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THE BIOCHEMICAL FUNCTIONS OF THE RETINOBLASTOMA BINDING PROTEIN 6 (RBBP 6) ISOFORMS IN METABOLIC REPROGRAMMING OCCURRING DURING CARCINOGENESIS

University of the Witwatersrand, Johannesburg, 2050, Molecular and cell biology.

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg 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 at any other University.

Signature:



Zanele Nsingwane

04 June 2018.

ABSTRACT

The Retinoblastoma binding protein 6 is dysregulated in most cancers, indicating it may play a role in metabolic reprograming- a hallmark of carcinogenesis. Its human isoforms have been shown to play diverse roles in apoptosis. This study aimed to elucidate biochemical roles of RBBP6 isoforms in metabolic reprogramming during carcinogenesis. *Drosophila melanogaster* wild type and p53 null mutants were treated with drug permutations of irinotecan (DNA damaging agent) and exogenous pyruvate to perturb metabolism. Moreover, using RT-PCR and Western blot expression profiles of SNAMA (*Drosophila* Orthologue of RBBP6) isoforms were shown followed by survival studies to investigate the effects of these drugs. Furthermore, using bioinformatics the domains of RBBP6 isoforms in various species were shown. Results indicate that RBBP6 isoforms show contrasting expression patterns. Furthermore, exogenous pyruvate protects the wild type flies from irinotecan toxicity while killing p53 null mutants. RBBP6 proves to be a potential druggable target for chemotherapy.

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QUOTATION

Knowledge can be communicated but not wisdom. One can find it, live it, be fortified by it, do wonders through it, but one cannot communicate and teach it.

Hermann Hesse.

RESEARCH OUTPUTS

Poster Presentation

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LIST OF ABBREVIATIONS

AIDS: Acquired immunodeficiency syndrome

AIF: Apoptosis Inducing Factor

ATP: Adenotriphosphate

ADP: Adenosine diphosphate

CAD: Caspase deoxyribonuclease

CANSA: Cancer Association in South Africa

cDNA: Complementary Deoxyribose Nucleic Acid

CDK: Cyclin Dependant Kinases

CO_2: Carbon dioxide

CPT-11: Camptothecin-11

DIAPI: Drosophila Inhibitor of Apoptosis

Dmp53: *Drosophila* melanogaster protein 53

DNA: Deoxyribose Nucleic Acid

DWNN: Domain with No Name

EDTA: Ethylene diamine tetraacetic acid

EtBr: Ethidium bromide

Fwd: Forward

GLUT: Glucose transporter

Hid: Head Involution Defective

Hr: Hour

IAPs: Inhibitors of Apoptosis

ICAD: Inhibitor of Caspase deoxyribonuclease

kDa: Kilo Dalton

Mdm2: Mouse double minute 2

Mei-41: Meiotic 41

Mins: Minutes

ml: Millilitres

MP: Methyl Pyruvate

O_2: Oxygen

OXPHOS: Oxidative Phosphorylation

P2P-R/PP-RR: Proliferation Potential-Related Protein

PACT: P53-Activated Cell Testes derivative

PAR: Poly- ADP-Ribose

PARP: Poly- ADP-Ribose Polymerase

PBS: Phosphate Buffer Saline

PCR: Polymerase Chain Reaction

PGM: Phosphoglycerate Mutase

PPP: Pentose Phosphate Pathway

Rb: Retinoblastoma

RBBP6: Retinoblastoma Binding Protein 6

RBQ-1: Retinoblastoma Binding Q protein 1

Rev: Reverse

RING: Really Interesting New Gene

RNA: Ribonucleic Acid

ROS: Reactive oxygen species

Rpr: Reaper

RT-PCR: Reverse transcription Polymerase Chain Reaction

SCO2: Synthesis of Cytochrome C Oxidase 2

SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

TAE: Tris-Acetate

TBE: Tris/Borate/EDTA

TIGAR: Tp53 Induced Glycolysis and Apoptosis Regulator

Topo 1: Topoisomerase 1

TP53: Tumour Protein 53

CHAPTER 1: INTRODUCTION

1.1 Cancer & Carcinogenesis: An overview

Cancer has been identified as one of the major health problems worldwide affecting individuals of every age (World Health Organization, 2015). There are variations between age of onset, growth rate and invasiveness. Globally, it kills more people than Malaria, AIDS, and Tuberculosis combined making it an urgent health problem. Cancer results when genetically damaged cells start proliferating uncontrollably. All cancers are due to abnormal cell proliferation, most of which is associated with aberrant cell cycle progression. Thus, damaged cells divide rapidly with their mutations bypassing the physiological checks and balances to prevent propagation of mutations (Hanahan and Weinberg, 2011).

Risk factors of cancer formation include: environmental influences such as, radiation and exposure to ultraviolet light. These factors yield mutagens that alter the DNA and present pyrimidine dimers, single and double strand breaks. Lifestyle factors such as lack of exercise, unhealthy diet, excessive alcohol consumption and smoking also increase risk of cancer. However, most of the recorded cancer cases arise from inherited genetic mutations (www.cancer.net). Epigenetics also plays a huge role in cancer formation where tumour suppressor genes become silenced by epigenetic factors like the hyper-methylation of CpG islands (Fearon, 1997; Haber and Harlow, 1997).

Cancer is the leading cause of death worldwide with approximately 14 million new cases recorded in 2012 (Ferlay et al., 2012). In 2015, there were 8.8 million cancer-related deaths globally. The world health organization estimates that within the next 20 years the number of new cancer cases will increase by 70% (World Health Organization, 2015). According to global cancer statistics, men had a higher incidence of cancer in 2012. In South Africa, one in four individuals is affected by cancer and over 100 000 people are diagnosed annually with a survival rate of only 60% (Cancer Association of South Africa, 2013).

When normal cells become diseased or damaged, they undergo a process called apoptosis (programmed cell death) whereby proliferation of these cells is curbed by induction of a genetically controlled death programme. On the other hand, cancer cells lack this ability and continue proliferating resulting in tumour growth. There are two types of tumours; benign and malignant tumours. Benign tumours are non-cancerous but can still cause problems when they grow and exert pressure on crucial organs, like lungs where they make breathing difficult. Malignant tumours are able to spread from their original environment (metastasis) and move to other parts of the body (invasion) which can be life threatening.

Carcinogenesis is a process where normal cells accumulate mutations and no longer respond to regulatory signals leading to an increase in abnormal cell proliferation. It consists of three major phases namely initiation, promotion and progression. During initiation, cells get exposed to carcinogens, causing changes to occur in a spontaneous manner, and further results in mutations in the cellular genome. The initiation phase is the major step of neoplasms (unusual growth of tissues). The mutated cell remains harmless until stimulated to continue dividing and cause cell imbalance. This stimulation occurs during the promotion phase (Devi, 2004).

During the progression phase, neoplastic conversion occurs, this is when pre-neoplastic cells commit themselves to develop as malignant tumours. This is made possible by gene mutations accumulating inside the growing pre-neoplastic cell clone. Consequently, changes in the neoplasm produce a number of malignant cells continuously (World Health Organization, 2015). Most cancers are diagnosed late and by the time treatment is initiated, the cancer has spread to other organs. If most cancers could be diagnosed early, it would be easy to treat them and the chances of curing them would increase significantly.

1.2 The use of irinotecan as a chemotherapeutic agent

Chemotherapy is the treatment of cancer using drugs to destroy cancer cells. Most cancer patients depend on chemotherapy. However, adverse side effects are frequent and include nausea, vomiting, hair loss, abdominal pains, and infections. Almost all chemotherapeutic drugs do not discriminate between cancer and normal

cells resulting in these side-effects. For example, patients undergoing chemotherapy lose hair, stem cells and intestinal muco-epithelial cells because they are highly proliferative and sensitive to anticancer drugs.

Camptothecin- a cytotoxic alkaloid first extracted from the Chinese tree *Camptotheca acuminate* was used widely as a chemotherapy drug before it was discontinued due to its adverse effects such as enteritis and cystitis (Schulz et al., 2009). Furthermore, it was also found to be insoluble in aqueous solution, thus its semi-synthetic version; Irinotecan (CPT-11) was developed (Schulz et al., 2009). Irinotecan is soluble in water and is associated with less severe side effects in comparison to Camptothecin.

Irinotecan is a DNA Topoisomerase I inhibitor that targets the rapidly dividing cancer during DNA replication (Liu et al., 2000). It is used for treatment of solid tumours such as breast, lung and colorectal cancer. Its mechanism of action involves the interaction with the enzyme topoisomerase 1. Topoisomerase 1 is an enzyme responsible for introducing temporary single strand breaks in the DNA during replication and then re-annealing them in a new confirmation, thereby relaxing the DNA supercoils. Irinotecan binds to topoisomerase 1-DNA complex and inhibits religation of the DNA strand, as a result lethal double strand, replication arrest and apoptosis occurs as shown in figure 1.1.

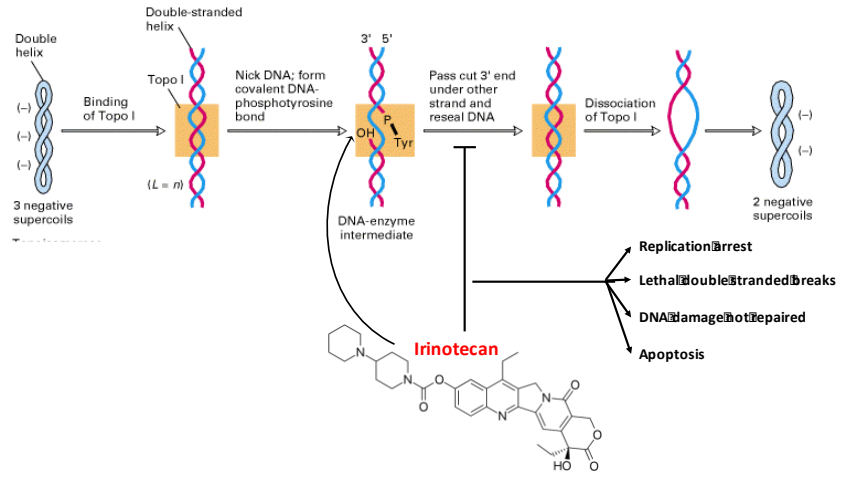


Figure 1.1: Irinotecan mechanism of action. Irinotecan binds to topoisomerase 1- DNA complex, formation of this complex inhibits religation of DNA strand, further resulting in replication arrest, lethal double strand breaks and apoptosis. This figure was created using Microsoft® PowerPoint.

Topoisomerase 1 is very important in *Drosophila melanogaster* development, it is found in increased quantities within developing embryos (Lee et al., 1993). A study by (Thomas et al., 2013) showed that most *Drosophila* stocks carry mutations in *Cyp6d2* which make them hypersensitive to Camptothecin and its analogues. Irinotecan is a DNA damaging agent and it's used in this study as such (Takeba et al., 2007).

1.3 Cellular metabolism in carcinogenesis

Cellular metabolism refers to the total biochemical processes that take place in living organisms either utilization or generation of energy. Modifications to these processes can be observed during carcinogenesis and aid in propagating cancer cell growth (Hanahan and Weinberg, 2011). All cells require energy and nutrients to grow and to proliferate. Glucose is the primary energy source for cells and is crucial throughout its life cycle. Most normal cells metabolise their glucose using the mitochondrial Oxidative phosphorylation (OXPHOS) which is more efficient than glycolysis. It gives rise to a net total of 36 ATP molecules compared to two molecules produced by glycolysis.

1.3.1 Glycolysis

Glycolysis is the primary metabolic pathway used by all organisms to breakdown glucose for energy production and it occurs in the cytoplasm. Glycolysis is a multistep process that involves the initial step of breaking down a six-carbon glucose molecule to two-carbon molecules of glyceraldehyde-3-phosphate and finally the formation of two pyruvate molecules that get oxidized under anaerobic conditions (Figure 1.2). NAD^+ molecules get reduced to NADH and two ATP molecules are produced in the process. The glycolysis pathway can be halted when all NAD^+ is converted to NADH. However, more NADH gets reconverted to NAD^+ when pyruvate is converted to lactate by the enzyme lactate dehydrogenase.

P53 controls glycolysis at multiple points such as directly repressing the expression of glucose transporters (GLUT 1 and GLUT 4) as well as GLUT 3 indirectly (see section 1.5).

1.3.2 Citric acid cycle/ tricarboxylic cycle

The citric cycle takes place in the mitochondria under aerobic conditions. The end product of glycolysis, pyruvate, enters the mitochondrion to be fully oxidized to acetyl-CoA, releasing one molecule of CO_2 facilitated by the enzyme pyruvate dehydrogenase. Acetyl-CoA enters the citric acid cycle and releases another two CO_2 molecules. High energy carriers bounce off from the citric acid cycle as acetyl CoA goes through the cycle.

1.3.3 Oxidative Phosphorylation

Oxidative phosphorylation (OXPHOS) also known as the electron transport chain takes place in the mitochondrion. It is responsible for synthesizing approximately 90% of total 36 ATP molecules in the cell. During the tricarboxylic cycle, NADH and $FADH_2$ are formed and then re-oxidized by mitochondrial membrane enzyme complexes which pass electrons from these oxidized molecules to an electron acceptor; oxygen (O_2), which is converted to H_2O. The energy that is released from this cycle drives the synthesis of ATP through ADP, Pi, and mitochondrial enzyme complexes. During this process, an increase in mitochondrial membrane potential occurs due to many apoptotic signals released in the cytoplasm. In addition, increased reactive oxygen species (ROS) production leads to oxidative stress and the release of pro-apoptotic factors such as cytochrome c, caspases and procaspases which further initiate apoptosis (Fleury et al., 2002).

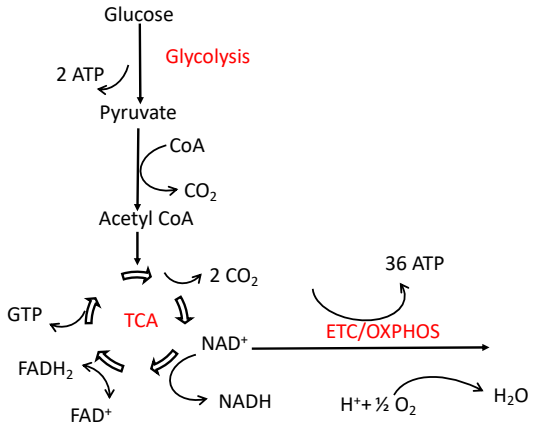


Figure 1.2: Metabolic pathway from glycolysis to OXPHOS. Glycolysis occurs in the cytoplasm and yields a net total of 2 ATP molecules. The citric cycle is the only cyclic pathway and takes place in the mitochondrion similarly to OXPHOS which produces a net total of 36 ATPs. Normal cells prefer this pathway. This figure was generated using Microsoft® PowerPoint.

1.3.4 The Warburg effect

Otto Warburg discovered that cancer cells prefer to metabolise glucose using the inefficient glycolysis pathway regardless of the abundant oxygen in the environment. This preference is known as the "Warburg effect" (Warburg, 1956). Thus, cancer cells rely on glycolysis as the main source of energy generation even in aerobic conditions. This is referred to as aerobic glycolysis and enables cancer cells to carry on malignant progression (Bensaad and Vousden, 2007). It is hypothesized that the major reason cancer cells prefer glycolysis to OXPHOS is because it generates ATP faster even though it yields less energy compared to OXPHOS. Also, cancer cells are sensitive to the apoptotic effect of intracellular reactive oxygen species (ROS) caused by oxidative stress in the mitochondrion. It is hypothesized in this thesis that reversal of this phenomenon by boosting OXPHOS might aid in inducing cell death in cancer cells thereby inhibiting growth of cancer cells. Moreover, this might enhance the growth of normal cells.

Apart from the Warburg effect there is also glutamine addiction where cancer cells become addicted to glutamine. In the 1950s, Harry Eagle studied the cell's nutritional requirements and discovered that cancer cells consume glutamine more than any other amino acid and that absence of exogenous glutamine results in cell dormancy further affecting cell viability (Eagle, 1955). Additionally, when cancer cells are starved of glutamine they become vulnerable since it is recognized as the primary mitochondrial substrate required for the maintenance of mitochondrial membrane potential (Wise and Thompson, 2010). This further indicates the tendency of cancer cells to reprogram their metabolism, thus, this presents a potentially effective therapeutic target.

1.4 Circumventing metabolic reprogramming: the role of Methyl pyruvate

Metabolic reprogramming occurs when cancer cells alter their normal metabolic properties to sustain themselves as they require rapid energy production to support their fast proliferation. The end-product of glycolysis (preferred energy generation pathway of cancer cells) is pyruvate and providing this substrate exogenously to cells might enable them to circumvent this pathway and thus leading to their death.

In this study, 3-Methyl pyruvate (MP), a derivative of pyruvate was administered to bypass the glycolytic pathway. Methyl pyruvate is lipophilic in nature, making it more membrane permeable and stable than pyruvate. These two aforementioned characteristics enable it to be more favoured by the TCA cycle than pyruvate (Nishida et al., 2014). Furthermore, when introduced into cells exogenously it boosts OXPHOS and obstructs the Warburg effect. As a result cancer cells are killed and normal cells survive. Since glycolysis is enhanced during carcinogenesis, the use of methyl pyruvate to stimulate OXPHOS which generates mitochondrial reactive oxygen species may be used for cancer therapy (Monchusi and Ntwasa, 2017).

1.5 Mammalian and *Drosophila melanogaster* p53: Overview

p53 is a tumour suppressor gene that encodes the protein product p53. It functions as a transcription factor that represses or stimulates the transcription of more than 50 distinct genes. Human p53 is a member of the super family of proteins that consists of the tumour suppressors Tp53, Tp63 and Tp73. It is the most frequently mutated gene in human cancers occurring in more than 50% of all cases.

In this study, *Drosophila melanogaster* is used as a model. Its p53 (Dmp53) null mutants are representative of some cancer genotypes. Human and *Drosophila* p53 share similar structural and functional characteristics. For example, Dmp53 is involved in cell cycle arrest in response to DNA damage (Steller, 2000) by interacting with genes such as: *p21*, *E2F*, *ATM*, *Rb*, and cyclin-dependant kinases- which are found both in humans and *Drosophila melanogaster* to mediate this process.

During cellular stress such as DNA damage, p53 is activated leading to an induction of a wide range of cellular response mechanisms which inhibit tumour development or repair the damaged DNA or induces apoptosis (Vogelstein et al., 2000; Vousden, 2006). DNA damage brings about p53 activation which promotes cell cycle arrest and transactivation of repairing enzymes. If DNA damage cannot be repaired, then apoptosis occurs (Riley et al., 2008).

Apoptosis in Drosophila melanogaster

In *Drosophila melanogaster*, apoptosis is crucial during embryogenesis. The induction of apoptosis involves the activation of three genes: reaper (*rpr*), head involution defective (*hid*) and *grim* (Mather et al., 2005). Reaper is an important effector of p53 induced apoptosis following DNA damage. It can be transcriptionally activated by a host of death inducing signals (Rodriguez et al., 1998). Reaper targets Dmp53 (White et al., 1994) and binds to the *Drosophila* inhibitor of apoptosis (DIAPI) which is capable of inhibiting *Drosophila* caspase activation. This activation is followed by the cleavage of ICAD (inhibitor of caspase deoxyribonuclease) to release free CAD by caspase 3, thereby stimulating apoptosis (Steller, 2000). When the three genes (*rpr*, *hid*, and *grim*) are expressed and functional, apoptosis is induced. When they are non-functional, it is inhibited (Grether et al., 1995; White et al., 1994).

Apart from p53 mediated apoptosis there are other forms of cell death occurring in cells that are p53 independent. This is important as this study utilized p53 null mutant *Drosophila* models to assess survival when toxicity was induced. One of such cell death mechanism is Parthanatos. Cell death by Parthanatos differs from processes like apoptosis and necrosis. It depends mainly on poly ADP-ribose-polymerase (PARP) activity, an enzyme that plays a role in the detection and initiation of immediate cellular response to metabolic, chemical or radiation induced single strand breaks. DNA damage or specifically single strand breaks causes the over activation of PARP which depletes cellular NAD+. NAD+ is a required substrate for ADP-ribose monomer production, its depletion further reduces cellular ATP to repair the damaged DNA. The depletion of ATP in the cell results in cell lysis and death. In addition PARP also possesses an ability to induce programmed cell death through the production of Poly (ADP-ribose)(PAR) which stimulates the release of apoptosis inducing factors(AIF) by the mitochondria (Fatokun et al., 2014).

1.5.1 P53 Domains & Structure

P53 has a molecular weight of approximately 53 kDa and is expressed as a tetramer protein **in vivo**. It has three domains, namely: the transactivational, DNA binding

and the tetramerization domains (Figure 1.3). The transactivational and DNA binding domains are located on the N-terminus of the *p53* gene while the tetramerization domain is located on the C-terminus. The transactivational domain plays a crucial role in binding the protein Mdm2 (negative regulator of p53) in mammals. The MDM2 homologue is present in several vertebrates and invertebrates, however it hasn't been identified in *Drosophila melanogaster*.

The DNA binding domain interacts with DNA directly by folding into a β sheet which further forms a scaffold for a large loop and helix motif. This domain contains mutation hot spots. Over 80% of cancer-causing p53 mutations are found on this domain (Petitjean et al., 2007). Mutated p53 is defective when it comes to binding DNA and thus transactivation is inhibited which leads to the inability to suppress cancer cell growth as a result increased tumour formation occurs (Pietenpol et al., 1994). The tetramerization domain is responsible for oligomerization of p53 subunits.

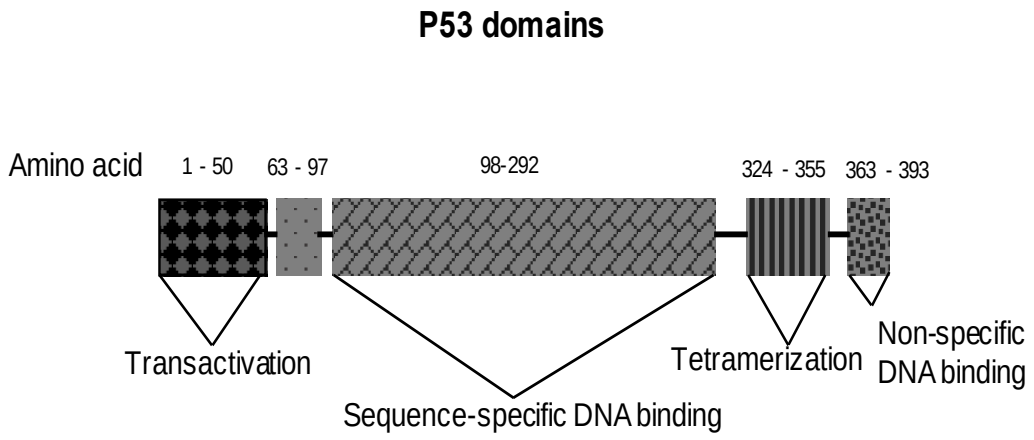


Figure 1.3: p53 domains with amino acid position. P53 comprises of three active domains known as the transactivation, DNA binding and the tetramerization domains. Adapted from (Ruttkay-Nedecky et al.,2013).

During low glucose levels, the survival of normal cells depends on the p53 activation pathway which induces the transcription of genes responsible for cell cycle arrest and DNA repair. The loss or mutations in p53 causes the protein to lose its ability to bind to its target DNA sequence and inhibit transcriptional activation of genes. Furthermore, these mutations alter the expression of important metabolic

enzymes, inhibit oxidative phosphorylation (leading to the Warburg effect) as well as oncogene activation (Pelicano et al., 2006).

The regulation of the cell cycle occurs via two pathways, depending on the stress levels of the cell. Under mild stress conditions low p53 is enough to induce the transcription of genes responsible for cell cycle arrest ($p21^{WAF1/\ CIP1}$), DNA repair (P53R2) as well as those responsible for protection against oxidative stress (TIGAR, Sestrins, GPX1 and ALDH4) (Bensaad and Vousden, 2007). When genes responsible for protection against oxidative stress are induced, decreased levels of intracellular ROS is observed. However, when there is elevated stress, increased p53 levels induce the transcription of pro-oxidant genes (PIG3, Proline oxidase) and repress the transcription of antioxidant genes (PGM, NQO1). This reaction leads to an increased intracellular ROS levels and eventually cell death occurs (Bensaad and Vousden, 2007).

The synthesis of cytochrome c oxidase 2 (SCO2) is important in the regulation of mitochondrial respiration by p53. SCO2 functions in regulating the cytochrome c oxidase complex. P53 induces the transcriptional expression of SCO2 and TIGAR (Tp53-induced glycolysis and apoptosis regulator) while repressing the expression of glucose transporter genes (GLUT1 and GLUT2) (Figure 1.4) (Bensaad et al., 2006). The expression of SCO2 and TIGAR is sufficient to slow down glycolysis and boost OXPHOS even at low levels. When p53 stimulates the expression of TIGAR, the result is the reduction of fructose-2, 6-bisphosphate further lowering glycolysis and activating the pentose phosphate pathway (PPP) which helps in reducing cell death that may be stimulated by oxidative stress. Down-regulating the expression of phosphoglycerate mutase (PGM) which is part of the glycolytic pathway slows down glycolysis, suggesting that cells may be forced to go through OXPHOS and proliferation inhibited.

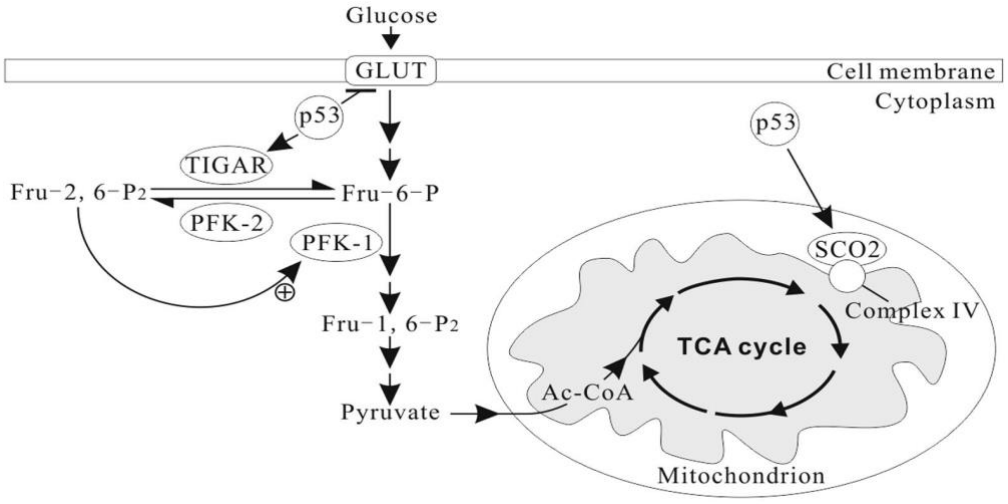


Figure 1.4: p53 regulation of glycolysis. P53 regulates mitochondrial respiration by blocking the expression of glucose transporter genes (GLUT1 and GLUT4) while stimulating TIGAR to inhibit cytoplasmic glycolysis and SCO2 to boost OXPHOS (Zheng, 2012).

Glucose metabolism in Drosophila melanogaster

Most metabolic processes in *Drosophila* are similar to those that are found in humans. *Drosophila* has been used to elucidate important characteristics of metabolic control conserved through evolution, giving new perceptions on more complicated vertebrate systems (Baker and Thummel, 2007). Its organ systems involved in uptake, storage, nutrient control, and metabolism are like those found in humans and other vertebrate species. For instance, the *Drosophila* fat body functions more like the mammalian liver and white adipose tissue. Also, the midgut of the *Drosophila* which is analogous to the human stomach and intestines is responsible for the digestion and absorption of food (Baker and Thummel, 2007). Oenocytes function in accumulating lipids and upon starvation act like the human hepatocytes (liver cells) in processing lipids. For nutrient metabolism, *Drosophila* have developed a way to retain enough sugar levels in their systems. Excess sugar is stored in the form of glycogen which can be easily accessed when sugar depletion occurs or a need for energy arises (Rusten et al., 2004; Wigglesworth, 1949).

Drosophila melanogaster has a glucose transport system similar to the one found in vertebrate species; although not much information was found on orthologues such as SCO2 and TIGAR which are very important in mammalian glucose

transport (Wang and Wang, 1993). More research focusing on SCO2 and TIGAR in *Drosophila* needs to be done. It will be important to know if the five GLUT receptors (GLUT1-5) found in mammals are also present in *Drosophila melanogaster* and if they have the same function. This study utilizes *Drosophila* as a model to further understand metabolism in cells.

1.6 Mouse Double Minute 2 (Mdm2): a p53 Negative regulator.

The *Mdm2* gene was first identified on double chromosomes of spontaneously transformed mouse 3T3 fibroblasts and was later discovered to be associated with the tumour suppressor p53 (Cahilly-Snyder et al., 1987; Momand et al., 1992). MDM2 is a 90 kDa protein which binds p53 thereby regulating its expression (Chen et al., 1996; Finlay, 1993; Momand et al., 1992). Mdm2 exists in vertebrate species such as mouse, human, golden hamster and rat. Mdm2 homologs have also been discovered in invertebrates such as the deer tick and trichoplax. However, an Mdm2 homologue in *Drosophila melanogaster* has not been identified.

1.6.1 Mdm2 and p53 interaction

There exists a negative feedback loop between Mdm2 and p53. Mdm2 is dependent on p53 binding to its promoter region to induce expression (Barak et al., 1993). An increase in Mdm2 expression is observed in normal cells, whereas there is a decrease during cellular stress such as DNA damage (Haupt et al., 2017). This increase in normal cells ensures p53 is kept at low levels. When this is the case, p53 is un-phosphorylated consequently enabling the interaction of p53 and Mdm2 to form a complex (p53/Mdm2) resulting in p53 degradation in the proteasome through the ubiquitin pathway (Figure 1.5) (Haupt et al., 1997; Momand et al., 1992). During DNA damage, p53 gets phosphorylated consequently inhibiting its binding to Mdm2.

dRad6 and Dmp53 interaction

In *Drosophila melanogaster* Dmp53 is regulated by the enzyme dRad6 which is found in humans as Rad6. It plays a role as a gene transcription regulator and an E2 ligase responsible for repairing damaged DNA. dRad6 maintains the low levels of Dmp53 under stress-free conditions through ubiquitination and 26S proteasomal

degradation. It negatively regulates Dmp53 turnover by the formation of the Dmp53/dRad6 complex which results in degradation of Dmp53 (Dohmen et al., 1991; Sung et al., 1991). Loss or mutations in the dRad6 inhibit Dmp53 degradation, thus affects development and *Drosophila* morphogenesis (Chen et al., 2011).

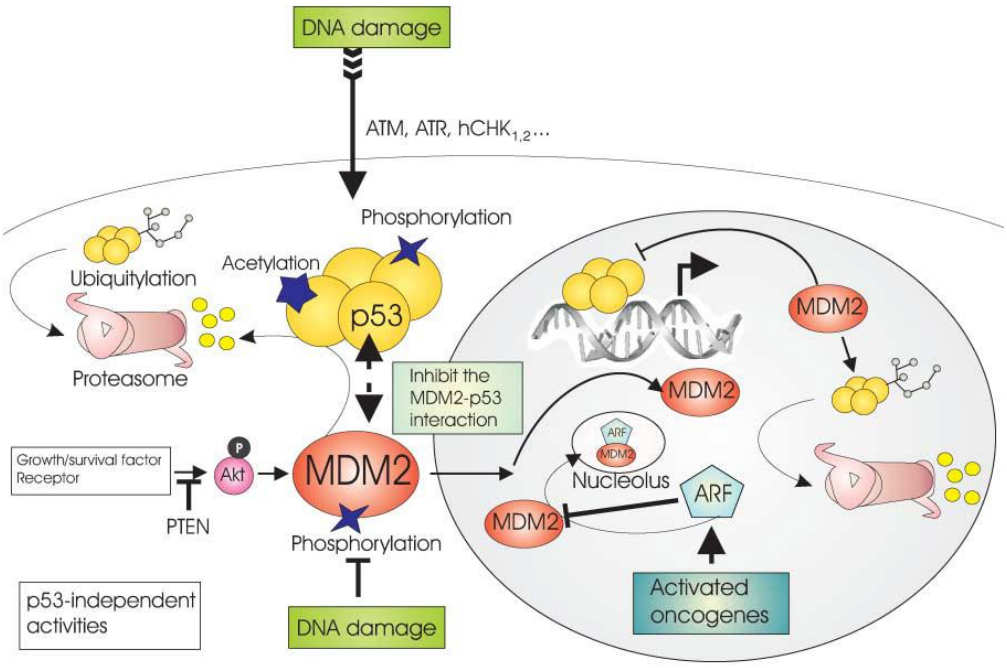


Figure 1.5: Mechanisms of p53 regulation by Mdm2. Mdm2 regulates p53 at numerous levels. P53 and Mdm2 are connected through an autoregulatory negative feedback loop where p53 induces the expression of Mdm2 which in turn targets p53 for degradation. Also, stress signals like DNA damage phosphorylates p53 further interfering with complex formation and inhibiting p53 degradation. Source: (Moll and Petrenko, 2003).

1.7 Retinoblastoma Binding Protein 6 and SNAMA structure

The Retinoblastoma Binding Protein 6 (RBBP6) is a 250 kDa multiprotein located on human chromosome 16p22.2. RBBP6 plays a role in various biological processes such as translation, transcription, development as well as ubiquitination. The RBBP6 family is present in all eukaryotes but not in prokaryotes and is identified by several names: Human (RBBP6) Mouse (p53-associated cellular protein testes derived PACT- or Proliferation potential-related protein -P2P-R), *Drosophila melanogaster* (SNAMA), *C.elegans* (RBPL-1) and Yeast (Mpe1p) which is involved in mRNA processing (Chen et al., 2013; Hull et al., 2015; Mather et al., 2005; Simons et al., 1997; Witte and Scott, 1997)Vo et al., 2001).

Most RBBP6 orthologues are negative regulators of p53. In mammals RBBP6 binds p53 and pRB tumour suppressors. It also promotes the degradation of p53 by enhancing Mdm2 activity (Sakai et al., 1995; Simons et al., 1997). However, SNAMA has not been shown to bind p53 although it has the p53-BD (p53 binding domain). Furthermore, SNAMA plays a role in apoptosis as well as in embryonic development (Mather et al., 2005). The mouse orthologue PACT is also a negative regulator of p53 and is crucial in development. Knock down of PACT results in early embryonic lethality just as loss of SNAMA leads to death of embryos (Li et al., 2007; Mather et al., 2005). RBBP6 and MDM2 share some structural and functional similarities.

1.7.1 RBBP6 Domains

The RBBP6 gene encodes a protein with multiple domains namely: Domain With No Name (DWNN), CCHC zinc finger, RING finger, Rb-binding domain, p53 binding domain, Proline-rich, Serine- Arginine rich (S-R) and the Nuclear Localisation signal (Mather et al., 2005; UniProt, 2002) (Figure 1.6). The DWNN domain is located on the N-terminus in mammals and is present in all species. The C-terminus consists of (Proline rich region (337-349 residues), SR (679-773 residues), Rb-binding (964-1120 residues) and p53-binding (1142-1727 residues (Simons et al., 1997; Witte and Scott, 1997). In *Drosophila melanogaster* SNAMA, a highly conserved N-terminal DWNN Catalytic Module (DCM) exists consisting of a ubiquitin-like domain DWNN, zinc finger motif as well as the RING finger-like motif. Additionally, the C-terminus consists of two lysine regions (Antunes, 2009; Mather et al., 2005).

The Domain with No Name (DWNN)

The DWNN is common in all isoforms and is located on the N-terminus. In mammals, it has a C-terminal GG motif which makes protease recognition possible and enables initiation of conjugation by cleaving between two glycine molecules (Pugh et al., 2006). In *Drosophila melanogaster*, the C-terminal GG motif is replaced by a proline rich region. The three dimensional structure of this domain has a ubiquitin-like fold and shares approximately 22% similarity with ubiquitin suggesting its involvement in the regulation of protein turn over in cells (Gao and Scott, 2002, 2003; Pretorius, 2007).

Zinc finger (CCHC type) and RING finger

Zinc fingers are small proteins that bind to zinc atoms possessing finger-like protrusions that facilitate interaction with their target molecule. This makes zinc essential for domain stability (Krishna, 2003). The CCHC zinc finger is the most common also known as the zinc knuckle and it possesses 40-60 amino acids (Miller et al., 1985). In *Drosophila melanogaster* almost all zinc fingers are involved in the development process and protein-protein synergy (Hart et al., 1996). The RING finger is a protein structural domain of zinc finger having an intrinsic E3 ubiquitin ligase which enable catalytic ubiquitin mediated proteasomal degradation of proteins like p53.

Most proteins that possess the RING finger are involved in the ubiquitin pathway where they bind ubiquitination enzymes (E1 ubiquitin activating, E2 ubiquitin conjugating and E3 ubiquitin ligating enzymes) and their substrates (Lorick et al., 1999). Firstly, ubiquitin is primed for action by an E1 ubiquitin activating enzyme, the activated ubiquitin is then transferred from E1 to E2 which acts as an escort for ubiquitin to its next destination- E3. E3 ubiquitin ligating enzymes are responsible for substrate recognition as well as promoting complex formations. Next, poly-ubiquitin chain on protein is formed, then proteasome binds and removes the poly-ubiquitin chain and unfolds protein which get degraded through a proteasome chamber (Joazeiro et al., 2000; Kappo et al., 2012). RING domains have conserved hydrophobic residues which are representative of the U-box domains, these are found in *Drosophila* isoforms (Figure 1.7). U box and RING domains are structurally similar and function as scaffolding molecules that recruit E2 in the ubiquitination pathway for degradation of proteins (Aravind et al., 2000).

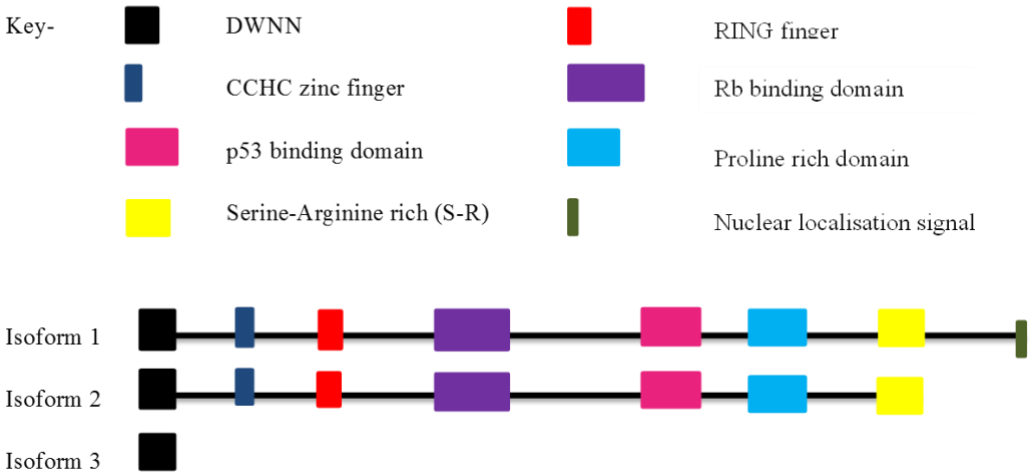


Figure 1.6: RBBP6 human isoforms and the domains. All isoforms have a common DWNN domain while isoform 1, 2 and 4 have the zinc finger, RING finger, Rb binding domain and p53 binding domain in common. This figure was generated using Microsoft® PowerPoint.



Figure 1.7: SNAMA domain structure. SNAMA A is the larger isoform and has the p53 and Rb binding domains like in the human RBBP6, SNAMA B has the DWNN and zinc finger motif which enable it to carry out certain functions. This figure was generated using Microsoft® PowerPoint.

RBBP6 isoforms

There are three RBBP6 isoforms in humans as shown in Table 1.1. They are derived from two mRNA transcripts, 1.1 and 6.1 kb. The 1.1 kb transcript encodes isoform 3 and the 6.1 kb transcript encodes isoform 1, Furthermore, alternative splicing of the 6.1 kb transcript gives rise to isoform 2. The length of Isoform 1 consists of 1792 amino acids, 18 exons and has a size of 202 kDa. It was shown to possess a ubiquitin ligase-like activity through its RING finger- domain by ubiquitinating YB-1 protein, thereby reducing its transactivational ability (Chibi et al., 2008). Isoform 1 is a full length isoform that binds both p53 and pRb (Sakai et al., 1995; Simons et al., 1997; Witte and Scott, 1997). It plays a pro-apoptotic role in human cancers. Isoform 2 is the second largest isoforms with an amino acid length of 1758, 16 exons and a mass of 197 kDa. Isoform 3 is the shortest and comprises of only the DWNN domain. It has an amino acid length of 118, 3 exons and a mass of 13 kDa (Di Giammartino et al., 2014; Dlamini et al., 2016; Simons et al., 1997). Isoform 3 is involved in the pro-apoptotic pathway in colon cancers also, it is speculated to compete with isoform 1 and 2 for binding sites.

SNAMA isoforms

SNAMA- Something that sticks like glue, is the name given to the *Drosophila melanogaster* homologue of RBBP6. It is located on chromosome 2 of the *Drosophila* genome and consists of two transcripts known as SNAMA A and SNAMA B (Figure 1.8). SNAMA A is the longest transcript and is 135 kDa, has ten exons in total while the short isoform- SNAMA B is approximately 55 kDa and has seven exons (Table 1.2). The two *Drosophila* isoforms Snama A and B are similar to the human isoform one and three respectively.

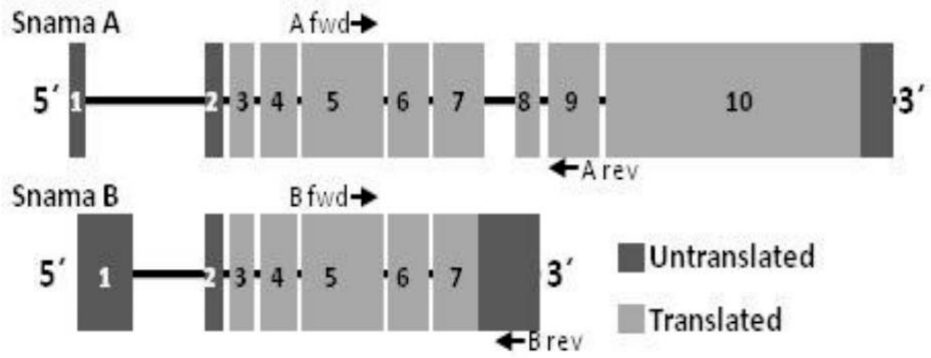


Figure 1.8: Snama genomic structure. Schematic diagram showing Snama A and B translated and untranslated regions as well as the exons in them. The two transcripts have different untranslated regions and have some exons in common. Snama A has ten exons whereas Snama B only has seven. Adapted from (Hull et al., 2015).

Table 1.1: Table depicting RBBP6 human isoforms, their lengths, molecular weight, exons and transcripts that code for the specific isoforms.

Isoform	Amino Acid Length	Mass (kDa)	Transcript	Exons
1	1792	202	6.1	18
2	1758	197	6.0	16
3	118	13	1.1	3

Table 1.2: Table depicting *Drosophila* melanogaster SNAMA isoforms, their lengths, molecular weight, and exons.

Isoform	Amino Acid Length	Mass (kDa)	Exons
SNAMA A	1231	135	10
SNAMA B	494	55	7

1.7.2 RBBP6 role in carcinogenesis

The expression of RBBP6 is increased in most cancers such as lung, oesophageal, and colorectal making it a potential biomarker for these diseases. For example, it has been observed that RBBP6 is overexpressed in colon cancers and that this corresponds to metastasis signifying its role as a potential diagnostic and prognostic

biomarker (Ntwasa 2016; Chen 2013). Furthermore, its upregulation in multiple cancers makes it a good target for cancer therapy. Additionally, RBBP6 is also implicated in the regulation of the cell cycle where its overexpression stimulate cell cycle arrest and induces apoptosis (Gao and Scott, 2002). The short isoform comprising of just the DWNN domain is down regulated in human cancers while the longer isoforms are upregulated (Mbita et al., 2012). A down regulation of isoform 1 usually occurs with apoptosis while a down regulation of isoform 3 coincides with carcinogenesis. In contrast, overexpression of isoform 3 inhibits 3′ end pre-mRNA cleavage in a similar way to siRNA-mediated knockdown of RBBP6 while overexpression of isoform 1 stimulate cell proliferation and leads to p53 degradation. The DWNN as a single module is also known to antagonize larger isoforms by competitive binding (Di Giammartino et al., 2014; Hull et al., 2015; Ntwasa, 2016).

1.7.3 RBBP6 interaction with p53

The human RBBP6 is an E3 ubiquitin-protein ligase which stimulates YBX1 ubiquitination which results in its degradation by proteasome (Chibi et al., 2008). It binds with the tumour suppressor proteins pRB and p53 (Sakai et al., 1995; Simons et al., 1997; Witte and Scott, 1997), suggesting its role in carcinogenesis, cell cycle as well as apoptosis. RBBP6 interacts with p53 through the DNA binding domain where it negatively regulates p53. It facilitates this process by functioning as a scaffold protein to assist in the formation of the p53/Mdm2 complex. This then results in an increase of Mdm2 mediated ubiquitination and degradation of p53 by the proteasome (UniProt, 2002).

SNAMA interaction with Dmp53

Drosophila melanogaster p53 (Dmp53) is the homologue of TP53 (Human p53). It is a sequence specific DNA binding protein that plays a role in inducing apoptosis in *Drosophila melanogaster* (Ollmann et al., 2000a). The DNA binding domain of Dmp53 has been found to be similar to that of p53 in mammals thus making it a likely mutation hotspot (Ollmann et al., 2000b).

Drosophila melanogaster p53 was isolated and cloned by three independent research groups. Their results showed that it was the only member of the p53 family

proteins in the fly (Mollereau and Ma, 2014) and it functions in a similar manner as the mammalian p53. For instance, in both mammalian p53 and *Drosophila* p53 the overexpression of the p53/Dmp53 induces apoptosis and the down regulation causes cells to be resistant to apoptosis. However, Dmp53 differs from p53 in that it does not induce a G1 cell cycle arrest and thus has no effect on the cycle (Ollmann et al., 2000a).

It has been proposed in the past that there might be an interaction between SNAMA and Dmp53. SNAMA might function as a suppressor of apoptosis and possibly degrade apoptosis activators such as Dmp53 (Mather, 2006). SNAMA also plays a role in maintaining embryo development and regulating apoptosis (Mather et al., 2005). The human RBBP6 and the mice homolog (PACT) play a crucial role in binding the non-mutated p53 and disrupt the p53 binding (Mather, 2006).

The role of SNAMA in embryogenesis

SNAMA plays an important role during the *Drosophila melanogaster* embryonic development. The deletion of SNAMA resulted in embryonic lethality, inhibition of cell cycle progression, and induction of abnormal apoptosis (Hull et al., 2015; Jones et al., 2006; Mather et al., 2005). Another role of SNAMA involves suppressing cell death for normal development in growing embryos (Hull et al., 2015). SNAMA is differentially expressed throughout *Drosophila* development, where its expression is increased during embryogenesis and decreased in adult flies (Mather et al., 2005). The expression of SNAMA occurs on the periphery of the cellular blastoderm and not in the endoreplicating cells on the interior of the embryo. It is also involved in antero-posterior (A-P) embryonic patterning which is crucial for development (Hull et al., 2015).

1.8 *Drosophila melanogaster* as a model for cancer studies

Drosophila melanogaster also commonly known as the fruit fly has been previously used as a model in scientific research. It has the following advantages: short life cycle of about ten days, production of many offspring, inexpensive and easy maintenance. It has four chromosomes and its genome consists of approximately 14 000 genes.

It has been observed that 50% of proteins found in *Drosophila* have mammalian analogues and approximately 75% of existing human disease genes have *Drosophila* homologues. Furthermore, several models of human diseases and the best candidate genes linked to human diseases have been established in flies. *Drosophila melanogaster* has contributed to the understanding of fundamental biological mechanisms through the discovery of systems like the notch, toll and hippo signalling pathways. For example the hippo signalling pathway (a primary growth control pathway in vertebrates) was discovered through studies with *Drosophila* tumour suppressor gene (Irvine and Staley, 2012). In this study the *Drosophila melanogaster* wild type and p53 null mutants were used to model normal cells and most cancers cells respectively.

1.9 Justification of study

Cancer is a global concern and kills more people than any other disease. It has major global economic impact costing billions of dollars annually for its management and treatment. This necessitates the development of new methods for treatment and management regimens. Targeting the metabolism of cancer cells provide a promising avenue to achieve this. Metabolic reprogramming has been identified as one of the hallmarks of cancer. Cancer cells prefer glycolysis (Warburg effect).

P53 is highly mutated in cancers and has been linked to the induction of the Warburg effect. RBBP6 and Mdm2 are E3 ubiquitin ligase proteins important for the negative regulation of p53 (Haupt et al., 1997; Li et al., 2007). They target the degradation of p53 through the ubiquitin pathway. While, interaction with p53 is known, the role of RBBP6 in metabolic reprogramming is not known. Targeting this interaction can help eradicate tumour cells (Chène, 2003). Understanding the role of RBBP6 during metabolic reprogramming may help identify targets. This study observes the expression of the RBBP6; mRNA and protein level for better understanding of their roles in metabolic reprogramming.

1.10 Aim

The aim of this study was to evaluate the biochemical role of SNAMA isoforms in metabolic reprogramming.

1.11 Objectives

The objectives of this study comprised of the following:

1. Comparing SNAMA isoforms to other invertebrates and vertebrates using sequence alignment tools.
2. Gene expression profiling of *Snama* transcripts at various treatments (irinotecan, methyl pyruvate and a combination of both) using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).
3. Protein expression profiling of SNAMA isoforms at various treatments using Western blot.
4. Investigate the effects of the various treatments on survival using Kaplan Meier survival plots.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

The materials used in this study are listed in the appendix section. Appendix A is comprised of laboratory equipment, manufacturer and model numbers of equipment (Table A1). Appendix B consists of the buffers and their compositions (Table A2). Appendix C, chemicals used as well as their supplier and catalogue numbers (Table A3). Appendix D consists of the aligned protein sequences for RBBP6 isoforms from different species. Appendix E comprise of survival analysis data.

2.2 Overview of methods used

In this chapter, methods utilized for this study are described. A bioinformatics study comparing RBBP6 isoforms in different vertebrate and invertebrate organisms was carried out. Sequences (FASTA format) were obtained from the National Centre of Bioinformatics Information (NCBI) and were aligned using Clustal Omega. *Drosophila melanogaster* was used as the model organism. The *Drosophila* strains used included; wild type Oregon R and p53 null mutant Psnama (Bloomington stock #6815 with a 3.3kb deletion at the p53 locus, y[1] w[1118]; TI {TI} p53 [5A-1-4] and Psnama, p53/w;cyo;TM6B.They were treated with 0.1 µM of irinotecan, 2.5% methyl pyruvate and a combination treatment of 5% methyl pyruvate and 0.1 µM irinotecan. Various combinations of irinotecan and methyl pyruvate were used to induce DNA damage and perturb glucose metabolism respectively. Flies were treated by mixing the drug permutations with yeast in blobs which were placed in the vials for three days and then allowed to recover for a further nine days. Flies were harvested, counted and subjected to Trizol and sample buffer for RNA and protein extraction respectively at 3-day intervals. Total RNA and protein extraction were done followed by RT-PCR and Western blot analysis respectively. Bands were quantified using densitometry software myImageAnalysis version 2.0 (Thermo Scientific) and statistical analysis was done using Microsoft® excel and GraphPad Prism 7 version 03. Statistix version 10 software was used to generate the Kaplan Meier survival plots following fly treatment. A summary of methods is shown in figure 2.1.

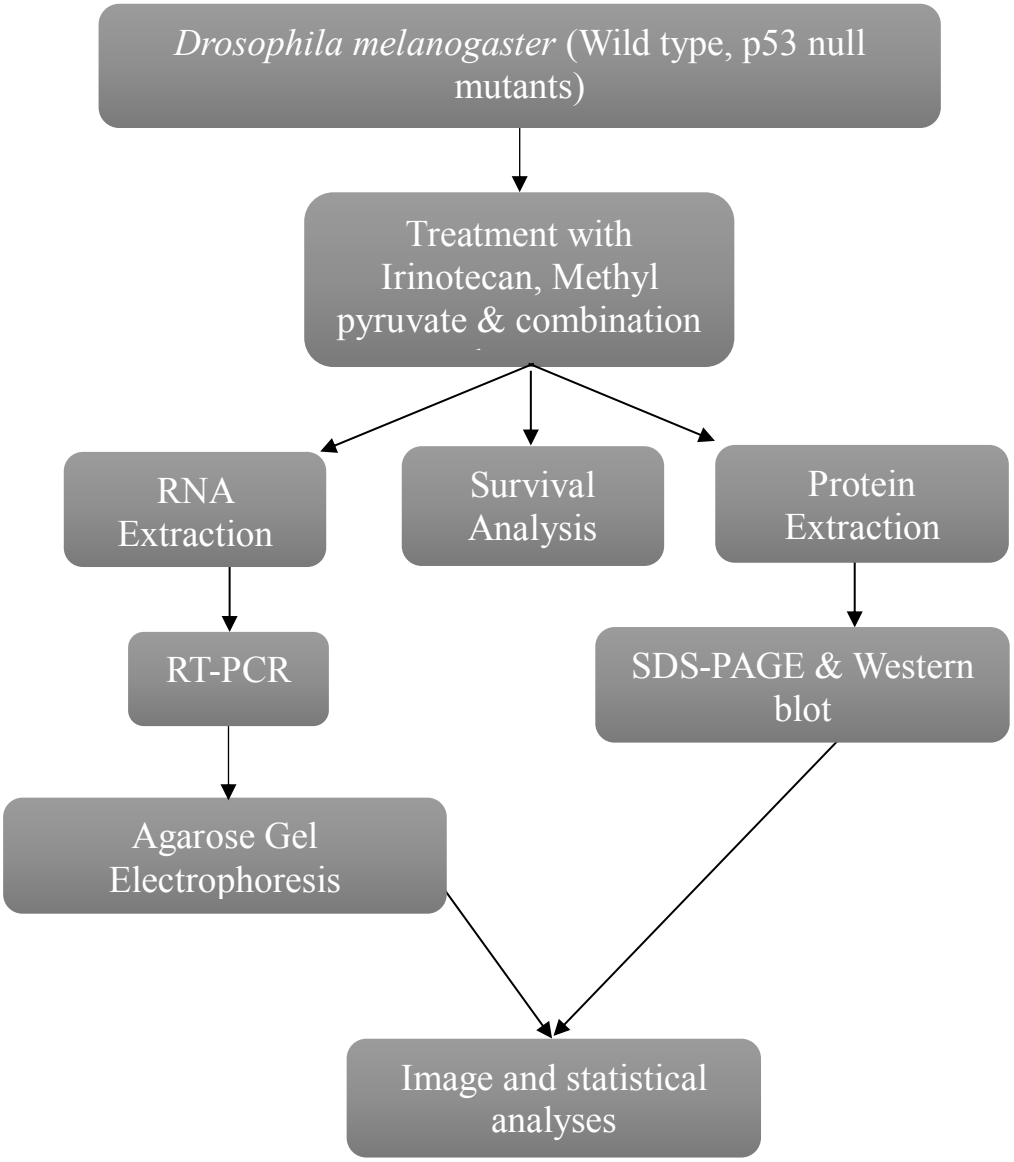


Figure 2.1: Flowchart showing experimental design for this research project. Bioinformatics analysis of RBBP6 isoforms was done to compare isoforms from different species. The *Drosophila melanogaster* wild type Oregon R and p53 null mutants (Psnama) were treated with irinotecan, methyl pyruvate and the combination treatment of both. RNA and protein were extracted, followed by RT-PCR, Western blot, and survival analysis.

2.3 Bioinformatics analysis

Protein sequences of the RBBP6 homologue isoforms of various species (Humans, Mice, *Drosophila melanogaster* and *Caenorhabditis elegans*) in FASTA format were obtained from the National Centre of Bioinformatics information (NCBI)

(https://www.ncbi.nlm.nih.gov). Sequences were then aligned using Clustal Omega (https://www.ebi.ac.uk).

2.4 *Drosophila melanogaster* strains: rearing and treatment

The *Drosophila melanogaster* strains – wild type Oregon R representing normal cells and the p53 null mutant flies (Psnama) representing a cancer phenotype were used. The Psnama mutants lack p53 but possess *Snama*, and phenotypically they can be identified with curly wings and red eyes. Flies were kept at 22ºC on a 12-hour cycle of lighting to ensure that all biological developments correspond to the time of day. Additionally, they were harboured in vials containing cornmeal agar and yeast as food supplement. For good health maintenance, flies were transferred to fresh vials on a weekly basis. Treatments administered comprised of 0.1 µM irinotecan, 2.5% methyl pyruvate as well as the combination of both (0.1 µM Irinotecan and 5% methyl pyruvate). 2.5% methyl pyruvate was used for individual treatment and 5% methyl pyruvate was used for the combination treatment. These concentrations were found to be optimal to perturb the metabolism in flies by (Hull et al., 2015).

2.5 RNA Extraction

Total RNA was isolated using the TRIzol method (Chomczynski, 1993). Trizol (500 µl) was added into 1.5 ml eppendorf tubes which had thirty flies and were crushed with sterilized homogenisers and incubated in ice for 5 mins. Following incubation, the homogenised sample was centrifuged at 12 000 rpm for 5 mins at 4ºC to separate the constituents of the sample according to weight. 100 µl of chloroform was added into the previously centrifuged mixture and was shaken vigorously until it turned milky. This was then incubated in room temperature for 10 mins after which it was centrifuged at 12 000 rpm for 15 mins at 4ºC. Subsequently, the top aqueous layer was carefully collected and placed into a fresh new eppendorf tube. 200 µl of isopropanol was then added to this aqueous solution and incubated at room temperature for 7 mins. Following incubation, centrifugation at 12 000 rpm for 10 mins at 4ºC was done and supernatant discarded. 200 µl of

cold 70% ethanol was added to the eppendorf tube and centrifuged at 12 000 rpm for 5 mins at 4ºC. The supernatant was then discarded and pellet left to air dry for 7 mins. To allow the pellet to dry properly, sample was placed in the heating block for 5 mins at 95°C. 50 µl of nuclease free water was added to pellet and tube placed in the heating block for 5 mins at 65ºC to allow dissolving. Afterwards, the sample was immediately incubated on ice for 10 mins. The Nanodrop® 1000 was used to determine the RNA concentration on all the samples. Absorbance at 260/280 was more than 1.8 in all samples. After RNA quantification, samples were stored at -20°C until required.

2.6 Reverse Transcription – Polymerase Chain Reaction

cDNA synthesis was performed using the RevertAid First Strand cDNA synthesis Kit #K1622 according to the manufacturer's protocol. About 2 µg of Total RNA was used across all samples. Samples were incubated for 60 mins at 42°C and the reaction terminated by heating at 70°C for 5 mins.

PCR was performed following the manufacturer's protocol provided on the PCR Mastermix booklet M0482S. Table 2.1 shows primers with specific sequences used for PCR.

Table 2.1: Oligonucleotides/ Primers and sequences used in this study.

PRIMERS	**SEQUENCE**
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′ ACTGATATCCATCCAGATAATG 3′

After all reagents had been added, the PCR tubes were placed in the thermal cycler and the program was set as shown in Table 2.2.

Table 2.2: RT-PCR Cycle conditions including the steps followed, temperature and time.

STEP	TEMPERATURE	TIME
Initial Denaturation	95ºC	30 secs
PCR cycles (30)	95ºC 45-68ºC 68ºC	15-30 secs 15-60 secs 1 min/kb
Final Extension	68ºC	5 mins
Hold	4ºC	-

2.7 Agarose gel electrophoresis

1% Agarose gel was prepared by mixing an appropriate amount of agarose powder with 1X TBE. The mixture was heated until the agarose had completely dissolved. 6 µl of 10 mg/ml ethidium bromide was added to the mixture to allow for DNA to be visible in UV light. The mixture was then poured into a tray with a comb which created wells, and it was allowed to cool at room temperature. Afterwards, the comb was carefully removed and the gel was placed into the electrophoresis tank containing 1X TBE. A total of 12 µl comprising 10 µl sample and 2 µl 6X loading dye was loaded into each well. 6µl of the 1kb plus DNA gene ruler (Thermo Scientific) was loaded into the first well and samples in subsequent ones. The gel was run at 100 volts for approximately 30 mins to an hour and later visualised using the Chemidoc system (Bio-Rad).

2.8 Protein Extraction

Protein extraction was performed using sample buffer (Table A2 in appendix B for constituents). Thirty flies were placed in 1.5 ml eppendorf tubes and left in ice for 5 mins. This was done in triplicates for wild type and duplicates for p53 null

mutants. 500 µl of sample buffer was then added and flies homogenised using plastic homogenisers. The homogenate was placed into a water bath at 95 ºC for 5 mins. Afterwards, homogenate was centrifuged at 12 000 rpm for 5 mins which allowed for separation into 2 phases. The upper aqueous phase was collected and transferred into clean eppendorf tubes and stored at -20ºC until use.

2.9 Protein Quantification.

Protein quantification was done using the Bradford protein assay. Five dilutions of the protein standard were prepared and the solutions were assayed in duplicates. The concentrations of the protein standards were as follows: 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml and 80 µg/ml. 160 µl of each sample solution, standard and blank were pipetted into separate microtiter plate wells. 40 µl of the dye reagent concentrate (Bio-Rad) was added into each well and the sample and reagent were mixed thoroughly using a pipette. Samples were incubated at room temperature for 5 mins and the absorbance was measured at 595 nm using a spectrophotometer (Thermo Scientific). Absorbances were exported to Microsoft excel and samples subtracted from blank and concentration calculated.

2.10 Sodium Dodecyl Sulphate-PAGE and Western Blotting

SDS-PAGE was done according to the protocol designed by (Laemmli, 1970). The samples were placed on a dry bath for 5 mins at 95 ºC and 20 µl of the protein samples (crude extract) were loaded onto the gel. For separation, cellular proteins were separated according to size and length at 100 volts for 1 hr 30 mins.

Following separation, proteins were transferred to PVDF membranes (Amersham Hybond-P) immersed in methanol, dH_2O and Towbin buffer for calibration. Transferring of proteins took place in a semi-dry blotter (Hoefer VE) for 1 hr 30 mins at constant current (280 mA). The membranes were then blocked with SuperBlock® protein blocking buffer (Thermo Scientific) for 1 hr at 4ºC to prevent non-specific binding of proteins. Three, 3 mins washes with 1 X PBS-Tween followed and membrane probed with a primary antibody (Table 2.3). The

membrane was washed five times in 1 X PBS-T for five mins per wash and then incubated at room temperature in an appropriate secondary antibody (Table 2.3) to specifically bind to the significant primary antibody for 1 hr. Five, 5 mins washes with 1 X PBS-T followed.

Substrate (Western Bright™ Quantum, Western blotting detection kit (advansta K-12042-D10)) was added to the membrane in a 1:1 ratio and incubated in the dark for 3 mins. The membrane was then analysed using the Bio-Rad imaging system.

Table 2.3: Antibodies that were used in this study as well as the dilutions and the catalogue numbers.

ANTIBODY	DILUTION	CATALOGUE NO.	SUPPLIER
pAB anti-RBBP6 rabbit + polyclonal (primary)	1:5000	NBP1-49535	Santa Cruz Biotechnology
Anti-rabbit IgG peroxidase raised in rabbit (secondary)	1:10 000	A-0545	Sigma
β Tubulin Goat polyclonal IgG (primary)	1:1000	K2206	Santa Cruz Biotechnology
Anti - Goat IgG (Whole molecule) peroxidase raised in rabbit (secondary)	1:2000	A8919	Sigma

2.11 Statistical Analyses

MyImageAnalysis version 2.0 (Thermo Scientific) was used for the quantification of both RT-PCR and Western blot bands. Their optical densities were exported to Microsoft Excel for normalisation, standard deviation, and mean calculation. Bar charts were generated using GraphPad Prism 7. version 03.

2.12 Survival Experiment

Fly treatment and counting

Twenty wild type (per group) and p53 null mutant flies of the same age were carefully distributed into separate labeled vials containing cornmeal and agar as food supplement. Five independent experiments were set up. The flies were exposed to various forms of treatments comprising; 0.1 μM irinotecan, 2.5% methyl pyruvate only and 0.1 μM Irinotecan + 5% methyl pyruvate for three days. Flies were transferred to fresh vials with drug free media for recovery which lasted for nine days. At 3-day intervals flies were counted. The number of dead and alive flies were tallied.

Kaplan-Meier Survivorship analysis

The number of dead and live flies were recorded over a period of 12 days. This raw data was then analyzed with help from Statistix version 10 software which generated Kaplan- Meier survivorship plots. The Kaplan-Meier survival plot is a non-parametric procedure used to measure the probability of an organism to survive in a given time frame whilst considering time in short intervals (Goel et al., 2010). It is often used in medical research to ascertain the number of subjects who survived after an intervention. The Kaplan Meier has some complications that go with it such as loss or death of subjects who fail to get to the end of the experiment, these are called "censored observations". The advantage of the Kaplan Meier plot includes the fact that it can estimate a survival curve with censored observations included. 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 Y-axis however shows the overall survival which is the percentage of subjects who haven't experienced the event to that point in time on the X-axis. The formula for calculating the probability of survival at a given time is;

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

CHAPTER 3: RESULTS

3.1 Overview of results

The aim of this study was to determine the biochemical roles of the Retinoblastoma binding protein 6 (RBBP6) isoforms in metabolic reprogramming associated with carcinogenesis. To accomplish this, a short bioinformatics study was first conducted to compare RBBP6 isoforms in vertebrate and invertebrate species. Domains of these were analysed. Furthermore, both wild type and p53 null mutant *Drosophila melanogaster* were treated with a combination of irinotecan (a DNA damaging agent) and exogenous pyruvate (bypasses glycolysis) to model chemotherapy combined with perturbation of metabolism. The flies were treated for 3 days and then transferred to drug-free media to allow recovery. The day of transfer is regarded as day 0. Moreover, survival analysis was carried out to investigate how exogenous pyruvate can be used to alleviate the side effects of DNA damage during chemotherapy.

Results obtained show that the Domain with No Name (DWNN) is conserved through all eukaryotic organisms but not in prokaryotes. In *Drosophila*, SNAMA A and B isoforms share the first four domains. RT-PCR show that at the mRNA level transcripts of *Snama* are expressed differentially in wild type and p53 null mutants following treatment with irinotecan, exogenous pyruvate and combination of both. Overexpression of *Snama A* is often accompanied by the downregulation of *Snama B* in both strains. Expression patterns of SNAMA at the protein level also show contrasting expression patterns (when SNAMA A is overexpressed SNAMA B is downregulated and vice versa) across all treatments and strains. SNAMA B show reduced expression when wild type flies are treated with exogenous pyruvate, whereas it is increased in p53 null mutants mainly when methyl pyruvate is combined with irinotecan. This indicates that the expression of *Snama* is independent of p53, also in some cases p53 is not involved in the transcription of *Snama* gene. Furthermore, this could also suggest that the splicing of isoforms is controlled. Survival analysis show that wild type flies survive more when treated with methyl pyruvate than with irinotecan treatment whereas p53 null mutants show decreased survival. This indicates that methyl pyruvate protects wild type flies from

irinotecan toxicity while being toxic to p53 null mutants. This is because irinotecan activates p53 and these flies lack p53, so they resist it while exogenous pyruvate enters the TCA and stimulates OXPHOS, further reversing the metabolic effect of these flies.

3.2 Comparison of invertebrate RBBP6 isoforms to vertebrates using sequence alignment tools

A short and precise bioinformatics study comparing RBBP6 isoforms in vertebrates (Homo sapiens RBBP6, Mice P2P-R) and invertebrates (*Caenorhabditis elegans* RBPL-1) to *Drosophila melanogaster* (SNAMA) was carried out. Sequences were obtained from the National Centre of Bioinformatics Information (NCBI) and were aligned using Clustal Omega (see appendix D). The results obtained show the different isoforms in the various organisms. Furthermore, the Domain With no Name is conserved through all species (Figure 3.1). SNAMA isoforms are observed to share the first four domains in common. The long isoform (SNAMA A) comprise the Rb and p53 binding domains which are essential in the cell cycle and have implications in carcinogenesis.

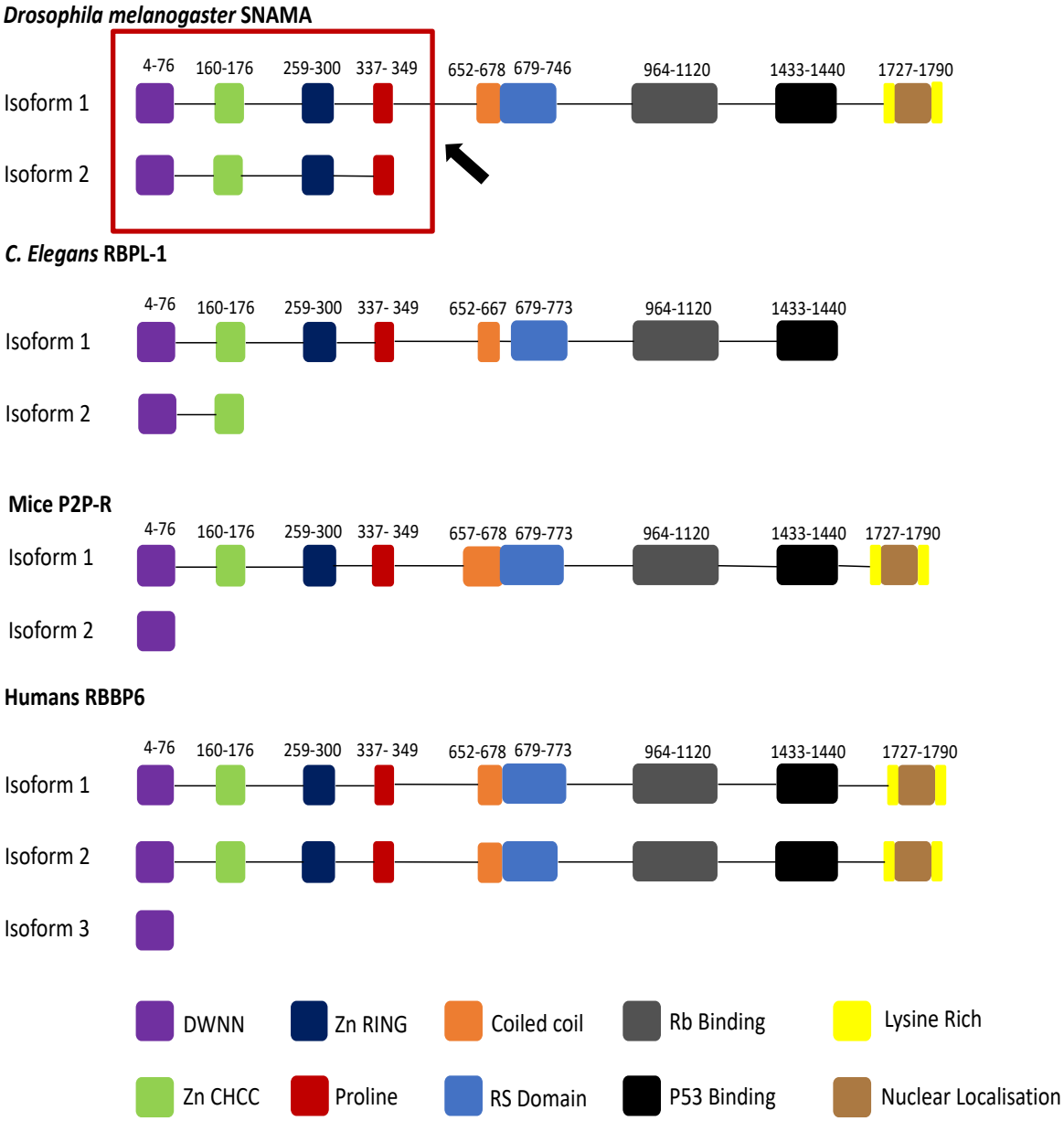


Figure 3.1: RBBP6 isoforms and domains of different vertebrate and invertebrate species. All orthologues have a common DWNN domain and most species have two isoforms (usually a long and short isoform) except for humans which have three. The arrow points at common SNAMA isoforms sharing first four domains. Sequences used to obtain domains were aligned using Clustal Omega. This figure was generated using Microsoft® PowerPoint.

3.3 Expression of SNAMA in wild type *Drosophila melanogaster* during treatment and recovery

To investigate the expression of *Snama* following DNA damage and upon recovery, RT-PCR was conducted. Furthermore the impact of boosting OXPHOS by using exogenous pyruvate was also assessed. Previous studies have shown that exogenous pyruvate enters the TCA cycle thereby boosting OXPHOS (Monchusi and Ntwasa, 2017). Total RNA was extracted from flies after treatment and during the recovery period. RT-PCR results indicate that the untreated wild type *Drosophila* expresses a higher amount of *Snama* A compared to *Snama B*. However, when flies are treated with irinotecan, *Snama B* is upregulated, while *Snama A* remained unchanged and all flies died (Figure 3.2 panel B). This suggests that these isoforms are differentially regulated by irinotecan treatment.

Wild type flies treated with methyl pyruvate overexpressed both isoforms during treatment and recovery (Figure 3.2 panel B and C). Interestingly, methyl pyruvate tends to upregulate the pro-proliferative *Snama A*. The combination of exogenous pyruvate with irinotecan resulted in longer survival- up to day 9 (Figure 3.2) indicating that exogenous pyruvate may protect normal cells against irinotecan-induced toxicity. It is also notable that methyl pyruvate was not able to reverse the depletion of *Snama* A by irinotecan. The expression of *Snama* B however, is sustained by methyl pyruvate. (Figure 3.2). Flies treated with both drug permutations were observed to survive till day 9 as indicated with the red borders in Figure 3.2. Those treated with only irinotecan or methyl pyruvate did not survive till day 9.

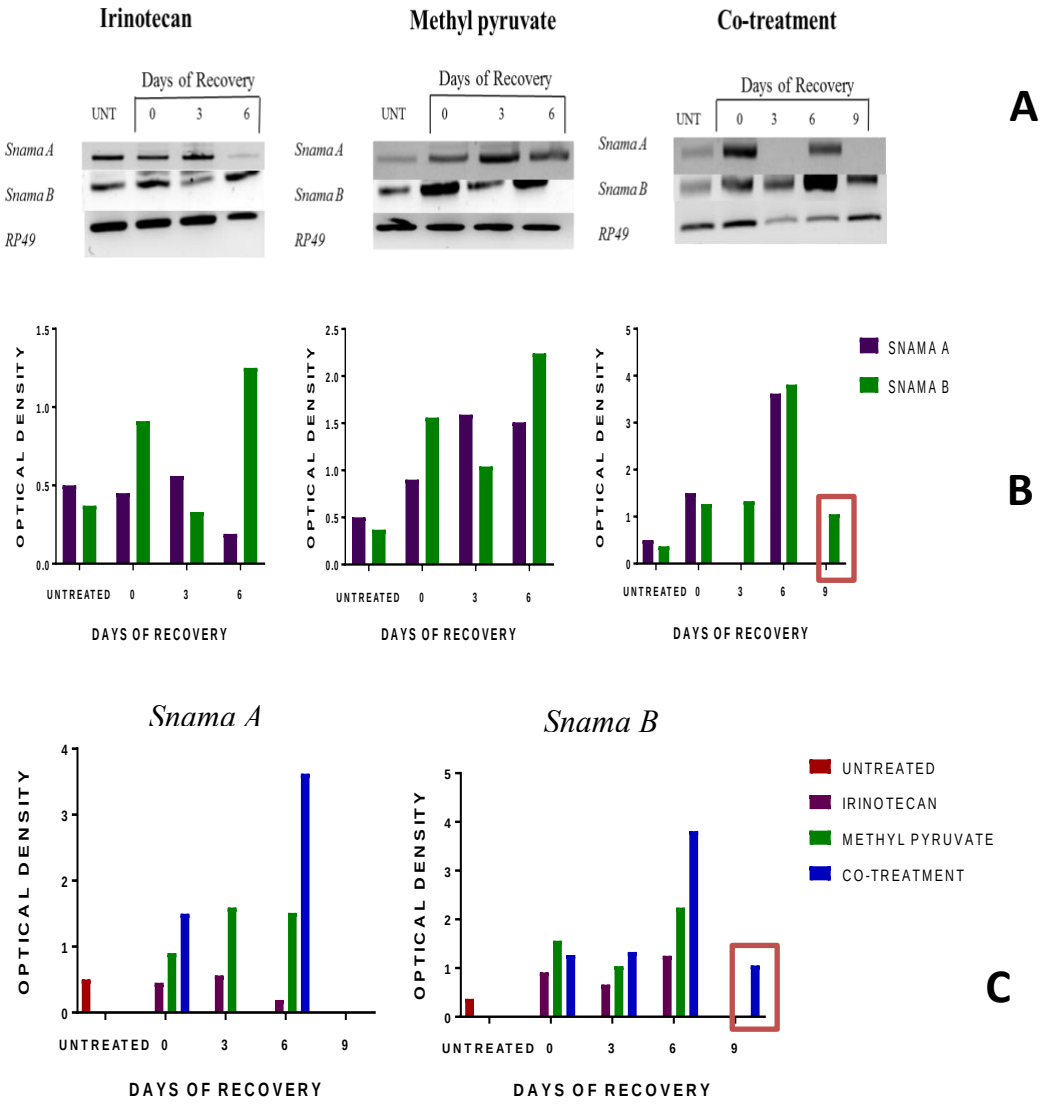


Figure 3.2: Differential gene expression of Snama A and B observed during treatment of wild type *Drosophila melanogaster*. (A) RT-PCR products run on an ethidium bromide stained agarose gel. (B and C) Bar charts were created by using GraphPad Prism 7 and results were normalised using *RP49*. UNT indicates the untreated sample and 0 the last day of treatment. Irinotecan, methyl pyruvate and combination of both were used to treat flies for three days and allowed nine days for recovery. During combination treatment flies live till day 9 (indicated by red borders).

3.4 Expression of *Snama* in p53 null mutants during treatment and recovery

Having seen the gene expression profiles of wild type *Snama* transcripts, we next investigated the expression patterns in p53 null mutants for comparative purposes. In this study the p53 null mutant *Drosophila melanogaster* were used to model cancer cells because of their mutated p53. Total RNA was extracted and thereafter RT-PCR was performed.

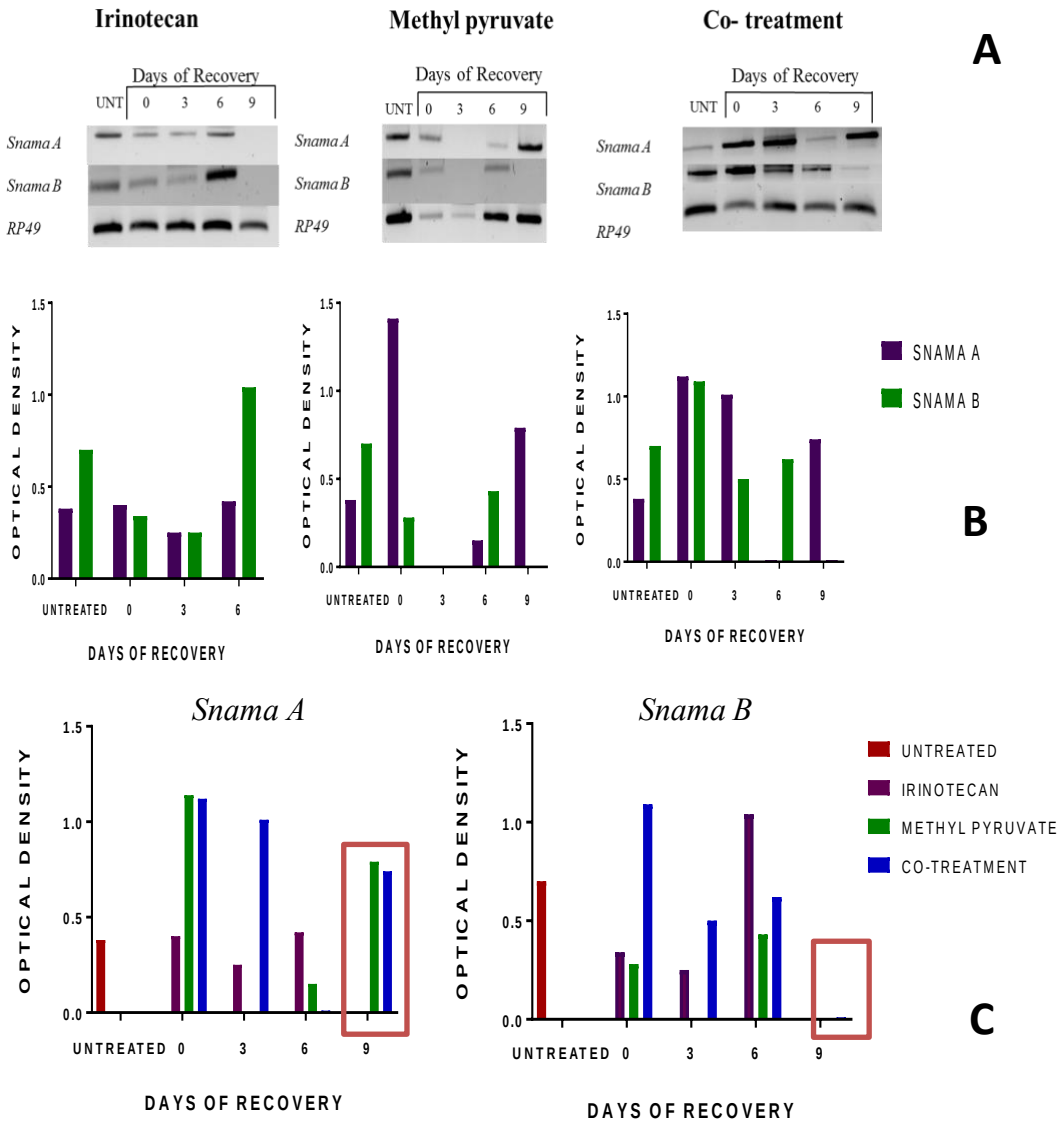


Figure 3.3: Differential gene expression of Snama A and B observed during treatment and recovery of p53 null mutant flies. (A) RT-PCR products run on an ethidium bromide stained agarose gel. (B and C) Bar charts were created by using GraphPad Prism 7 and results were normalised using RP49. UNT indicates the untreated sample and 0 the last day of treatment. Irinotecan, methyl pyruvate and combination of both were used to treat flies for three days and allowed nine days for recovery. Exogenous pyruvate increases the

expression of the pro-proliferative Snama A and flies lived till day 9 while Snama B was downregulated (indicated by red borders).

Results obtained show that Irinotecan treated flies lived longer although no expression of transcripts were observed at day 9 (Figure 3.3), suggesting that p53 null mutants are resistant to irinotecan. Methyl pyruvate treatment upregulated the pro-proliferative Snama A as previously observed in wild type flies. Combination of irinotecan with exogenous pyruvate treatment upregulated the expression of Snama A throughout treatment and recovery and flies lived till day 9 (Figure 3.3).

3.5 Expression of SNAMA protein in wild type *Drosophila melanogaster* during treatment and recovery

Although RT-PCR showed the varying expression of both transcripts, protein production levels were investigated to compare with mRNA expression as they sometimes do not correlate. Proteins are known to be more stable thus it was important to investigate the protein expression pattern of SNAMA in p53 null mutants further elucidating the biochemical roles of RBBP6 isoforms in metabolic reprogramming which takes place following DNA damage. Wild type flies were treated with irinotecan, methyl pyruvate and the combination of both. Protein was extracted at determined days (three-day interval from 0-9) and Western blotting performed. Three independent experiments were carried out and they show that wild type *Drosophila melanogaster* displayed contrasting expression patterns of SNAMA isoforms following treatment with irinotecan, methyl pyruvate and the combination of both.

Irinotecan treated flies tend to downregulate both isoforms although SNAMA B is more downregulated while SNAMA A is at higher levels during recovery (Figure 3.4). Combination treatment with exogenous pyruvate maintains this balance. Treatment with exogenous pyruvate causes downregulation of both isoforms in recovery. This downregulation was accompanied by increased survival of wildtype flies compared to other treatments (Figure 3.4, Figure 3.7) indicating the positive role methyl pyruvate might play in protecting and enhancing survival in normal cells.

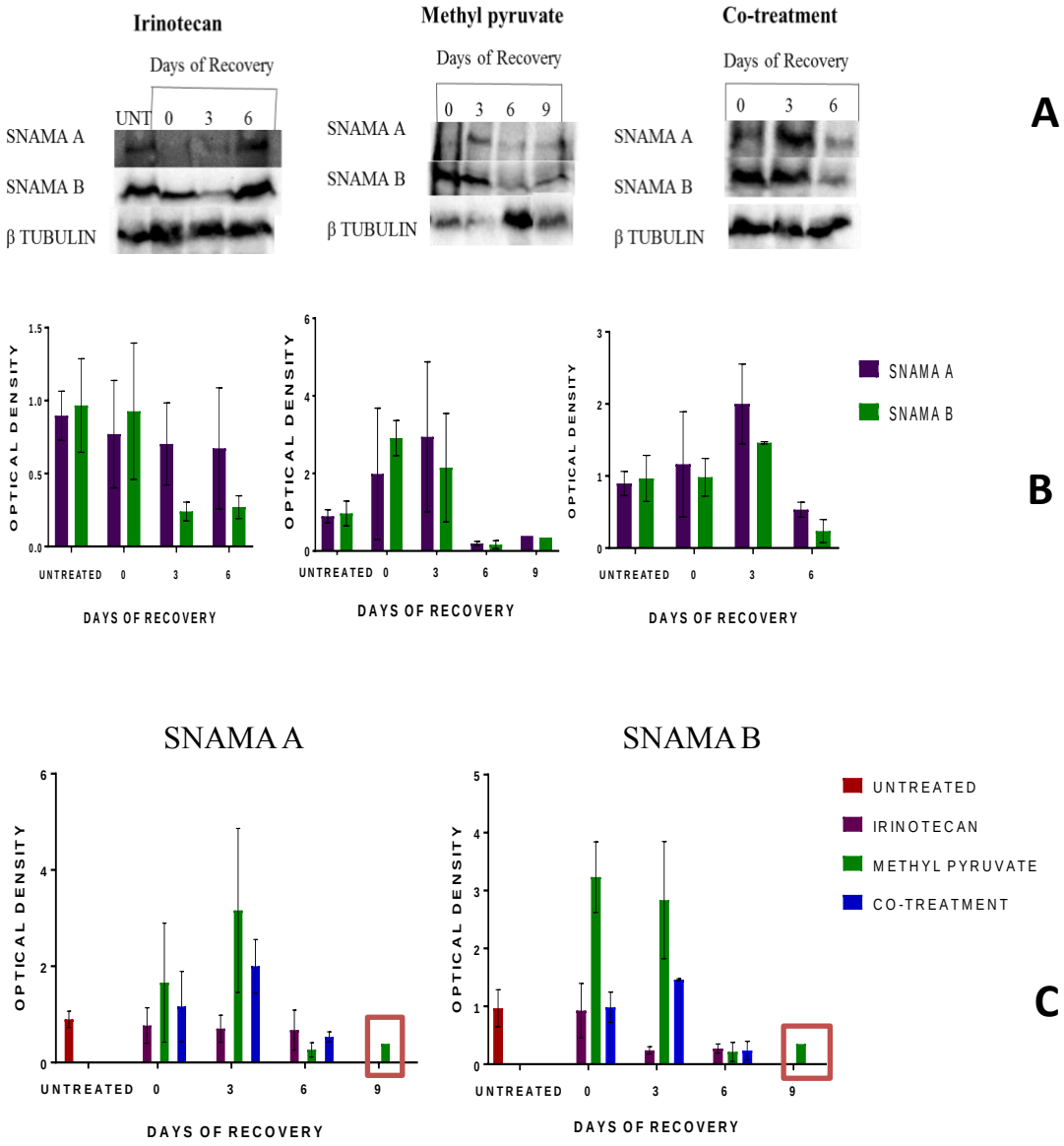


Figure 3.4: Differential protein expression of SNAMA A and B observed during treatment and recovery of wild type flies. (A) Blots showing protein expression of SNAMA isoforms. (B and C) Densitometry analysis were created by using GraphPad Prism 7 and results were normalised using β tubulin as the reference protein. UNT indicates the untreated sample and 0 the last day of treatment. The error bars respresent the standard deviation between samples. Irinotecan, methyl pyruvate and combination of both were used to treat flies for three days and allowed nine days for recovery. Methyl pyruvate underexpresses SNAMA B which correlate with survival (Indicated by red border).

3.6 Expression of SNAMA protein in p53 null mutants *Drosophila melanogaster* during treatment and recovery

Protein expression in p53 null mutants was investigated and compared with wild type flies. Flies were treated with irinotecan, methyl pyruvate and the combination of both. Protein was extracted, and Western blot was performed. Two independent experiments were carried out, and they show that when p53 null mutant flies are untreated SNAMA B is overexpressed and treatment with irinotecan results in a downregulation throughout recovery (Figure 3.5).

Irinotecan suppressed the expression of both isoforms in p53 null mutants while SNAMA B dominated. Contrary to SNAMA B, p53 null mutant flies shows a downregulation of SNAMA A when untreated and during treatment while an upregulation during recovery (Figure 3.5). It appears that isoforms show contrasting expression patterns. Methyl pyruvate treatment shows that SNAMA A hardly varies staying at low levels while SNAMA B tends to increase. SNAMA B is overexpressed especially in combination treatment

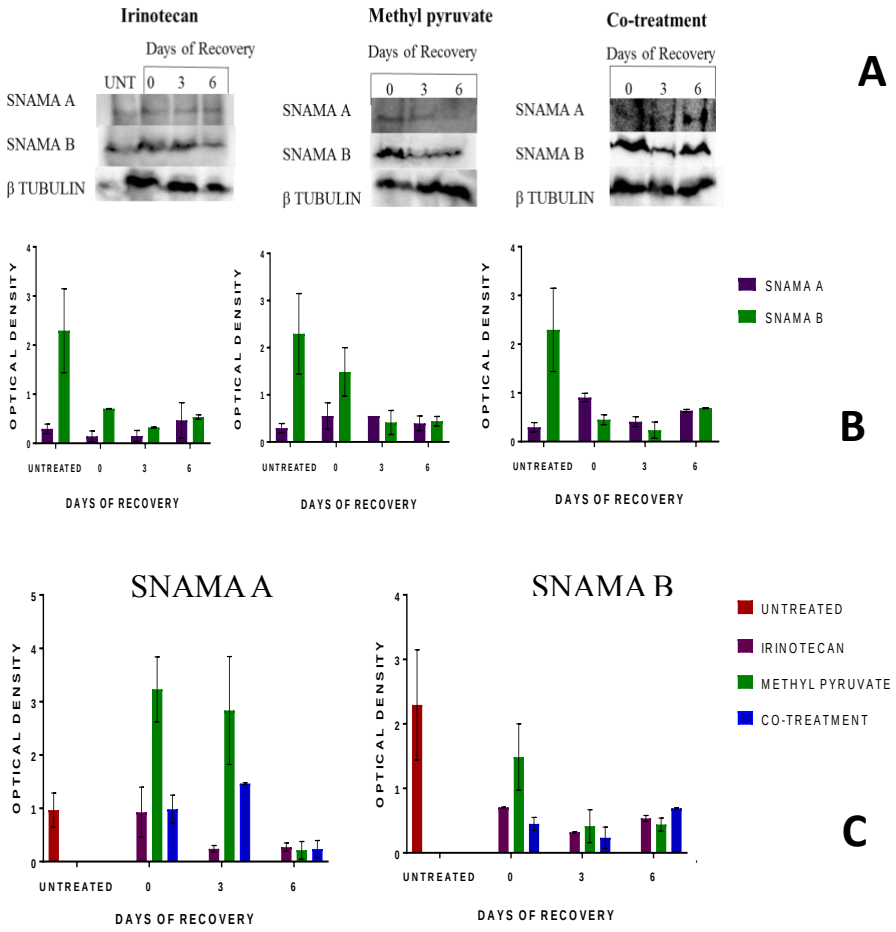


Figure 3.5: Differential protein expression of SNAMA A and B observed during treatment and recovery of p53 null mutant flies. (A) Blots showing protein expression of SNAMA isoforms. (B and C) Bar charts were created by using GraphPad Prism 7 and results were normalised using β tubulin as the reference protein. UNT indicates the untreated sample and 0 the last day of treatment. The error bars respresent the standard deviation between samples. Irinotecan, methyl pyruvate and combination of both were used to treat flies for three days and allowed nine days for recovery. Isoforms show contrasting expression patterns.

3.7 Comparison of *Snama* A and B in both wild type and p53 null mutants during treatment and recovery

mRNA expression levels are often used to speculate functional differences that occur at the protein level although they don't always correlate to protein levels due to the occurrence of post-transcriptional regulation (Greenbaum et al., 2003). Expression patterns of both Snama isoforms in normal and p53 null mutant flies were compared to gain better insight into their roles in DNA-damage response. Flies were treated with irinotecan and exogenous pyruvate followed by Reverse Transcriptase Polymerase Chain Reaction as well as Western blot (Figure 3.6, Table 3.1).

The results obtained shows that wild type flies have contrasting expression pattern to p53 null mutants when treated with irinotecan alone or in combination with exogenous pyruvate. Exogenous pyruvate tends to upregulate Snama A. When Snama A is overexpressed, Snama B is downregulated at various treatments in both wild type and p53 null mutants (Figure 3.6 i-iii). This indicates that transcription of Snama is not completely dependent on p53 and the splicing of transcripts may be differentially controlled. At the protein level, reversed differential expression patterns across all treatments and strains were observed. Also, treatment with methyl pyruvate reduced the expression of SNAMA B in wild type flies which correlates with survival (Figure 3.6 v). In p53 null mutants however, SNAMA B was overexpressed especially in cotreatment (Figure 3.6 vi).

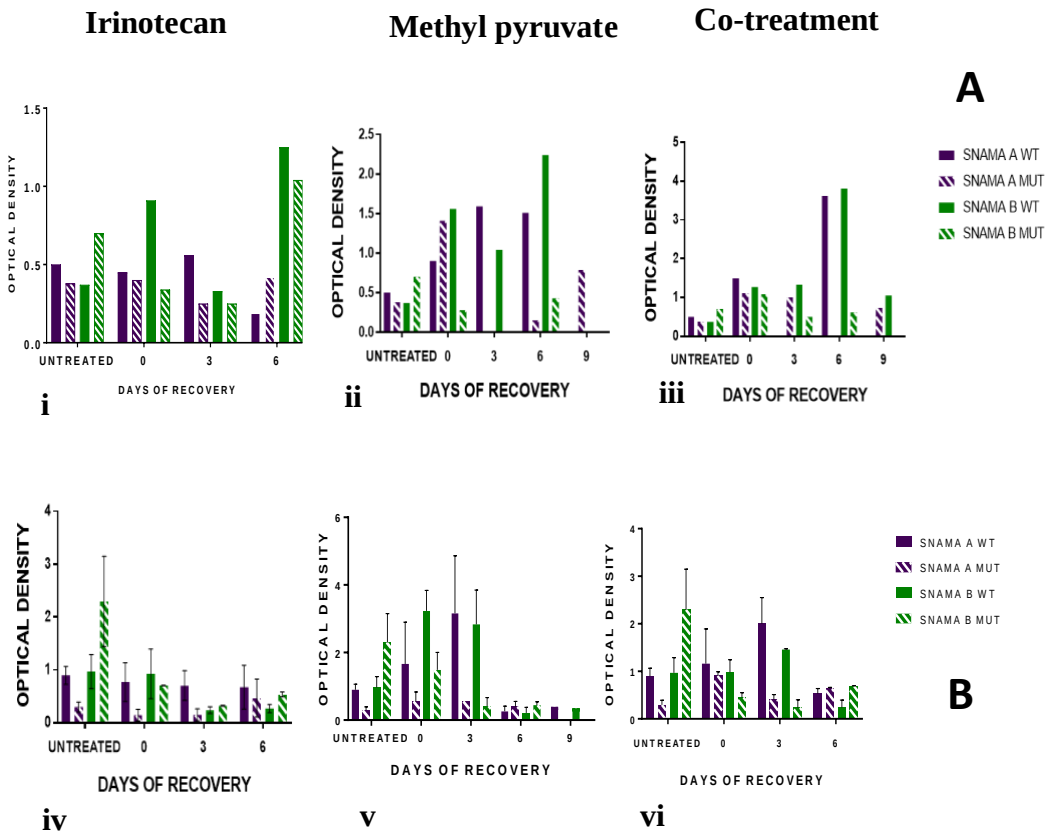


Figure 3.6: Gene (A) and protein (B) expression of Snama isoforms from wild type and p53 null mutant Drosophila melanogaster during treatment and recovery. Graph i-iii represent gene expression of *Snama A* and *B* in irinotecan (i), methyl pyruvate (ii) and the combination treatment (iii). Graph d-f on the other hand represent protein expression of isoforms in irinotecan (iv) methyl pyruvate (v) and combination treatment (vi).

Table 3.1: Regulation of SNAMA isoforms during treatment and recovery.

mRNA expression					
Snama A			***Snama B***		
Wild type		**P53 null mutants**	**Wild type**		**P53 null mutants**
Irinotecan	Up (T), Down (R)	Up (T), Up (R)	IR	Up (T), Up (R)	Down (T), Up (R)
Methyl Pyruvate	Up (T), Up (R)	Up (T), Up (R)	MP	Up (T), Up (R)	Down (T), Down (R)
Co-Treatment	Up (T), Up (R)	Up (T), Up (R)	COT	Up (T), Up(R)	Up (T), Down (R)
Protein expression					
SNAMA A			**SNAMA B**		
Wild type		**P53 null mutants**	**Wild type**		**P53 null mutants**
Irinotecan	Down (T), Down (R) D	Down (T), Up (R) D	IR	Up (T), Down (R) D	Down (T), Down (R) D
Methyl Pyruvate	Up (T), Down (R) S	Up (T), Up (R) D	MP	Up (T), Down (R) S	Down (T), Down (R) D
Co-Treatment	Up (T), Down (R) D	Up (T), Up (R) D	COT	Up (T), Down (R) D	Down (T), Down (R) D

T- Treatment
R- Recovery
D- Dead before day 9
S- Survived till day 9
Up- Up regulated
Down- Down regulated

3.8 Survival Analysis of wild type and p53 null mutants

The survival trends of wild type and p53 null mutants were studied to investigate the effects of exogenous pyruvate during DNA damage. Flies were treated with irinotecan, methyl pyruvate and the combination of both for three days and allowed nine days recovery period and counted in three-day intervals. Results obtained show that the untreated wild type had a better survival trend than all three treatments as expected. Furthermore, exposure to exogenous pyruvate increases survival in wild type flies compared to irinotecan treated (Figure 3.7). When methyl pyruvate is

combined with irinotecan this also improved survival in wild type flies. This indicates that methyl pyruvate protects wild type flies from DNA damage.

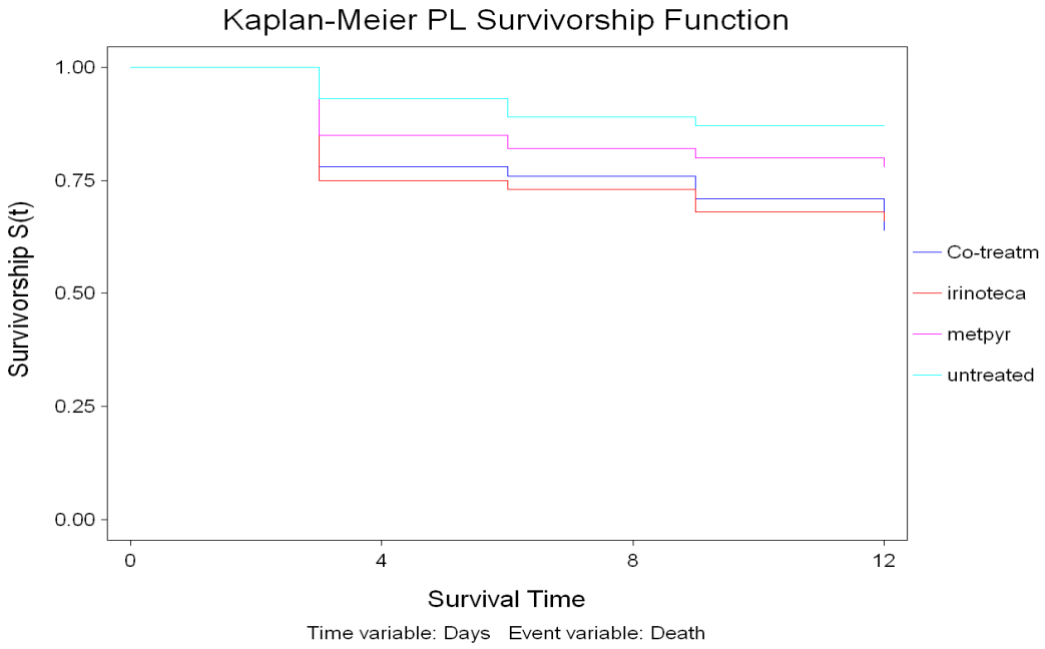


Figure 3.7: Survival analysis of wild type Drosophila melanogaster. Untreated flies showed better survival than all treatments as expected. Methyl pyruvate increases survival of flies compared to irinotecan. Figure was generated using Statistix 10 software.

In p53 null mutants, combination treatment with methyl exogenous pyruvate and irinotecan results in less survival during early recovery days (kills p53 null mutants) compared to irinotecan alone (Figure 3.8). This indicates that exogenous pyruvate induces toxicity in p53 null mutants.

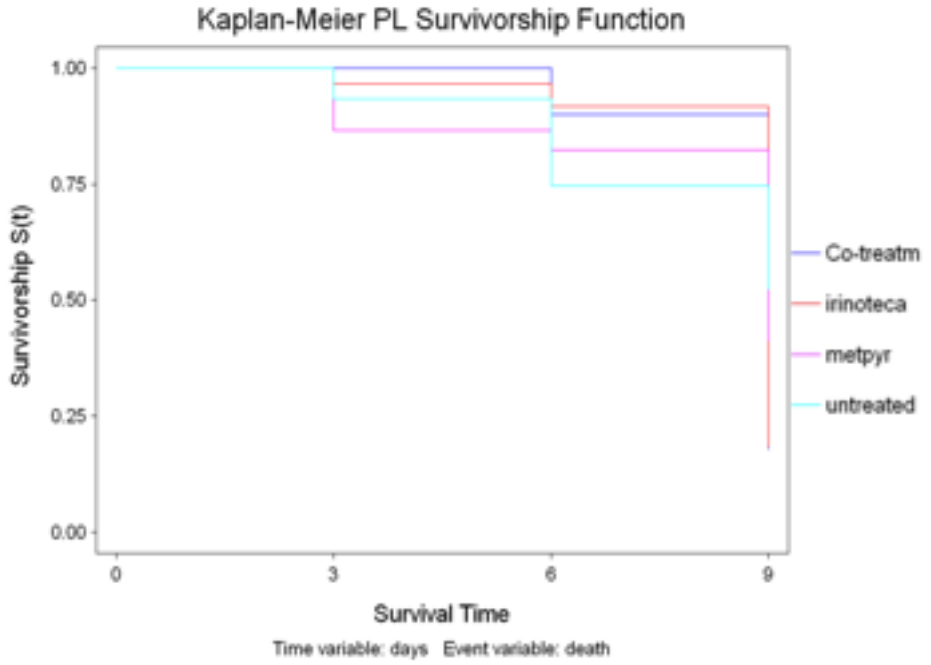


Figure 3.8 Survival analysis of p53 null mutant Drosophila melanogaster. P53 null mutants have a better survival trend when treated with irinotecan compared to methyl pyruvate which results in less survival. Figure was generated using Statistix 10 software.

CHAPTER 4: DISCUSSION

4.1 Overview

Retinoblastoma binding protein 6 is an important protein in embryonic development and in carcinogenesis. This study investigated the roles of SNAMA (the Drosophila orthologue) isoforms in metabolic reprogramming occurring during carcinogenesis. Wild type and p53 null mutant Drosophila melanogaster were used as a model for normal and cancer cells respectively. Comparison of RBBP6 isoforms in vertebrate and invertebrates was done using sequence alignment tools. Survival analysis was carried out using Kaplan Meier plot to investigate the role of exogenous pyruvate in protecting normal cells during irinotecan induced DNA damage. Evidence suggest that p53 mutation can induce the Warburg effect, this study observed this can be reversed by exogenous pyruvate. Moreover, expression patterns of the isoforms were investigated using Reverse Transcriptase Polymerase Chain Reaction and Western blot analysis.

We found that the two Drosophila melanogaster isoforms, SNAMA A and B are expressed contrastingly depending on the stress they encounter and may play roles in carcinogenesis. They may probably be involved in the apoptotic pathway where they either stimulate or repress it. SNAMA B probably antagonizes SNAMA A leading to its reduced expression. Similarly, just like in humans (specifically in polyadenylation) where the smaller isoform 3 which is structurally similar to SNAMA B antagonizes the larger isoform by competitive binding. SNAMA B may also function like the human isoform 3 whereas SNAMA A may function like Mdm2. This suggestion is made following the observation that they share similar structural domains and function in inhibiting p53. Furthermore, we also observed that bypassing the glycolytic pathway by treatment with methyl pyruvate affects the expression of SNAMA isoforms leading to divergent effects on normal and p53 null mutants. This change in expression brought about by methyl pyruvate correlated with increased survival in normal flies undergoing chemotherapy and decreased survival in p53 null mutant flies indicating that methyl pyruvate might probably induce apoptosis in a p53 independent manner.

4.2 SNAMA isoforms show contrasting expression patterns following irinotecan induced DNA damage

During carcinogenesis, cancer cells prefer glycolysis circumventing OXPHOS. This study investigated possible roles that SNAMA isoforms (Drosophila orthologue of RBBP6) may play during DNA damage and metabolic reprogramming. This was done by observing SNAMA expression profile of wild type and p53 null mutants following treatment with irinotecan (to induce DNA damage) and exogenous pyruvate (to boost OXPHOS). Reverse Transcriptase Polymerase Chain Reaction and Western blot analysis were carried out. It was observed that both isoforms show contrasting expression patterns in wild type and p53 null mutants across all treatments. SNAMA A is increased in untreated wild type and reduced in untreated p53 null mutant flies. On the other hand, SNAMA B is reduced in untreated wild type and increased in untreated p53 null mutants, showing differential expression. Interestingly, in untreated p53 null mutants SNAMA A is decreased whilst SNAMA B is increased. This suggests that both isoforms may probably play roles that vary in the presence or absence of p53. SNAMA B showed reduced expression when wild type files were treated with methyl pyruvate, whereas it was increased in p53 null mutants mainly when methyl pyruvate was combined with irinotecan. This suggests that the absence of p53 increases the pro-apoptotic SNAMA A, indicating that the expression of SNAMA is not completely dependent on p53. Thus, SNAMA B is the probable pro-apoptotic isoform and SNAMA A anti-apoptotic.

SNAMA shows some striking similarities with MDM2. P53 activates the transcription of MDM2 and upon expression MDM2 negatively regulates p53. Work done by Hull and colleagues (2015) showed that Snama has a p53 binding domain suggesting it can possibly transcriptionally activate Snama. Nonetheless, there are situations where p53 can be upregulated without Snama transcripts being upregulated. Also, situations where p53 is absent and Snama transcripts fluctuates. This indicates that there are some cases where p53 is not involved in the transcription of Snama gene. Since Snama A and B are spliced products, it is also possible their splicing is also controlled.

SNAMA A and B probably have different roles and their function may counteract one another. In this study, we observed that during the overexpression of Snama A, Snama B is underexpressed and vice versa especially in p53 null mutant flies further suggesting they may possibly be involved in divergent roles. This is not surprising as it has been observed that the different isoforms of the human RBBP6 play different roles. For example, the human isoform 3 of RBBP6, which comprises only the DWNN domain plays a role in regulating the cell cycle at G2/M and its down regulation reduces apoptosis, while the under expression of isoform 1 leads to reduced cell proliferation. These roles counteract one another (Mbita et al., 2012; Ntwasa, 2016). The possibly different roles that may be played by SNAMA isoforms might be due to the fact that although they share similar binding domains, they also differ in some. However, their similarities in domain structures might also lead to competitiveness for binding sites. Due to their differences in domain structure, for example the nuclear localisation domain only present in SNAMA A, they may function in different locations within the cell. SNAMA A may probably function in the nucleus while SNAMA B in the cytoplasm.

This study suggests that SNAMA B might have an antagonistic role towards SNAMA A. In humans, the short isoform structurally similar to SNAMA B antagonises the larger isoform by competitive binding to the cleavage stimulation factor (CstF) complex that is involved in the 3' end cleavage and polyadenylation of pre-mRNAs (Di Giammartino et al., 2014). In Drosophila melanogaster, a similar trend was observed where SNAMA B confidentially appears to under- express SNAMA A. A hypothetic model of how SNAMA isoforms antagonizes one another and how they may probably be involved in apoptosis is shown in the diagram below (Figure 4.1). It illustrates how the inhibition of SNAMA A by SNAMA B represses Dmp53 activation which leads to reduced apoptosis. However, in p53 null mutants it is suspected that addition of exogenous pyruvate might induce apoptosis independently of p53 following the intrinsic (death) pathway.

Further elucidation of the roles of SNAMA isoforms especially in regard to apoptosis is crucial as it might help in the discovery of novel treatment strategies.

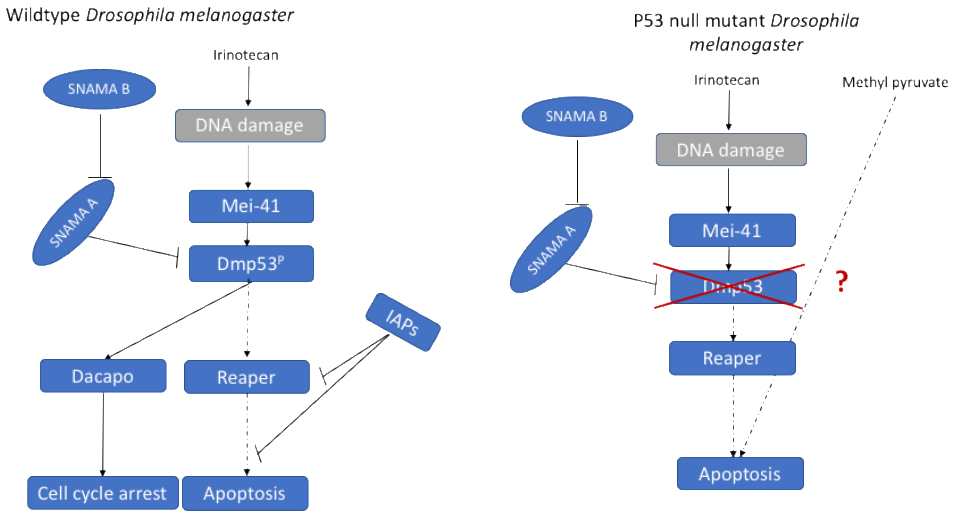


Figure 4.1: Hypothetic model of how SNAMA isoforms may produce the antagonistic roles. This schematic diagram shows the apoptotic pathway where irinotecan induces DNA damage which then stimulates Mei-41, then Dmp53 induction either activates the cell cycle arrest pathway or apoptosis. In wild type SNAMA B inhibit SNAMA A which then inhibits Dmp53 leading to reduced apoptosis. In p53 null mutants, cell death may occur independently of p53 with help from methyl pyruvate.

4.3 SNAMA may be a functional homolog of Mdm2

Several studies have observed the roles of MDM2 in cancers. Of note is its overexpression in several tumours, in fact it is suggested to be a potential biomarker in this regard. This overexpression has also been linked to poor prognosis, metastasis and drug resistance. However, during cancer treatment, the expression of MDM2 decreases and when it is knocked down cancer cells die (Jones et al., 1995; Shi and Gu, 2012). This study has used p53 null mutant flies as models of cancers as p53 is mutated and non-functional in over 50% of cancers. Using Reverse Transcriptase Polymerase Chain Reaction and Western blot analysis, the biochemical roles of SNAMA isoforms were investigated. Flies were treated with irinotecan, methyl pyruvate and the combination of both and left to recover.

Results showed molecular changes in treated and recovering flies. Chemotherapy (irinotecan treatment) and treatment with methyl pyruvate were observed to reverse

expression of both SNAMA isoforms compared to untreated wild type and p53 null mutant flies. This reversal of expression correlates to poor survival in p53 null mutant flies indicating p53 independent apoptosis. The similarity of expression patterns of SNAMA and MDM2 in normal and cancerous cells is striking suggesting SNAMA may in fact act as a functional homolog of MDM2. It is interesting to note that there is currently no identified homolog of MDM2 in Drosophila melanogaster.

Both RBBP6 and MDM2 proteins are nuclear proteins that comprise the E3 ligase activity and catalyse ubiquitination substrates such as p53 (Chibi et al., 2008). It has been observed that in mammals MDM2 and RBBP6 are structurally similar, possessing domains such as the RING finger, p53 binding and pRb binding sites. Both proteins are important during embryogenesis, deletion or knockdown of SNAMA leads to death of embryos (Hull et al., 2015). The same with absence of PACT (RBBP6 homolog in mice) results in early death and they die with a posterior phenotype. This phenotype is rescued when both p53 and PACT are knocked down (Jones et al., 1995). Similarly, it was observed when both SNAMA and p53 were absent flies lived (Nweke, 2015) indicating the absence of apoptosis.

This study proposes that SNAMA likely functions similarly to MDM2 due to the identification of some structural and functional similarities. For example, both SNAMA and MDM2 are negative regulators of p53 and are both important in development. However, the mechanism of action of SNAMA is still not fully understood.

The possible role of SNAMA as a functional homolog of MDM2 further strengthens its proposal as a good target for cancer treatment.

4.4 Exogenous pyruvate protects wild type flies from irinotecan toxicity while killing p53 null mutants

The commitment of cancer cells to the Warburg effect is a hallmark of cancer. Reversing this metabolic shift can help in killing cancer cells while saving normal cells. The effects of circumventing the glycolytic pathway was investigated in this

study. Wild type and p53 null mutant Drosophila melanogaster were treated with irinotecan and methyl pyruvate. Irinotecan was used to induce DNA damage while exogenous pyruvate used to perturb metabolism and bypass the glycolytic pathway.

Results showed that when wild type and p53 null mutants were treated with irinotecan did not live past day six of recovery suggesting an unbiased toxicity to normal and cancer cells. Irinotecan targets rapidly dividing cells. Rapid division of cells can also be observed in normal cells especially during DNA replication. For example, cells in the hair, blood, epithelial, bone marrow, get targeted due to their fast replicating nature. During chemotherapy, p53 gets activated by irinotecan (Takeba et al., 2007), consequently inducing apoptosis. The toxicity of irinotecan to normal cells have been identified in several studies (Xu, 2002) and is noted to be one of the cons of chemotherapy.

In this study, it was further observed that the introduction of exogenous pyruvate, increased survival in wild type flies compared to irinotecan treatment which killed them. On the other hand, p53 null mutants were less susceptible to irinotecan than wildtype. Since irinotecan activates p53 and null mutants lack p53, they become resistant to it and survive longer, however, exogenous pyruvate remarkably kills them. Since p53 is involved in the regulation of the glycolytic pathway, bypassing it might decrease p53 dependent apoptosis and thus increase survival. On the other hand, p53 null mutant flies didn't survive when treated with methyl pyruvate suggesting methyl pyruvate induces p53 independent apoptosis. Cancer cells prefer to metabolise glucose using the inefficient glycolysis pathway even in an abundant presence of oxygen. This is called the Warburg effect. In contrast, normal cells prefer mitochondrial OXPHOS which yields a net total of 36 ATP molecules compared to only two in glycolysis. This study exposed flies to exogenous pyruvate, thus providing the end product of glycolysis and consequently bypassing the pathway.

In cancer cells and models, exogenous pyruvate treatment reverses the metabolic reprogramming and is thus toxic to them and enhances the expression of isoforms. The observations of the effect of exogenous pyruvate correlates with work done by

Monchusi and Ntwasa (2017) in which exogenous pyruvate protected the normal lung fibroblast MRC-5 cells from irinotecan toxicity but killed cancer cells.

4.5 Conclusion

This study has shed light on the possible roles of RBBP6 isoforms during metabolic reprogramming and carcinogenesis. Further evidence that *Drosophila melanogaster* homologue of RBBP6- SNAMA- may be a functional homologue of MDM2 is provided and thus a negative regulator of p53. Furthermore, SNAMA isoforms display contrasting expression patterns depending on the stress brought upon them. The expression of these isoforms is further affected during the circumvention of metabolic reprogramming. Moreover, bypassing the glycolytic pathway by the use of exogenous pyruvate has diverse effects in normal cells (wild type flies) and a cancer model (p53 null mutant flies), having protecting effects on the former and killing the latter. These observations hint at the crucial role of RBBP6 in carcinogenesis and as a potential druggable target for cancer therapy. Future studies validating the roles of RBBP6 investigating the use of RBBP6 as a target in cancer therapy may help in the discovery of novel treatment and management regimens.

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APPENDIX

Appendix A: Lab equipment

Table A1: Laboratory equipment, manufactures and model number of equipment used in this study.

LAB EQUIPMENT	MANUFACTURER & MODEL NO.
Biofuge pico (centrifuge machine)	Heraeus Instruments D-31520
Computer Controlled Electrophoresis power supply	Bio-Rad 3000Xi
Digital dry bath	Labnet D1100-230v
GeneAmp PCR System	Perkin Elmer 2400
Gel doc System	Bio-Rad XR+
INJECT+ MATIC sleeper®	Geneve CG-1207
Laboratory labelling system	LAB XPERT™ XC-500-461
Magnetic stirrer	Freed Electric MH4-9517
Microwave Oven	KIC MWS- 900M
Motor	Heidolph 50300
Water bath	Julabo p 130
Weighing balance	Precisa XT220A

G10 Gyrotory® Shaker	New Brunswick Scientific (U.S.A) 3,430,926
Junior Orbit Shaker	LAB-LINE 3521 3522-1
Nanodrop® spectrophotometer	Thermo Fischer Scientific USA 1000
Hoefer VE blotting module	Amersham Biosience UK

Appendix B: Buffers

Table A2: Buffers and their compositions used in this study.

BUFFERS	**COMPOSITION**
2X SDS Sample buffer	1 M Tris-HCl buffer (pH 6.8) 50% Glycerol, 10% SDS 0.5% β-Mercaptoethanol 4.4 % Bromophenol blue
1X PBST	0.05 M Phosphate Buffers Tween-20 5.67 M NaCl 0.01 M KCl
5X Running buffer	25 mM Tris 192 mM glycine 1% SDS
Tris-HCL pH 6.8	0.5 M TRIS (hydroxymethyl) aminomethane
Tris-HCL pH 8.8	1.5M TRIS (hydroxymethyl) aminomethane
10% Ammonium persulphate	100mg Ammonium Persulphate + 1ml dH20
10% SDS	10% SDS
TOWBIN / Transfer buffer (pH8.3)	25 mM Tris base 192 mM glycine 0.1%SDS 20% (v/v) methanol on day of use.

Appendix C: Chemicals

Table A3: Chemicals, catalogue numbers as well as suppliers used in this study.

CHEMICALS	**CATALOGUE NO.**	**SUPPLIER**
2- Mercapto Ethanol	M-3148	SIGMA
Acrylamide	A3699	SIGMA
Acetic acid glacial	2789	MERCK
Agar Agar Powder	1037407	MERCK
Agarose Powder	50004	WhiteSci
Ammonium persulphate	A0118|O00500	ASSOCIATED CHEMICAL ENTERPRISES
Bio-rad protein assay	500-0006	BIO-RAD
Ethanol absolute	20821.330	VWR CHEMICALS
Glycerol	2676500LC	MERCK
Glycine	G8898	SIGMA
Irinotecan hydrochloride	L1406-	SIGMA
Methanol	781114	RAD-CHEM
Methyl 4- hydroxybenzoate	H5501	SIGMA

Methyl pyruvate	371173	SIGMA
Nuclease free water	129117	QIAGEN
Phosphate Buffered Saline	P4417	SIGMA
PCR Mastermix 2X	M0482S	NEW ENGLAND BIOLABS
Streptomycin sulfate salt	A9518	SIGMA
Propan-2-ol	5075040LC	VWR CHEMICALS
Sodium Dodecyl Sulphate	442444H	VWR CHEMICALS
Super block PBS blocking	37580	THERMO SCIENTIFIC
TEMED	T 8133	SIGMA
Tris (hydroxymethyl) amiomethane	T 1503	SIGMA
TWEEN 20	DB0560	BIO BASIC INC.
TRI Reagent	T9424	SIGMA

Appendix D: Aligned Sequences of RBBP6 isoforms from different organisms

KEY:

Human (RBBP6)

A1 Isoform 1 ⟶ (NP_008841.2)

A2 Isoform 2 ⟶ (NP_061173.1)

A3 Isoform 3 ⟶ (NP_1160152)

Mice (P2P-R)

A4 Isoform 1 ⟶ (NP_035377.2)

A5 Isoform 2 ⟶ (NP_778188.1)

***Drosophila* (SNAMA)**

A6 Isoform A ⟶ (NP_611884.1)

A7 Isoform B ⟶ (NP_001246487.1)

***C. elegans* (RBPL-1)**

A8 Isoform 1 ⟶ (NP_001032975.1)

A9 Isoform 2 ⟶ (AJ34990.1)

CLUSTAL O(1.2.4) multiple sequence alignment

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A8      MSSIHYKFRAELDYKTLQFDGLHIRGEQLVREICAKENL-KLELFELQLQNAHTKKTYSD
A9      MSSIHYKFRAELDYKTLQFDGLHIRGEQLVREICAKENL-KLELFELQLQNAHTKKTYSD
A6      -MSVHYKFKSTLNFDTITFDGLHISVGDLKREIVQQKRLGKIIDFDLQITNAQSKEEYKD
A7      -MSVHYKFKSTLNFDTITFDGLHISVGDLKREIVQQKRLGKIIDFDLQITNAQSKEEYKD
A3      MSCVHYKFSSKLNYDTVTFDGLHISLCDLKKQIMGREKL-KAADCDLQITNAQTKEEYTD
A5      MSCVHYKFSSKLNYDTVTFDGLHISLCDLKKQIMGREKL-KAADSDLQITNAQTKEEYTD
A4      MSCVHYKFSSKLNYDTVTFDGLHISLCDLKKQIMGREKL-KAADSDLQITNAQTKEEYTD
A1      MSCVHYKFSSKLNYDTVTFDGLHISLCDLKKQIMGREKL-KAADCDLQITNAQTKEEYTD
A2      MSCVHYKFSSKLNYDTVTFDGLHISLCDLKKQIMGREKL-KAADCDLQITNAQTKEEYTD
          .:**** : *::.*: ******   :* ::*  ::.* *    :**: **::*: *.*

A8      D-ELIPRNSSIIVQRFPRKDAAKVQKVQAGVNSGMVNQLDATS--------SFLDPSSHI
A9      D-ELIPRNSSIIVQRFPRKDAAKVQKVQAGVNSGMVNQLDATS--------SFLDPSSHI
A6      DGFLIPKNTTLIISRIPIAHPTKK--GWEP--PAAENAFSAA----------PAKQDNFN
A7      DGFLIPKNTTLIISRIPIAHPTKK--GWEP--PAAENAFSAA----------PAKQDNFN
A3      DNALIPKNSSVIVRRIPIGGVKSTSKTYVI--SRTEPAMATTKAVCKNTISHFFYTLLLP
A5      DNALIPKNSSVIVRRIPIGGVKSTSKTYVI--SRTEPVMGTTKAVCKNTITLFLHNCFYL
A4      DNALIPKNSSVIVRRIPIGGVKSTSKTYVI--SRTEPVMGTTKAIDDASASISLAQLTKT
A1      DNALIPKNSSVIVRRIPIGGVKSTSKTYVI--SRTEPAMATTKAIDDSSASISLAQLTKT
A2      DNALIPKNSSVIVRRIPIGGVKSTSKTYVI--SRTEPAMATTKAIDDSSASISLAQLTKT
        *  ***:*:::*: *:*     .               : ::

A8      SSAEFENMDEAERLNHIRDQSTRAYDQSNFRRRQPGIMTGPPPPTYTCNRCSQPGHWYKN
A9      SSAEFENMDEAERLNHIRDQSTRAYDQSNFRRRQPGIMTGPPPPTYTCNRCSQPGHWYKN
A6      MDLSKMQGTEEDKIQAMMMQSTVDYDPKTYHRIKGQSQVGEVPASYRCNKCKKSGHWIKN
A7      MDLSKMQGTEEDKIQAMMMQSTVDYDPKTYHRIKGQSQVGEVPASYRCNKCKKSGHWIKN
A3      L-------------------------------------------------------------
A5      YNVSVT--------------------------------------------------------
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A4      ANLAEANASEEDKIKAMMSQSGHEYDPINYMK---KTLVGPPPPSYTCFRCGKPGHYIKN
A1      ANLAEANASEEDKIKAMMSQSGHEYDPINYMK---KP-LGPPPPSYTCFRCGKPGHYIKN
A2      ANLAEANASEEDKIKAMMSQSGHEYDPINYMK---KP-LGPPPPSYTCFRCGKPGHYIKN

A8      CPMLNT---------KRTTGIPSQELMETTV-DDPDAMMHPSGKYVIPIMHWKARQETLA
A9      CPMKLQ---------APKTKVKREDKKRDDR----------------------------
A6      CPFVGGKDQQ---EVKRNTGIPRSFRDKPDAAEN------ESADFVLP------------
A7      CPFVGGKDQQ---EVKRNTGIPRSFRDKPDAAEN------ESADFVLP------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      CPTNGDKNFESGPRIKKSTGIPRSFMMEVKDPNMKGAMLTNTGKYAIPTIDAEAYAIGKK
A1      CPTNGDKNFESGPRIKKSTGIPRSFMMEVKDPNMKGAMLTNTGKYAIPTIDAEAYAIGKK
A2      CPTNGDKNFESGPRIKKSTGIPRSFMMEVKDPNMKGAMLTNTGKYAIPTIDAEAYAIGKK

A8      RK---NEDGSSSPAQTSRKVPPELLCPICQSLFKEAIVTSCCGNSYCADCIEARILDPDN
A9      ------------------------------------------------------------
A6      ------------AVQNQEIPEDLICGICRDIFVDAVMIPCCGSSFCDDCVRTSLLESED
A7      ------------AVQNQEIPEDLICGICRDIFVDAVMIPCCGSSFCDDCVRTSLLESED
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      EKPPFLPEEPSSSSEEDDPIPDELLCLICKDIMTDAVVIPCCGNSYCDECIRTALLESDE
A1      EKPPFLPEEPSSSSEEDDPIPDELLCLICKDIMTDAVVIPCCGNSYCDECIRTALLESDE
A2      EKPPFLPEEPSSSSEEDDPIPDELLCLICKDIMTDAVVIPCCGNSYCDECIRTALLESDE

A8      QKCPGADCGKDISITSIIPNKTLRDAAAAWLSATGPGAPTTPQIVPEPEQ------IRIR
A9      ------------------------------------------------------------
A6      SECPDCK-EKNCSPGSLIPNRFLRNSVNAFKNETGYNKSAAKPAAVKNEEKPPVEKEVEK
A7      SECPDCK-EKNCSPGSLIPNRFLRNSVNAFKNETGYNKSAAKPAAVKNEEKPPVEKEVEK
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      HTCPTCH-QNDVSPDALIANKFLRQAVNNFKNETGYTKRLRKQLPPPPPPVPPPRPLMQR
A1      HTCPTCH-QNDVSPDALIANKFLRQAVNNFKNETGYTKRLRKQLPPPPPPIPPPRPLIQR
A2      HTCPTCH-QNDVSPDALIANKFLRQAVNNFKNETGYTKRLRKQLPPPPPPIPPPRPLIQR

A8      ---IGLK---------------------APSSSQSQITPSGISPGST--------LVQQQ
A9      ------------------------------------------------------------
A6      -------KPVAEV-EPEETEV-------KPEKQ-KESETNGSNPPKS------ESPEPPA
A7      -------KPVAEV-EPEETEV-------KPEKQ-KESETNGSNPPKS------ESPEPPA
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      NLQPLMRSPISRQQDPLMIPVTSSSAHSAPSISSLTSNPSALAPSVSGNPSSAPAPVPDI
A1      NLQPLMRSPISRQQDPLMIPVTSSSTHPAPSISSLTSNQSSLAPPVSGNPSSAPAPVPDI
A2      NLQPLMRSPISRQQDPLMIPVTSSSTHPAPSISSLTSNQSSLAPPVSGNPSSAPAPVPDI

A8      T---------------------------TLTSV-SSGTSL--------------------
A9      ------------------------------------------------------------
A6      TTEPS-QKEKDKYDSDYEDNITIKMPQPAADS----------------------------
A7      TTEPS-QKEKDKYDSDYEDNITIKMPQPAADS----------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      TATVSISVHSEKSDGPFRDSDNKLLPAAALTSEHSKGASSIAITALMEEKGYQVPVLGTP
A1      TATVSISVHSEKSDGPFRDSDNKILPAAALASEHSKGTSSIAITALMEEKGYQVPVLGTP
A2      TATVSISVHSEKSDGPFRDSDNKILPAAALASEHSKGTSSIAITALMEEKGYQVPVLGTP

A8      ----------------------SAQPS---------------------------------
A9      ------------------------------------------------------------
A6      ----------------------TTVPSKRSPSYSHRSES---------SHRRDRSDYVSD
A7      ----------------------TTVPSKRSPSYSHRSES---------SHRRDRSDYVSD
A3      ------------------------------------------------------------
```

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A5      ------------------------------------------------------------
A4      SLLGQSLLHGQLIPTTGPVRINAARPGGGRPGWEHSNKLGYLVSPPQQIRRGERSCYRSI
A1      SLLGQSLLHGQLIPTTGPVRINTARPGGGRPGWEHSNKLGYLVSPPQQIRRGERSCYRSI
A2      SLLGQSLLHGQLIPTTGPVRINTARPGGGRPGWEHSNKLGYLVSPPQQIRRGERSCYRSI

A8      ------------NVNPSIPGIPLASQVPSM----------------VQDVSLPPPQLRQE
A9      ------------------------------------------------------------
A6      HDHKHQ--RPS--KSESVNKDRSLLPLPIGTLPSYQGHMMAESEEARRSSAYKPPY--MQ
A7      HDHKHQ--RPS--KSESVNKDRSLLPLPIGTLPSYQGHMMAESEEARRSSAYKPPY--MQ
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      NRGRHHSERSQRTQGPSLPATPVFVPVPPP-------------------PLYPPPPHTLP
A1      NRGRHHSERSQRTQGPSLPATPVFVPVPPP-------------------PLYPPPPHTLP
A2      NRGRHHSERSQRTQGPSLPATPVFVPVPPP-------------------PLYPPPPHTLP

A8      LPPGIPGLPQFGLPPPGVPGLSATVLPQHQSMPLNYGM----PLFSAGFPPAVSSVPRPS
A9      ------------------------------------------------------------
A6      MQRGPPPMHMMS---HHMPAYNNGFNNMGQRPPLSYVPYQNQSVHPMRAPYGSAGGGMNM
A7      MQRGPPPMHMMS---HHMPAYNNGFNNMGQRPPLR------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      LPPGVPPP-----------QFSPQF-PPGQPPPAGYSV------PPPGFP------PAPA
A1      LPPGVPPP-----------QFSPQF-PPGQPPPAGYSV------PPPGFP------PAPA
A2      LPPGVPPP-----------QFSPQF-PPGQPPPAGYSV------PPPGFP------PAPA

A8      AISDEWN-------------------------AFLQNKDRNSSRR---DHKDRTR-----
A9      ------------------------------------------------------------
A6      NMSQPFQSPNLASIYQGVAAKVGSGPIDDPLEAFN---RIMKEKE--RKKVDRFR-----
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      NISTPWVSSGVQTAHSNTIPTTQAPPLSR--EEFYREQRRLKEEEKKKSKLDEFTNDFAK
A1      NLSTPWVSSGVQTAHSNTIPTTQAPPLSR--EEFYREQRRLKEEEKKKSKLDEFTNDFAK
A2      NLSTPWVSSGVQTAHSNTIPTTQAPPLSR--EEFYREQRRLKEE----------------

A8      ------RKDRHDSRSRRRRDSSSSSSMSSSSS-DEDERR----RKRREK--ESSKKRRSV
A9      ------------------------------------------------------------
A6      ------SSDRHRSRSPDRQRHRFKSPMY-----EKDNSRDNLKDKRP-------------
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      ELMEYKKIQKERRRSFSRSKSPYSGSSYSRSSYTYSKSRSGSTRSRSYSRSFSRSHSRSY
A1      ELMEYKKIQKERRRSFSRSKSPYSGSSYSRSSYTYSKSRSGSTRSRSYSRSFSRSHSRSY
A2      ------------------SKSPYSGSSYSRSSYTYSKSRSGSTRSRSYSRSFSRSHSRSY

A8      EK--ERPRRADEHRRDRDRDRERDRDRSHRDVRSSGRSKDIKASSSHRRDRDDARRKDRR
A9      ------------------------------------------------------------
A6      --------------------RSRERKREHSYERHIRHPRSSRQPNDGS------------
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      SRSPPYPRRGRGK-----SRNYRSRSRSHGYHRSRSRSPPYRRYHSRSRSPQAFRGQSPT
A1      SRSPPYPRRGRGK-----SRNYRSRSRSHGYHRSRSRSPPYRRYHSRSRSPQAFRGQSPN
A2      SRSPPYPRRGRGK-----SRNYRSRSRSHGYHRSRSRSPPYRRYHSRSRSPQAFRGQSPN

A8      RDDVRKKERREKREEEDDDQKTKDAESKDEDEIDIDGIIAEYGNVQA-------------
A9      ------------------------------------------------------------
A6      --KSPGG--RIKRSGH-----RRSASP--KPGY-----------KSDYRD----------
A7      ------------------------------------------------------------
```

```
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      KRNVPQG--ETEREYF-----NRYREV--PPPYD---IKAYYGRSVDFRDPFEKERYREW
A1      KRNVPQG--ETEREYF-----NRYREV--PPPYD---MKAYYGRSVDFRDPFEKERYREW
A2      KRNVPQG--ETEREYF-----NRYREV--PPPYD---MKAYYGRSVDFRDPFEKERYREW

A8      ---------QDTDGDAQVADENAQNDEDSTSPKEESVSPKPDETHEEDADEEPVDEEM--
A9      ------------------------------------------------------------
A6      ---------KPY---NKP-----------SAPKTEAVEPPPPGFEPLQLTDED-------
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      ERKYREWYEKYYKGYAVG---------AQPRPSANREDFSPERLLPLNIRNSPFTRGRRE
A1      ERKYREWYEKYYKGYAAG---------AQPRPSANRENFSPERFLPLNIRNSPFTRGRRE
A2      ERKYREWYEKYYKGYAAG---------AQPRPSANRENFSPERFLPLNIRNSPFTRGRRE

A8      --DTEVVVRDKSIDPVYQAMETSEAEVAETKEESVPVEEDEEPENHDEDVEDHKKEKESQ
A9      ------------------------------------------------------------
A6      GYRNKH-----------PTS---------------------------------SEA--SQ
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      DYAAGQSHRNRNLGGNYPEK---------------------------------LSTRDSH
A1      DYVGGQSHRSRNIGSNYPEK---------------------------------LSARDGH
A2      DYVGGQSHRSRNIGSNYPEK---------------------------------LSARDGH

A8      SSTINTADEDDESSKKMKKHKKSKKNKKHHRKEEDGDEDEERKRKKHKKHKKEKKSKKEK
A9      ------------------------------------------------------------
A6      SSK-------GDSSK----------------KRGENR----HEEAPRKRHRSRSISKEPK
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      NAKDNPKSKEKESEN----------------VPGDGK----GN--KHKKHRKRRKGEE--
A1      NQKDNTKSKEKESEN----------------APGDGK----GN--KHKKHRKRRKGEE--
A2      NQKDNTKSKEKESEN----------------APGDGK----GN--KHKKHRKRRKGEE--

A8      RDE--DEDDLDTEKKEKK-------------KERKYQDDK--DEDSNRKE--------PR
A9      ------------------------------------------------------------
A6      PNDSNYRSLTPPAKITTP---------------------------------KMTAAQLRQR
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      -----SESFLNPELLETSRKCRESSGIDETKTDTLFVLPSRDDATPVRDEPMDAESITFK
A1      -----SEGFLNPELLETSRKSREPTGVEENKTDSLFVLPSRDDATPVRDEPMDAESITFK
A2      -----SEGFLNPELLETSRKSREPTGVEENKTDSLFVLPSRDDATPVRDEPMDAESITFK

A8      SLDDEDKVELDKNFA---DKKEKKKGKWNEDEEDIFEDRKEELPKESDRRDRKDRRHDDE
A9      ------------------------------------------------------------
A6      E---------SSPK-----TPEKSHDDYLTAKARIM-----------------ASQPVI
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      SVSDKDKREKDKPKVKSDKTKRKSDGSATAKKDNVLKPSKGPQEKVDGDREKSPRSEPPL
A1      SVSEKDKRERDKPKAKGDKTKRKNDGSAVSKKENIVKPAKGPQEKVDGERERSPRSEPPI
A2      SVSEKDKRERDKPKAKGDKTKRKNDGSAVSKKENIVKPAKGPQEKVDGERERSPRSEPPI

A8      DDRKERRHERDSQKIDEQDRKKERKRDRETEAYD-SDKLQAPKTKVKREDKKRDDRKDYE
A9      ------------------------------------------------------KDYE
A6      NDTEME-----------------TNVGKENKA----------KSPLSKDRK----KKKKD
```

```
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      KKAKEEATKIDSVKPSSSSQKDEKVTGTPRKAHSKSAKEHQEAKPAKDEKV----KKDCS
A1      KKAKEETPKTDNTKSSSSSQKDEKITGTPRKAHSKSAKEHQETKPVKEEKV----KKDYS
A2      KKAKEETPKTDNTKSSSSSQKDEKITGTPRKAHSKSAKEHQETKPVKEEKV----KKDYS

A8      RDRERRKDDYEKEKS-------KRKESDRDNEKEKQREKEVEKEHEKDRK------EKRK
A9      RDRERRKDDYEKEKS-------KRKESDRDNEKEKQREKEVEKEHEKDRK------EKRK
A6      KDKAERKKNKKDK---------RAKKEKGDRQKKSSSVNRSDSDINNSSLMNESNYK---
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      KDIKSEKPASKDEKAKKP-EKNKLLDSKGEKRKRKTEEKSVDKDFESSSM-KISKVEGTE
A1      KDVKSEKLTTKEEKAKKPNEKNKPLDNKGEKRKRKTEEKGVDKDFESSSM-KISKLEVTE
A2      KDVKSEKLTTKEEKAKKPNEKNKPLDNKGEKRKRKTEEKGVDKDFESSSM-KISKLEVTE

A8      IVEKESEKPRKSVHERMQKADSSTSSSSRTTTAPSLERKPVSFTVA-SSKPTTNIRV---
A9      IVEKESEKPRKSVHERMQKADSSTSSSSRTTTAPSLERKPVSFTVA-SSKPTTNIRV---
A6      VLS---PRA-----------------------------------------QSPSIEINAA
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      IVK---PSPKRKMEGDVE----------------KLERTPEKDKIASSTTPAKKIKLNRE
A1      IVK---PSPKRKMEPDTE----------------KMDRTPEKDKISL-SAPAKKIKLNRE
A2      IVK---PSPKRKMEPDTE----------------KMDRTPEKDKISL-SAPAKKIKLNRE

A8      ------------------------------------------------RQYSSSSST
A9      ------------------------------------------------RQYSSSSST
A6      ---QL-------------------------------------------------------
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      TGKKIGNAENASTTKEPSEKLESTSSKIKQEKVKGKAKRKVAGSEGSSSTLVDYTSTSST
A1      TGKKIGSTENISNTKEPSEKLESTSSKVKQEKVKGKVRRKVTGTEGSSSTLVDYTSTSST
A2      TGKKIGSTENISNTKEPSEKLESTSSKVKQEKVKGKVRRKVTGTEGSSSTLVDYTSTSST

A8      KEQEDEERSKRDRRKKDETDVESIGEKEKKSSSRKVPKESVDVKHKSTKIKFDL------
A9      KEQEDEERSKRDRRKKDETDVESIGEKEKKSSSRKVPKESVDVKHKSTKIKFDL------
A6      --SPT-----HNATENVNPKSHSILTVGAASDDNLGPRSKLSEANSVNLSKWEIDENILG
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      GGSPV-----RKSEEKTDTKRTVIKTMEEYNNDNTAPAEDVIIMIQVPQSKWDKDDFESE
A1      GGSPV-----RKSEEKTDTKRTVIKTMEEYNNDNTAPAEDVIIMIQVPQSKWDKDDFESE
A2      GGSPV-----RKSEEKTDTKRTVIKTMEEYNNDNTAPAEDVIIMIQVPQSKWDKDDFESE

A8      ------------------------------------------------------------
A9      ------------------------------------------------------------
A6      LEDSSKK-AAGASDDPSEITSDVLRKAENAIFAKAINAIRPMEFQ---------VII---
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      EEDVKTTQPIQSVGKPSSIIKNVTTKPSATAKYTEKESEQPEKLQKLPKEASHELMQHEL
A1      EEDVKSTQPISSVGKPASVIKNVSTKPSNIVKYPEKESEPSEKIQKFTKDVSHEIIQHEV
A2      EEDVKSTQPISSVGKPASVIKNVSTKPSNIVKYPEKESEPSEKIQKFTKDVSHEIIQHEV

A8      ------------------------------------------------------L----
A9      ------------------------------------------------------------
```

```
A6      NS--------KDNSKDRSVVRSDKDRSSSPRRNNSS----RSVKDRLGTK-----ISN--
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      RSSKGSASSEKGRAKDREHSGSEKDNP--DKRKSGAQPDKESTVDRLSEQGHFKTLSQSS
A1      KSSKNSASSEKGKTKDRDYSVLEKENP--EKRKNSTQPEKESNLDRLNEQGNFKSLSQSS
A2      KSSKNSASSEKGKTKDRDYSVLEKENP--EKRKNSTQPEKESNLDRLNEQGNFKSLSQSS

A8      ------------------------------------------------------------
A9      ------------------------------------------------------------
A6      DRSRSRDKSKGRRRAARSSDDDANRGRSDRH--------GSRKRDNR---SRDRAAPSE-
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      KETRTSEKHES---VRGSSNKDFTPGRDKKVDYDSRDYSSSKRRDERGELARRKDSPPRG
A1      KEARTSDKHDS---TRASSNKDFTPNRDKKTDYDTREYSSSKRRDEKNELTRRKDSPSRN
A2      KEARTSDKHDS---TRASSNKDFTPNRDKKTDYDTREYSSSKRRDEKNELTRRKDSPSRN

A8      ------------------------------------------------------------
A9      ------------------------------------------------------------
A6      ----------KRQERSYK----------RSSPEDDKLRRQNKEQSESKHG-----KHDQN
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      KESLSGQKSKLREERDLPKKGAE-SKKSNSSPPRDKKPHDHKAPYETKRPCEETKPVDKN
A1      KDSASGQKNKPREERDLPKKGTGDSKKSNSSPSRDRKPHDHKATYDTKRPNEETKSVDKN
A2      KDSASGQKNKPREERDLPKKGTGDSKKSNSSPSRDRKPHDHKATYDTKRPNEETKSVDKN

A8      ------------------------------------------------------------
A9      ------------------------------------------------------------
A6      NSDDSDRRA--AKNTKSSDSRVVSSVTAVVAPPKPCRPDNPFRKFVD-------------
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      SGKEREKHAAEARNGKESSG---GKLPCIPNPPDPPMEKELAAGQVEKSAVKPKPQLSHS
A1      PCKDREKHVLEARNNKESSG---NKLLYILNPPETQVEKEQITGQIDKSTVKPKPQLSHS
A2      PCKDREKHVLEARNNKESSG---NKLLYILNPPETQVEKEQITGQIDKSTVKPKPQLSHS

A8      ------------------------------------------------------------
A9      ------------------------------------------------------------
A6      ----------------------TSSSSSLVVKYDNTI------------QKE------GA
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      SRLSSDLTRETDEAAFEPDYNESDSESNVSVKEEEAVASISKDLKEKTTEKAKESLTVAT
A1      SRLSSDLTRETDEAAFEPDYNESDSESNVSVKEEESSGNISKDLKDKIVEKAKESLDTAA
A2      SRLSSDLTRETDEAAFEPDYNESDSESNVSVKEEESSGNISKDLKDKIVEKAKESLDTAA

A8      ------------------------------------------------------------
A9      ------------------------------------------------------------
A6      SSDNGMEHRKQR--------------DKKLKKHSK-YSSTDSLKSEKRKDPKSKKKSKIL
A7      ------------------------------------------------------------
A3      ------------------------------------------------------------
A5      ------------------------------------------------------------
A4      ASQPGADRSQSQSSPSVSPSRSHSPSGSQTRSHSSSASSAGSQDSKKKKKKKEKKKHKKH
A1      VVQVGISRNQSHSSPSVSPSRSHSPSGSQTRSHSSSASSAESQDSKKKKKKKEKKKHKKH
A2      VVQVGISRNQSHSSPSVSPSRSHSPSGSQTRSHSSSASSAESQDSKKKKKKKEKKKHKKH

A8      ------------------------------------------------
```

```
A9      ------------------------------------------------
A6      KKKKKSKK--------------------------------------
A7      ------------------------------------------------
A3      ------------------------------------------------
A5      ------------------------------------------------
A4      KKHKKHKKHAGADGDVEKSQKHKHKKKAKKNKDKE--KEKDDQKVRSVTV
A1      KKHKKHKKHAGTEVELEKSQKHKHKKKSKKNKDKEKEKEKDDQKVKSVTV
A2      KKHKKHKKHAGTEVELEKSQKHKHKKKSKKNKDKEKEKEKDDQKVKSVTV
```

Appendix E: Survival analysis data

Statistix 10

Kaplan-Meier Product-Limit Survival Distribution
Time Variable: Days
Event Variable: Death
Group Variable: Group

Group = Co-treatment

Time	Died	Cen-sored	At Risk	Lower 95% C.I.	S(t)	Upper 95% C.I.	SE S(t)	H(t)
3	22	0	100	0.7007	0.7800	0.8430	0.0414	0.2485
6	2	0	78	0.6690	0.7600	0.8323	0.0427	0.2744
9	5	0	76	0.6180	0.7100	0.7875	0.0454	0.3425
12	7	64	71	0.5474	0.6400	0.7233	0.048 0	0.4463

Group = irinotecan

Time	Died	Cen-sored	At Risk	Lower 95% C.I.	S(t)	Upper 95% C.I.	SE S(t)	H(t)
3	25	0	100	0.6702	0.7500	0.8158	0.0433	0.2877
6	2	0	75	0.6370	0.7300	0.8064	0.0444	0.3147
9	5	0	73	0.5869	0.6800	0.7607	0.0466	0.3857
12	2	66	68	0.5643	0.6600	0.7442	0.0474	0.4155

Group = methyl pyruvate

Time	Died	Cen-sored	At Risk	Lower 95% C.I.	S(t)	Upper 95% C.I.	SE S(t)	H(t)
3	15	0	100	0.7745	0.8500	0.9034	0.0357	0.1625
6	3	0	85	0.7350	0.8200	0.8821	0.0384	0.1985
9	2	0	82	0.7124	0.8000	0.8660	0.0400	0.2231
12	2	78	80	0.6905	0.7800	0.8492	0.0414	0.2485

Group = untreated

Time	Died	Cen-sored	At Risk	Lower 95% C.I.	S(t)	Upper 95% C.I.	SE S(t)	H(t)
3	7	0	100	0.8655	0.9300	0.9648	0.0255	0.0726
6	4	0	93	0.8157	0.8900	0.9367	0.0313	0.1165
9	2	0	89	0.7912	0.8700	0.9220	0.0336	0.13 93
12	0	87	87					

Kaplan-Meier Product-Limit Survival Distribution
Time Variable: days
Event Variable: death
Group Variable: group

Group = Co-treatment

Time	Died	Cen-sored	At Risk	Lower 95% C.I.	S(t)	Upper 95% C.I.	SE S(t)	H(t)
3	0	10	30					
6	2	8	20	0.7117	0.9000	0.9704	0.0671	0.1054
9	8	2	10	0.100 8	0.1800	0.3006	0.1146	1.7148

group = irinotecan

Time	Died	Cen-sored	At Risk	Lower 95% C.I.	S(t)	Upper 95% C.I.	SE S(t)	H(t)
3	1	9	30	0.8366	0.9667	0.9939	0.0328	0 .0339
6	1	9	20	0.7324	0.9183	0.9788	0.0565	0.0852
9	8	2	10	0.1029	0.1837	0.3061	0.1167	1.6946

group = Methyl-Pyruvate

Time	Died	Cen-sored	At Risk	Lower 95% C.I.	S(t)	Upper 95% C.I.	SE S(t)	H(t)
3	4	6	30	0.7165	0.8667	0.9435	0.0621	0.1431
6	1	9	20	0.6315	0.8233	0.9269	0.0725	0.1944
9	5	5	10	0.2415	0.4117	0.6060	0.1351	0. 8875

group = untreated

Time	Died	Cen-sored	At Risk	Lower 95% C.I.	S(t)	Upper 95% C.I.	SE S(t)	H(t)
3	2	8	30	0.7934	0.9333	0.9808	0.0455	0.0690
6	4	6	20	0.5586	0.7467	0.8728	0.0911	0.2921
9	3	7	10	0.3146	0.5227	0.7232	0.1256	0.6488