyruvate + irinotecan) and treatment with irinotecan alone had similar survival trends (Figure 3.3).

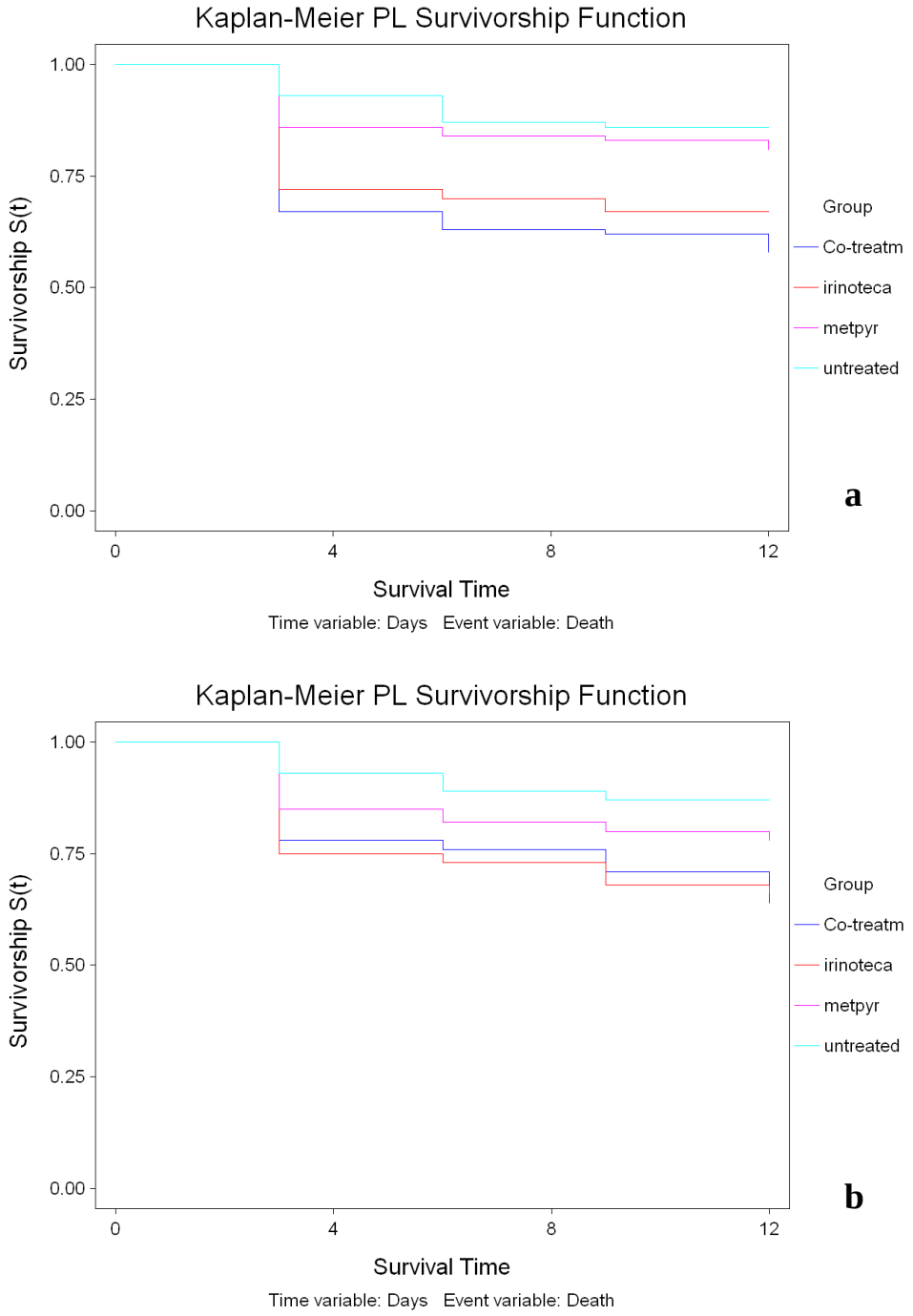
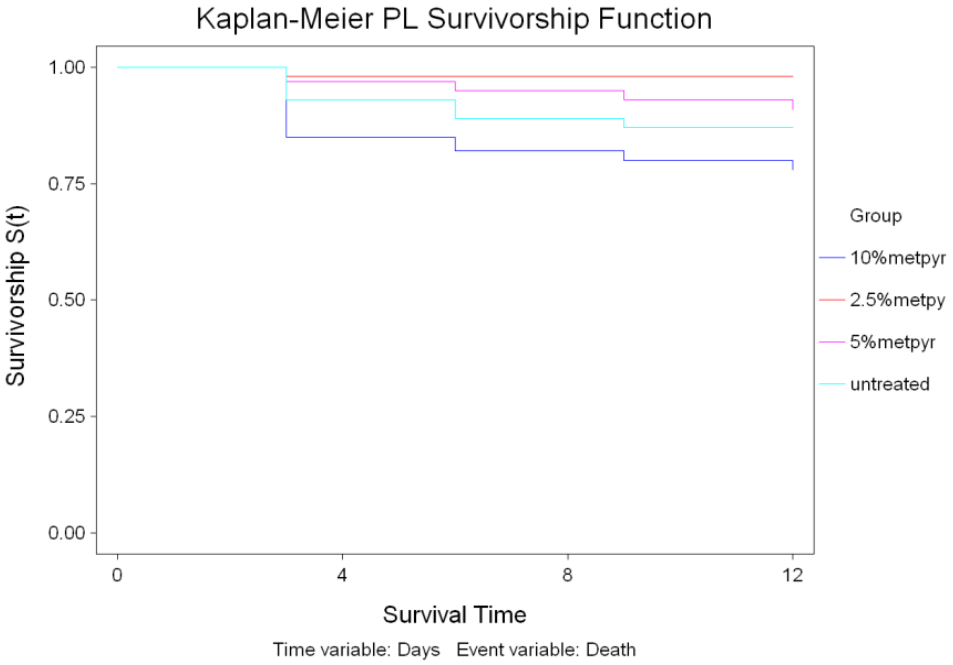


Figure 3.3: Survival analysis of male and female adult flies with different treatments. In both males (a) and females (b), treatment with 1mM irinotecan and 10% methyl pyruvate seems to be toxic to the flies. The plot indicates different groups; co-treatm (10% methyl pyruvate + 1mM irinotecan), irinoteca (1mM irinotecan), metpyr (10% methyl pyruvate only).

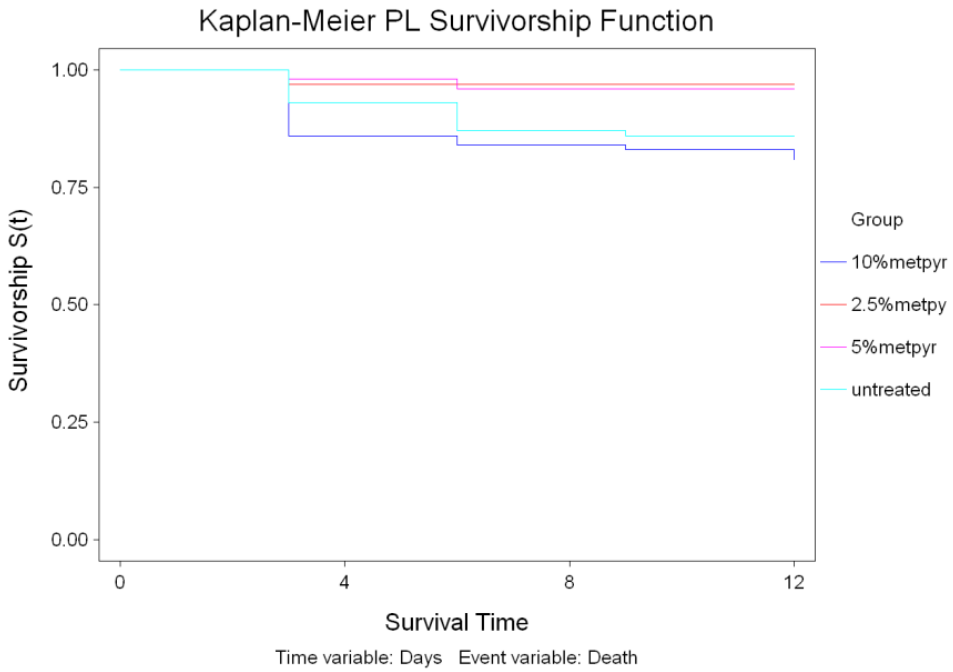
Since it is possible that high dosage of methyl pyruvate seems to induce toxicity, its concentration was varied to 2.5%, 5% and 10% in another experiment

performed with 800 flies. After five independent experiments, it was observed that male flies treated with 2.5% had the highest survival rate, followed by flies treated with 5% methyl pyruvate (Figure 3.4a). Flies treated with 2.5% and 5% methyl pyruvate seem to have greater survival rates compared to untreated flies. At 10% concentration of methyl pyruvate, the flies had the least survival rates just as observed by (Hull and Ntwasa, 2010). The best survival trend can be observed in flies treated with 2.5% methyl pyruvate only, suggesting that this concentration is optimal. The same trend was also observed in the female flies in which flies treated with a concentration of 2.5% had the highest survival rate and those treated with 10% had the lowest (Figure 3.4b).

After ascertaining that reducing the concentration of methyl pyruvate given to flies somehow increases their survival rates the obvious next step was to also vary concentration of methyl pyruvate when combined with irinotecan. After a 3 day treatment and a 9 day recovery period in which flies were transferred to fresh vials, it was observed that at a combined treatment of 10% methyl pyruvate and irinotecan the effects were toxic as observed by (Hull and Ntwasa, 2010) but at 5% methyl pyruvate concentration the flies seem to have a high survival rate and fared even better than untreated flies (Figure 3.5 a,b), suggesting that this concentration is optimal when combined with the DNA damaging agent; irinotecan.

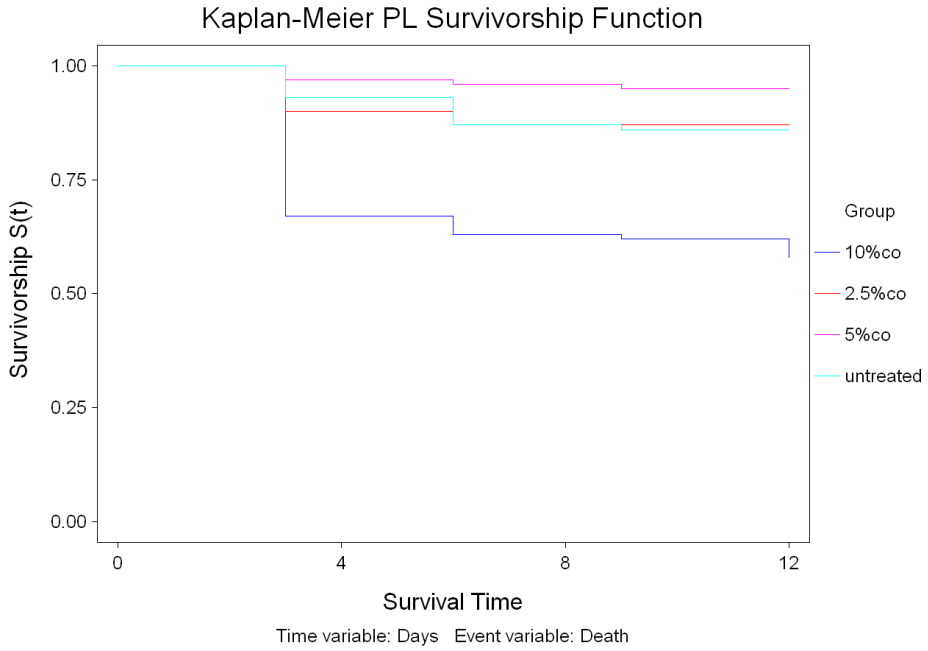


a

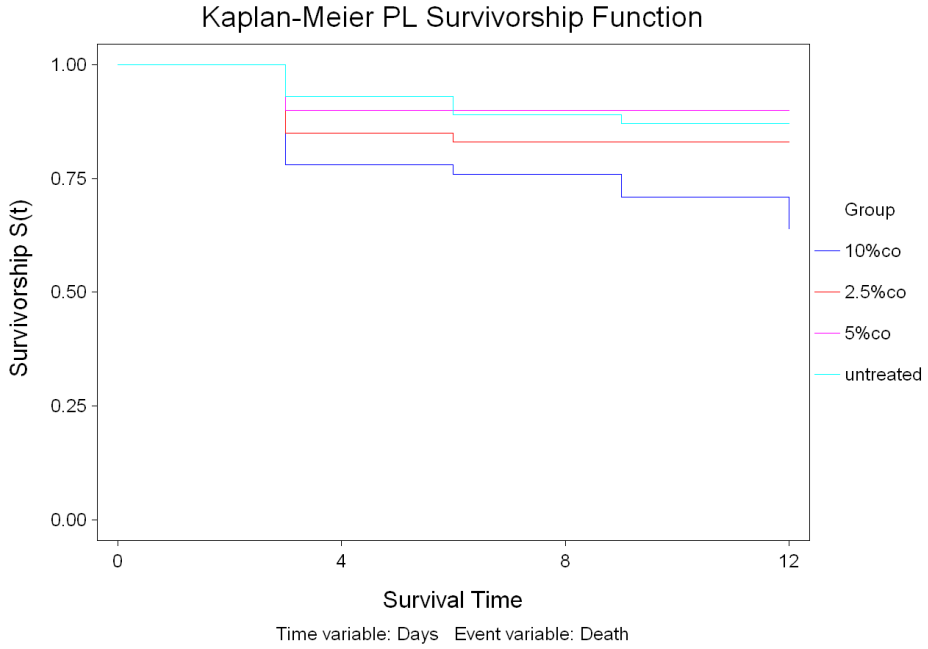


b

Figure 3.4: Survival analysis of adult male and female flies treated with methyl pyruvate alone. The plot indicates different groups; 10% metpyr (10% methyl pyruvate only), 2.5% metpyr (2.5% methyl pyruvate only), 5% metpyr (5% methyl pyruvate only) and untreated.



a



b

Figure 3.5: Survival analysis of adult male and female flies when treated with methyl pyruvate and irinotecan. The plot indicates different groups; 10%co (10% methyl pyruvate + 1mM irinotecan), 2.5%co (2.5% methyl pyruvate + 1mM irinoteca), 5%co (5% methyl pyruvate + 1mM irinotecan) and untreated.

3.5 Molecular analysis of the effects of different concentrations of methyl pyruvate on the expression of *Snama* isoforms and the Reaper gene (*Rpr*).

Survival analysis showed that different methyl pyruvate concentrations affect the survival rate of adult flies and this prompted further investigations on the effects methyl pyruvate at the molecular level. Adult flies were treated with 2.5% methyl pyruvate (the observed optimal concentration) and 10% methyl pyruvate (the observed toxic concentration). We observed that *Snama A* was upregulated during 10% methyl pyruvate treatment and *Snama B* upregulated during treatment with 2.5% methyl pyruvate (Figure 3.6).

Rpr, an apoptosis inducer, becomes upregulated during treatment with both 2.5% and 10% methyl pyruvate.

RP49, seem not to be expressed during recovery at 2.5% methyl pyruvate treatment and this result was consistent after triplicate experiments. Therefore, we hypothesize that at those conditions the levels of RNA RP49 might be too low to be seen.

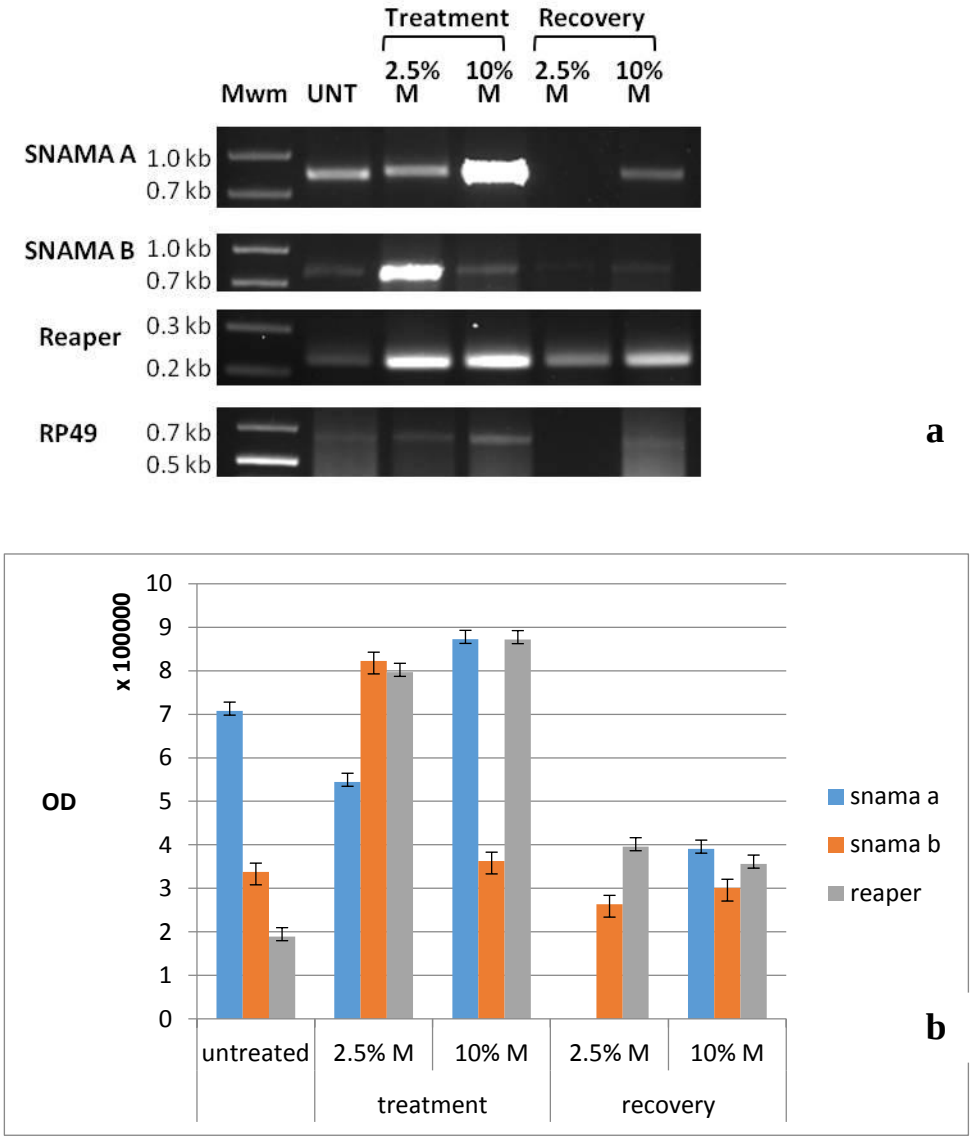


Figure 3.6: Expression of *Snama* isoforms and *Rpr* during treatment with methyl pyruvate. (a) Agarose gel depicting expression of the various genes at various conditions. (b) Densitometry reading of 3 independent experiments normalized by RP49 expression. (M= methyl pyruvate)

3.6 Molecular analysis of Adult male and female flies when Methyl Pyruvate is combined at different concentration with irinotecan.

We investigated the effects on the molecular level of a combined treatment of irinotecan with 5% methyl pyruvate (optimal concentration) and 10% methyl pyruvate (toxic concentration). This investigation was prompted in order to

observe what changes occur on the molecular level when these treatments were administered. Changes on the expression levels of *Snama* isoforms and the reaper (*rpr)* gene were observed.

Three independent experiments showed that the levels of *Snama A* (the longer isoform) were unchanged during the combined treatment with 5% methyl pyruvate and also during recovery and that at 10% methyl pyruvate, however, *Snama* B levels seem to slightly increase at 5% methyl pyruvate and decreased slightly at 10% (figure 3.7). Significantly, there is an observed slight decrease of the levels of the *reaper* (*rpr*) gene during recovery at treatment with 5% methyl pyruvate.

RP49 (Ribosomal protein 49) is a house keeping gene in the *Drosophila.* Figure 3.7 shows the expression of RP49 in all categories of treatment except in the untreated category, even after 3 independent experiments. This might be due to the use of male flies (which have been observed to show low *RP49* expression levels). Furthermore, other transcripts (*Snama A*, *Snama B* and *Rpr* are clearly detectable in the same sample confirming the presence of mRNA.

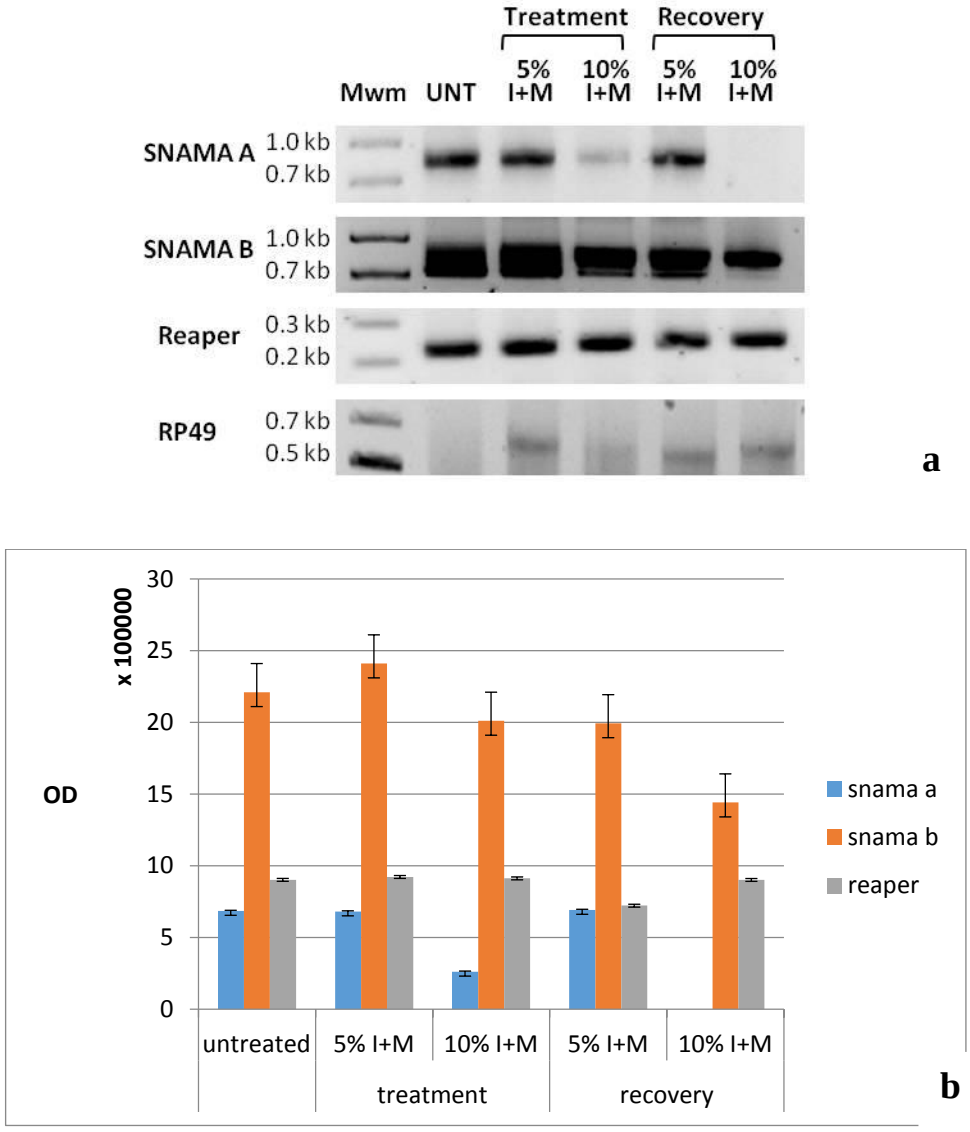


Figure 3.7: Expression of *Snama* isoforms and *rpr* during a combined treatment of irinotecan and methyl pyruvate. (a) Agarose gel depicting expression of the various genes at various conditions. (b) Densitometry reading of 3 independent experiments normalized by RP49 expression. (M=methyl pyruvate).

3.7 Survival analysis of *p53*$^{-/-}$/*Snama*$^{-/-}$ Adult flies when treated with irinotecan and methyl pyruvate.

We investigated the effects of irinotecan and methyl pyruvate treatments on adult flies in which p53 and *Snama* have been knocked down. Flies were treated for 3 days and then left to recover for 6 days. During this 9-day period, flies were counted every 3 days to ascertain dead and living flies. Untreated mutant flies

(untmut) seem to have a higher survival rate compared to untreated wild type flies (untwt). Irinotecan-treated mutant flies (irnmut) also survived more than irinotecan-treated wild type flies (irnwt). Interestingly, both wild type (irn5mpwt) and mutant flies (irn5mpmut) treated with a combination therapy of irinotecan had the same survival patterns which was equivalent to those observed in untreated wild type flies. The patterns for both irn5mpwt and irn5mpmut are consistent with patterns of untreated wildtype flies (untwt) so their patterns lie behind the untwt yellow line; therefore they are unseen in this plot (figure 3.8).

1mM of irinotecan and the optimum concentration of 5% methyl pyruvate were used in this experiment.

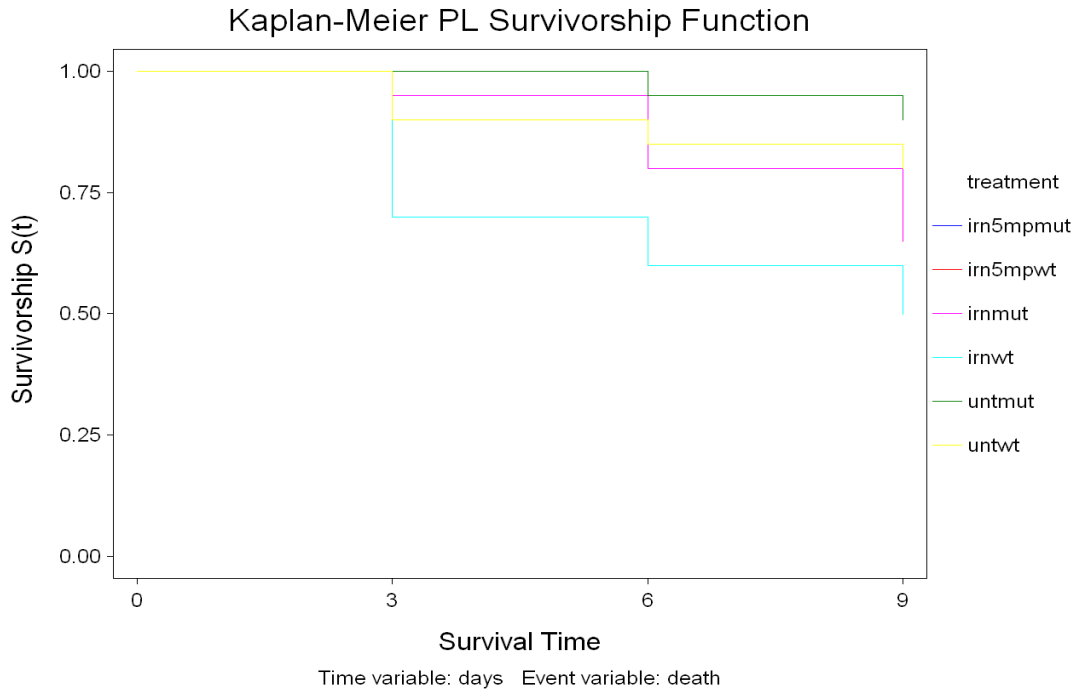


Figure 3.8: Survivorship plots of *p53*$^{-/-}$/*Snama*$^{-/-}$ adult flies during treatment. Treatment categories include; Untreated mutant flies (untmut), untreated wild type flies (untwt), Irinotecan-treated mutant flies (irnmut) irinotecan-treated wild type flies (irnwt), mutant flies treated with irinotcan + methyl pyruvate (irn5mpmut), wild type flies treated with irinotcan + methyl pyruvate (irn5mpwt).

3.8 Cloning of SNAMA isoforms.

In preparation for *in vitro* transcription, we cloned both *Snama* isoforms (A and B). Primers for both isoforms were design in such a way as to carefully distinguish both isoforms. Primers for *Snama A* were designed from its 10th exon and primers for *Snama B* were designed from its 3′ UTR. These regions are unique to the isoforms. After cloning, both samples were linearized using restriction enzymes *Sal1* for *Snama A* and *AatII* for *Snama B* (Figure 3.9).

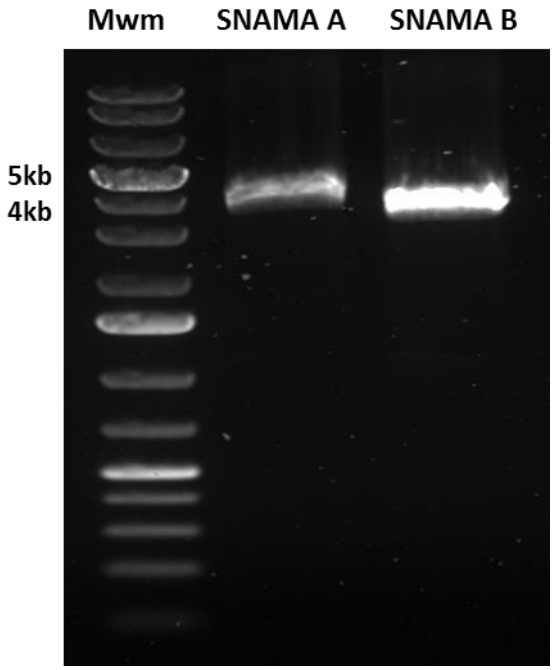


Figure 3.9: Linearized *Snama A* and *B*. Cloned SNAMA isoforms are about 4kb in length. The plasmid used was pGEM-T, which is 3kb in length and the inserts are both about 1kb in length.

4. Discussion

In this study, the existence of two SNAMA isoforms has been confirmed. It is also demonstrated that they are expressed differentially during embryogenesis. Furthermore, these isoforms are differentially regulated upon genotoxic stress using DNA damaging agents often used during chemotherapy. A potentially important aspect of this study is the evidence that when the glycolytic pathway is bypassed by the use of methyl pyruvate, beneficial effects can be observed in normal cells that are exposed to the chemotherapeutic agents. Another important aspect of this study is the observation that indeed SNAMA may have equivalent biological roles to Mdm2, the prototypical negative regulator of p53.

The differential expression of SNAMA isoforms during development suggests that they play unique roles. The temporal expression patterns suggest that the isoforms are required at different stages during embryogenesis. For example, the absence of *Snama A* during the formation of the cellular blastoderm suggests that it is not required at this stage. It has also been observed that the *Snama* transcripts present during the early stages of embryogenesis were deposited by the female during oogenesis as early embryos do not transcribe genes. Furthermore, these observations suggest that the females overexpress *Snama* during oogenesis.

During DNA damage, the differential expression of SNAMA might also suggest different roles. It is postulated that SNAMA may function by interacting with p53 in the same manner as the human (RBBP6) and mouse (PACT) orthologues. It is therefore suggested that this interaction somehow negatively regulates p53 levels

as it has been observed that higher levels of SNAMA coincide with decreased levels of p53. It has also been observed in the current study that the *p53*$^{-/-}$/*Snama*$^{-/-}$ and the *p53*$^{-/-}$/*Mdm2*$^{-/-}$ phenotypes are similar. This observation further strengthens the notion that SNAMA interacts and regulates p53 just like Mdm2 does in vertebrates.

It has also been shown that by bypassing the glycolytic pathway we relieve stress during chemotherapy. An increase in survival trend observed when methyl pyruvate is administered during chemotherapy may be due to p53-dependent control of the glycolytic pathway with consequent apoptosis since p53 interposes at different points in the glycolytic pathway. Furthermore, upregulation of *Snama* isoforms during this treatment is significant as evidence suggests that the overexpression of SNAMA further reduces elevated p53 levels during chemotherapy, thus alleviating apoptotic death.

4.1 *Snama* isoforms are differentially expressed throughout development.

Previous studies have shown that SNAMA plays a critical role in embryonic development. When *Snama* was knocked out, lethality of the embryo occurred (Mather et al., 2005). Recently, a second isoform of *Snama B* was discovered through bioinformatics analysis performed by Flybase developers (Dos Santos *et al* 2015). This isoform shares the first 7 exons with *Snama A* (Figure 4.1).

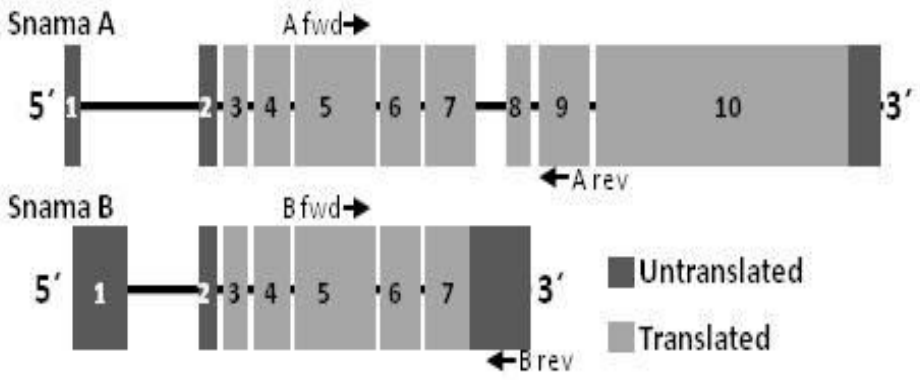


Figure 4.1: Schematic representations of *Snama A* and *Snama B* showing their exons and untranslated regions. The darker regions are the untranslated regions and the lighter regions are the translated regions as indicated in the figure. Arrows indicate primers that were used in this study to amplify the isoform specifically.

Source: Hull et. al (submitted)

RT-PCR showed that the 2 isoforms are expressed during development differentially. During the early stages of embryogenesis, embryos do not express *Snama* and the gene expressed is maternally derived. However, at the E2 stage (3-6 hr), when the formation of cellular blastoderm begins, *Snama A* is undetectable by RT-PCR and may not be required. The maternal *Snama B* persists during this period suggesting that it is required for an extended time when compared to *Snama A*. It is significant that *Snama A* is then expressed zygotically by the embryo at the E3 stage (6-9hrs) onwards whereas, *Snama B* is not. At this stage a new wave of cell proliferation is known to occur in the embryo driving gastrulation. This implicates *Snama A* in cellular proliferation which is the major process driving this developmental stage. The expression of both isoforms differentially during development may also indicate that they play crucial and different roles.

4.2 SNAMA isoforms are differentially expressed during DNA damage

When wild type flies are treated with irinotecan, p53 is up-regulated in adult flies except in virgins where it is undetectable by RT-PCR. On the other hand, both isoforms of SNAMA are expressed differentially (figure 3.2). It is interesting that the upregulation of both *Snama A*, and *Snama B* are matched with increases in p53 levels of expression. This may suggest activation of *Snama* by p53. However, there is no evidence, currently, suggesting that p53 can activate transcription of *Snama*. This study shows that during treatment with the DNA damaging agent the dominant expression in adults was that of p53 whereas the dormant expression in virgins was that of *Snama*. It is interesting that the expression patterns during recovery are similar in males and in virgins and differing with adult females. This is important because in a previous study, females seemed to respond differently to males with females showing a more severe phenotype (Hull and Ntwasa, 2010).

4.3 Methyl pyruvate has beneficial effects during chemotherapy

Irinotecan inhibits the topoisomerase 1 enzyme that regulates unwinding and rejoining of DNA strands during DNA replication (Liu et al., 2000). This inhibition enables it to kill rapidly dividing cells such as cancer cells. However, due to the fact replication is a normal physiological process and due to the fact some cells, such as reproductive cells and hair follicle cells also divide rapidly, irinotecan is also lethal to normal cells especially the rapidly dividing ones. Treatment with a DNA damaging agent induces p53-dependent apoptosis (Morris and Geller, 1996). It is well known that treatment with irinotecan is toxic which has been confirmed in this study with the low survival rates observed. These deaths are likely to be as a result of a p53-dependent apoptosis that is induced by

irinotecan. The interposition of p53 at various points in the glycolytic pathway enables critical components to be tightly regulated and also results in p53-induced apoptosis. Bypassing this pathway might ensure that p53-induced apoptosis is avoided. This makes sense since, methyl pyruvate treatment in this study has been shown to be beneficial as it increases survival rate during chemotherapy. This theory can be supported by the fact that SNAMA is upregulated during treatment with methyl pyruvate thus reducing p53-dependent apoptosis. SNAMA, is suggested to play a role as a repressor of apoptosis or in negatively regulating an apoptosis regulator (Mather et al., 2005). Similarly, the mouse homologue PACT was shown to negatively regulate p53 in an Mdm2 dependent manner (Li et al., 2007). Since p53 levels are increased during DNA damage, it is suggested that the upregulation of SNAMA decreases the levels of p53. This stipulation is derived from the fact that overexpression of the RBBP6 mouse homologue, PACT, results in the Mdm2-dependent degradation of p53 (Li et al., 2007). As this study has shown, an increased survival trend is observed with methyl pyruvate treatment; this can also be explained by the overexpression of SNAMA which we suggest helps decrease p53 levels thus reducing p53-mediated apoptosis. These results suggest that methyl pyruvate relieves genotoxic stress and that the clinical application of this may help increase the survival of patients undergoing chemotherapy.

4.4 SNAMA probably plays similar roles to Mdm2

In the current study it has been shown that when the p53 and *Snama* genes are knocked down simultaneously, there is an increase in survival rate. p53 regulates apoptosis and this means that without it p53-dependent apoptosis cannot occur.

Consequently, cells live longer when this pathway is disrupted by the absence of both p53 and *Snama*. Significantly this phenotype is identical to that of the *p53*$^{-/-}$/ *Mdm2*$^{-/-}$. The similarity of the *p53*$^{-/-}$/ *Mdm2*$^{-/-}$ and *p53*$^{-/-}$/*Snama*$^{-/-}$ phenotype is no co-incidence as SNAMA and Mdm2 both possess some structural similarities like the RING finger domain and the p53 and Rb binding sites. These structural features may enable SNAMA to be able to function in the degradation of p53. The increase in survival trends observed in *p53*$^{-/-}$/*Mdm2*$^{-/-}$ by (Kubbutat et al., 1997) and in p53$^{-/-}$/Snama$^{-/-}$ in the current study might be because of the absence of the apoptosis inducer p53 and a crucial role played by SNAMA in p53-dependent apoptosis. It is therefore tempting to say that SNAMA might function as Mdm2 does.

5. Conclusion

Contrary to initial beliefs, it is now known that *Snama* is a broad gene with multiple transcipts. The expression of *Snama* isoforms at different periods in the developmental stages hints about their distinctive roles. Also, the expression of *Snama* during genotoxic stress with a possible interaction with p53 makes it a possible target for anti cancer drugs.

The beneficial effects of methyl pyruvate observed in the current study can be applied clinically in treatment of cancer patients during chemotherapy.

6. Future work

The current study has shown the temporal expression patterns of the isoforms during development, however, future studies on the spatial expression of the isoforms by *in situ* hybridization should be carried out. Knowledge about their cellular localization may help in elucidating their roles. Also using a technology that can produce mutants possessing a single *Snama* isoforms can also help in finding out the unique roles each of the isoforms play.

It is now known that *Snama* is expressed during genotoxic stress and this study suggests that *Snama* might play the same role as Mdm2. Future studies regarding the interaction between *Snama* and p53 on how expression of p53 is affected by the absence of *Snama*. This can be done by the knocking down of *Snama* and analysing by Western blot analysis the expression levels of p53.

It is also now known that by bypassing the glycolytic pathway, stress during chemotherapy is relieved probably because of the circumvention of p53-

dependent apoptosis. In future studies this has to be confirmed by conducting apoptotic assays.

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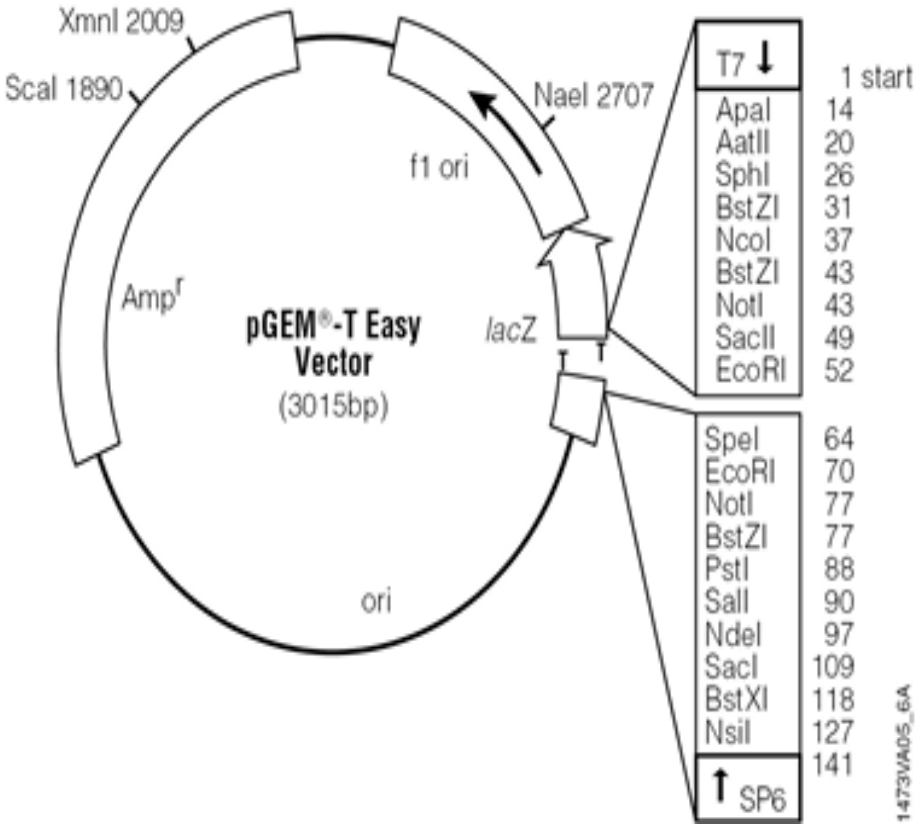
Appendix A, pGemT Easy vector.



Appendix B, 1kb Plus DNA Ladder.

