

**Investigating how survivin expression affects
the cell death response induced by UV
irradiation**

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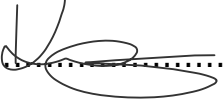
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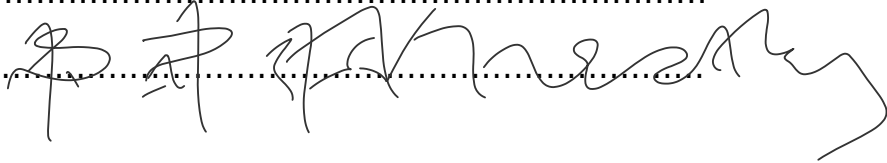
Plagiarism Declaration

I confirm that all the work in this thesis is my own and I take full responsibility for it. All collaborative efforts have been recognised in the text or in the acknowledgements.

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Abstract

Survivin is a multi-functional protein with documented roles in both cell death and proliferation. Over-expression of survivin has been observed in several cancer types and due to its pro-survival activities, it is an ideal target for onotherapy. However, there are still many unanswered questions surrounding the role of survivin in cancer. Furthermore, a cancer specific mitochondrial pool of survivin exists that is thought to be important for its role in inhibiting apoptosis, as the mitochondria are common sites for apoptosis initiation. To support this, previous research has shown that removal of its bona fide mitochondrial targeting sequence represses the anti-apoptotic function of survivin. Therefore, the aim of this thesis was to investigate the role of mitochondrial survivin in protecting cancerous cells from UV-induced apoptosis.

When assayed for its ability to inhibit apoptosis, wild-type survivin showed a greater reluctance to enter the intrinsic pathway when compared to control cells. Furthermore, of those cells that entered apoptosis, only a small proportion died as a result. However, immunoassays identified a reduction in the fission protein DRP1 in untreated Svn¹⁻¹⁰GFP samples, but a significant increase following exposure to UVC. Elevated DRP1 was initially thought to promote mitochondrial fission, an early step in the intrinsic pathway, but fluorescence microscopy revealed no difference between the mitochondrial networks of cells expressing GFP and SvnGFP. Collectively, the data presented highlights a potential role for mitochondrial survivin in apoptosis inhibition through its ability to alter mitochondrial metabolism and interact with several proteins involved in both the intrinsic pathway and anastasis.

Key Words

Survivin, Cancer, Apoptosis, Mitochondria, Cytochrome c, UV

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List of Abbreviations

ABK	Aurora-B kinase
ADP	Adenosine diphosphate
AIF1	Apoptosis-inducing factor 1
APAF1	Apoptotic protease-activating factor 1
ATG12	autophagy-related protein Atg12
ATM	Ataxia Telangiectasia mutated
ATP	Adenosine triphosphate
BAK	Bcl-2 homologous antagonist/killer
BAX	Apoptosis regulator BAX
BCL2	Apoptosis regulator Bcl-2
BCL2L11 (BIM)	Bcl-2-like protein 11
BCLX	Bcl-2-like protein 1
BDB	Borealin/Dasra-B
BH	Bcl-2 homology (domain)
BH3	Bcl-2 homology domain 3
BID	BH3-interacting domain death agonist
BIR	Baculovirus Inhibitor of Apoptosis Repeat
BSA	Bovine Serum Albumin
CAP	Checkpoint associated protein
CASP3	Caspase 3
CASP7	Caspase 7
CASP8	Caspase 8
CASP9	Caspase 9
CDE	Cell Cycle-Dependent Element
CDK	Cyclin-dependent kinase
CDKN2A (p14)	Cyclin-dependent kinase inhibitor 2A
cDNA	Complementary DNA
CHK2	Serine/threonine-protein kinase Chk2
CHR	Cell Cycle Homology Region
CLAP	Chymostatin, Leupeptin, Antipain, Pepstatin A
CPC	Chromosomal Passenger Complex
CYC	Cytochrome c
DAPI	4',6-diamidino-2-phenylindole

DISC	Death-inducing signalling complex
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DRP1	Dynamin-related protein-1
DSB	Double Strand Break
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron Transport Chain
FADD	Fas-associated death domain
FADH	Flavin adenine dinucleotide
FIS1	Mitochondrial fission protein-1
G1	Growth (phase) 1
G2	Growth (phase) 2
GF	Growth factor
GFP	Green Fluorescent Protein
GPX	Glutathione peroxidase
GSK3β	Glycogen synthase kinase 3 β
HBXIP	Hepatitis B virus X-interacting protein
HeLa	'Henrietta Lacks' (immortalised cervical cancer line)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF1A	Hypoxia-inducible factor 1-alpha
HR	Heptad repeat
HRP	Horseradish peroxidase
HSP	Heat shock protein
HTRA2 (OMI)	High temperature requirement protein A2
IAP	Inhibitor of Apoptosis
IMC	Invasion-Metastasis Cascade
INCENP	Inner centromere protein
L-OPA1	Long form (OPA1)
MCL1	Myeloid leukaemia cell differentiation protein 1
MDM2	E3 ubiquitin ligase Mdm2
MFN1	Mitofusin-1
MFN2	Mitofusin-2
MOMP	Mitochondrial Outer Membrane Permeabilisation

MPTP	Mitochondrial Permeability Transition Pore
MRE11	Meiotic recombination 11 homolog 1
MRN	MRE11–RAD50–NBS1 (complex)
mRNA	Messenger RNA
MTDR	MitoTracker™ Deep Red
MTR	MitoTracker™ Red
MTS	Mitochondrial Targeting Sequence
NA	Numerical Aperture
NADH	Nicotinamide adenine dinucleotide
NBS1	Phosphopeptide-binding Nijmegen breakage syndrome protein 1
NOTCH1	Neurogenic locus notch homolog protein 1
NSCLC	Non-small cell lung cancer
OMA1	metalloendopeptidase OMA1
OPA1	Optic Atrophy-1
OXPHOS	Oxidative phosphorylation
p21	Cyclin-dependent kinase inhibitor 1
PARC	TP53-associated parkin-like cytoplasmic protein
PBA	PBS/BSA/Azide (buffer)
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PINK1	PTEN-induced kinase-1
PKA	Protein kinase A
PM	Plasma membrane
PMAIP1 (NOXA)	Phorbol-12-myristate-13-acetate-induced Protein 1
PMSF	Phenylmethylsulfonyl fluoride
PUMA	Bcl-2-binding component 3
qPCR	Quantitative Polymerase Chain Reaction
RAD50	ATP-binding cassette–ATPase
RB1	Retinoblastoma protein
RBL1	Retinoblastoma-like protein 1
RBL2	Retinoblastoma-like protein 2
RI	Refractive Index
RNA	Ribonucleic acid

ROS	Reactive Oxygen Species
RT	Room Temperature
S	Synthesis (phase)
SAC	Spindle Assembly Checkpoint
SDS	Sodium Dodecyl Sulphate
SDW	Sterile Distilled Water
SMAC	Second mitochondrial derived activator of caspases
S-OPA1	Short form (OPA1)
SQSTM1	sequestosome 1
ssDNA	Single-stranded DNA
Svn	Survivin
tBID	Truncated BH3-interacting domain death agonist
TBS	Tris Buffered Saline
TCA	Tricarboxylic Acid (cycle)
TME	Tumour microenvironment
TNFR	Tumour necrosis factor receptor
TP53	Cellular tumour antigen TP53
TRADD	TNFR1-associated death domain
UTR	Untranslated Region
UV	Ultra-violet (radiation)
VDAC	Voltage-dependent anion-selective channel
XAF1	XIAP associated factor 1
XIAP	X-linked Inhibitor of Apoptosis
YME1L	ATP-dependent zinc metalloprotease YME1L1

CHAPTER 1: Introduction

1.1 Overview

Survivin over-expression has been documented in numerous types of cancer. Research has revealed that survivin is found in a cancer specific mitochondrial pool (Dohi, Beltrami, *et al.*, 2004), but little is known about the extent of its role in these organelles. It is well documented that over-expression of survivin aids in the evasion of cancer cells from cell death (Tamm *et al.*, 1998), and the mitochondrion are a major signalling hub for the initiation of the intrinsic pathway of apoptosis (Bohovych and Khalimonchuk, 2016). Therefore, the aim of this thesis is to investigate the role of survivin in apoptosis inhibition and to understand how its localisation to the mitochondria is involved in this function.

1.2 Tissue Homeostasis

The constant re-modelling of tissues is maintained by balancing cell division with cell death. The management of cell sub-populations is therefore carefully controlled by environmental cues that respond to regional changes in the cellular milieu, either to promote or reduce proliferation. Due to this, division and death are essential components of the homeostasis stronghold that upholds tissue integrity and function.

Programmed cell death (PCD) is defined as the physiological response of cells targeted for degradation, that are either no longer required or that have become dysfunctional (Gavrilescu and Denkers, 2003). The cell cycle, on the other hand, provides a means of generating new, healthy cells that can fill the spaces left behind following completion of cell death programmes. Combined, these two processes help to maintain tissue homeostasis by removing cells that threaten tissue health and replacing them with cells that promote tissue viability.

Due to the essential role that these pathways play in tissue homeostasis, any change in the activity of one pathway that is not matched by an equal, and opposite change in the other, will likely to lead to severe pathological states that can result in tissue malfunction, either due to excessive cell division or insufficient cell death.

1.3 The Cell Cycle

One major pathway that requires strict biochemical control is the cell cycle. During this process all cellular contents and genetic information, derived from a single parent cell, is duplicated and shared evenly between two identical daughter cells. Due to the importance and complexity of this event, controls are in place which prevent deviation in the event of malfunction. Such errors could result in abnormalities which, if left unresolved, can have catastrophic consequences (Hartwell and Weinert, 1989; Malumbres, 2014).

The cell cycle consists of two stages, interphase and mitosis. During interphase the cell progresses through various coordinated stages, known commonly as growth 1 (G1), synthesis (S), and growth 2 (G2) which

involve the duplication of cellular components, such as organelles, replication of deoxyribonucleic acids (DNA), and growth to maturity, respectively (Bishop and Weinberg, 1996). Following these preparative steps, the cell is ready to undergo mitosis (Figure 1), a complex series of events that results in chromosome alignment and segregation, ready for cleavage via cytokinesis to form two identical daughter cells (Cooper, 2000).

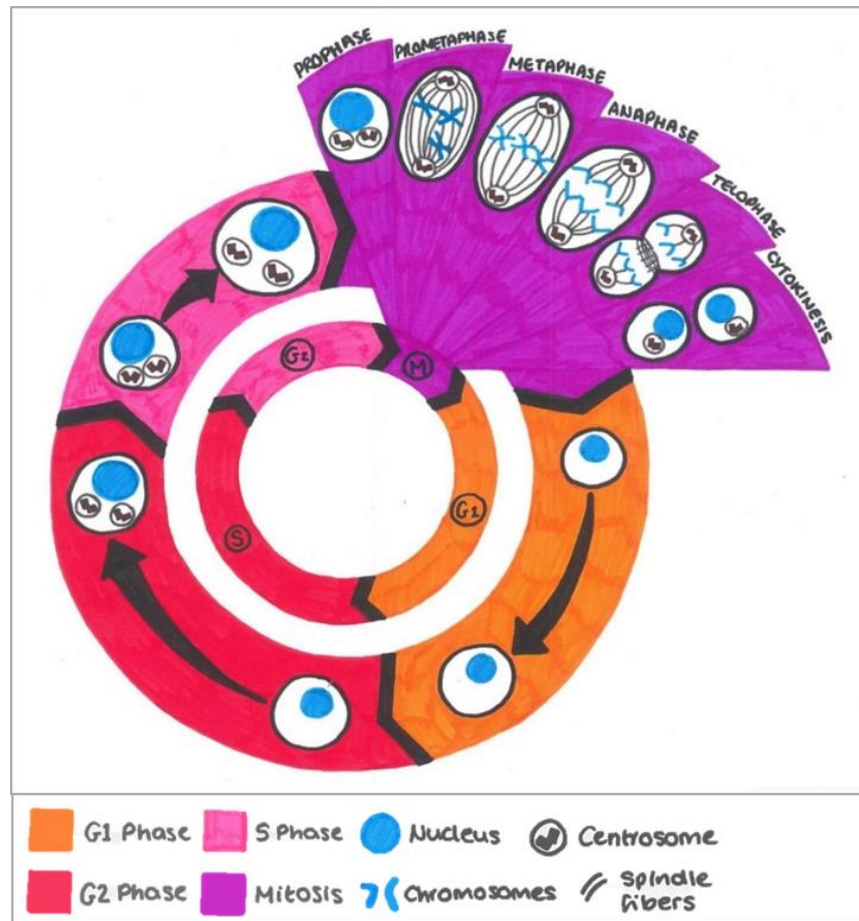


Figure 1 – The Cell Cycle. *Interphase consists of three stages known as G1, S and G2. During these stages the cell orchestrates several processes that result in replication of all cellular contents as well as development of the centrosome. Following completion of interphase, the cell enters mitosis where replicated contents are shared equally between the two opposite poles of the cell by the action of spindle fibres. Initiation of cytokinesis promotes subsequent cleavage of the mitotic cell resulting two identical daughter cells. Drawn by author (adapted from Zitouni et al., 2014).*

1.3.1 Checkpoint-Mediated Control

To maintain thorough control, checkpoints are positioned throughout the cell cycle (Figure 2) which prevent progression to the next stage, should

cells not obtain the pre-requisites to pass each checkpoint (Hartwell and Weinert, 1989; Schönthal, 2004). If such requirements are not met the cells progression through the cycle is halted until a solution can be found, whether that be repair of cellular damage, removal of stress stimuli, or restoration of essential growth factor (GF), hormone or nutrient availability (Pietenpol and Stewart, 2002). At this point, any cellular damage that cannot be repaired will activate apoptosis. Such control is orchestrated by an evolutionarily conserved apparatus that links the cell cycle with both intra and extracellular signalling pathways that monitor the surrounding environmental conditions, to determine cell fate.

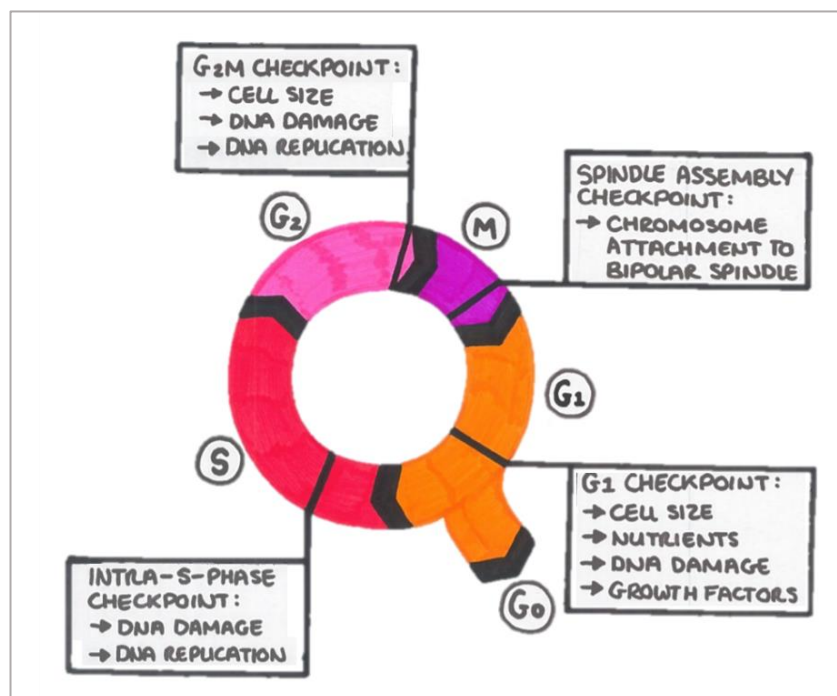


Figure 2 – Cell Cycle Checkpoints. Progression of cells through each phase of the cell cycle is regulated by a variety of checkpoints. (1) The G₁ checkpoint ensures that the cell has enough GFs to commit to cell cycle completion; lack of such factors could result in entry to G₀. (2) The Intra-S-phase checkpoint identifies sites of DNA damage and will prevent initiation of DNA replication if such damage is detected. (3) Prior to entry into mitosis, the G₂-M checkpoint assesses the progress of the cell to ensure it is adequate in size and that no errors have occurred during replication. (4) Finally, the spindle assembly checkpoint monitors formation of attachments between the chromosomes and spindle fibres during prometaphase. G₁ (orange), S (red), G₂ (pink) and mitosis (M; purple). Drawn by author.

1.3.1.1 Checkpoint-Associated Proteins

Along with checkpoint-mediated control, progression between each phase is also regulated by a plethora of checkpoint-associated proteins (CAPs)

which detect changes, such as DNA damage (Figure 3) or increased GF availability, and relay that information to various signalling cascades that either facilitate or inhibit cell cycle progression (Schönthal, 2004).

Of the many CAPs present during the cell cycle, the most commonly referred to are the tumour suppressor proteins, cellular tumour antigen p53 (TP53) and Retinoblastoma protein (RB1). Both TP53 and RB1 are recognised as coupling sites for cell proliferation and apoptosis (Vermeulen, Berneman and Van Bockstaele, 2003). These proteins monitor progression through the cell cycle and ultimately promote its continuation or target cells for destruction (Weinberg, 1995; Kruiswijk, Labuschagne and Vousden, 2015). Without this, damaged cells would be able to proliferate uncontrollably, as seen in the development of tumours where these proteins are commonly mutated (Bishop and Weinberg, 1996; Pietenpol and Stewart, 2002; Kim, Zhang and Lozano, 2015).

Another example of CAPs are cyclin-dependant kinases (CDKs) which modulate the activity of other cell cycle proteins, markedly RB1 (Lew and Kornbluth, 1996; Adams, 2001; Vermeulen, Berneman and Van Bockstaele, 2003). The activity of these holoenzymes is dependent upon their association with cyclins; regulatory subunits that are required for enzymatic activity of CDKs (Bishop and Weinberg, 1996; Malumbres, 2014).

It is worth noting that any defects in the regulation and maintenance of cell cycle checkpoints can result in cell pathology which is commonly associated with tumorigenesis (Pietsenpol and Stewart, 2002).

1.3.2 Interphase

G1 Phase – DNA Integrity and Cell Size

Cell replication begins with the induction of G1 phase, where the cell maintains its metabolic activity and grows continuously, without initiating DNA replication. This is because the cell must first commit to cell cycle completion before DNA can be duplicated. In concordance with this, the first checkpoint, referred to as the “restriction point”, is positioned at the end of G1 phase. Here, the cell must decide whether there are enough

nutrients and GFs in the environment to support energy requirements through the cell cycle (Barnum and O'Connell, 2014). If there are enough GFs available, then the cell will be able to pass through the restriction point and continue into the DNA replication phase (S phase). However, if there are insufficient GFs the cell will arrest, and enter the quiescent phase (G0) where it will remain until stimulated to re-enter (Pietenpol and Stewart, 2002).

S Phase – DNA Synthesis

During early S-phase the cell encounters a second checkpoint referred to as the "intra-S-phase" checkpoint. This checkpoint ensures the stable formation of the replisome, a complex formed from DNA polymerase and associated replication enzymes, and successful initiation of DNA replication (Maga, 2013). It achieves this by halting the cell cycle when progression of either of these two events is blocked (Barnum and O'Connell, 2014).

When DNA replication is initiated, the double-stranded DNA (dsDNA) must be unwound to expose its single-stranded counterparts (ssDNA). This is achieved by the activity of DNA helicase and allows for primers, short pieces of ribonucleic acid (RNA) generated by DNA primase, to bind to each strand of ssDNA. Primers aid in the binding of DNA polymerase and replication enzymes to the ssDNA which leads to the elongation of DNA and eventually, the synthesis of two new strands of ssDNA, complementary to both original strands (Lew, 2003; Harper and Brooks, 2005). This generates two new, complete pieces of dsDNA that consist of one original strand of ssDNA, known as the "template" strand, and one newly synthesised ssDNA strand.

In the event that an error occurs during DNA replication, a protein called replication protein A will bind to the exposed ssDNA, preventing it from forming secondary structures, and most importantly signalling to the cell that an error has occurred and that the cell cycle must be arrested until this error can be dealt with (Chen, Lisby and Symington, 2013; Borgstahl *et al.*, 2014; Deng, 2015).

G2 Phase – DNA Damage and Mitotic Entry

Following DNA replication and maturation of the cell, the G2-M checkpoint regulates progression from G2 into mitosis. As with the S-phase checkpoint, the G2-M checkpoint prevents cells entering mitosis if it senses either un-replicated or damaged DNA (Cooper, 2000; Stark and Taylor, 2004). Should either of these occur, TP53 and RB1 work together to down-regulate the activity of several proteins that are essential for G2 and mitosis (e.g. cyclins and cyclin-dependent kinases) in order to cause cell cycle arrest (Figure 3) (Hwang and Muschel, 1998; Adams, 2001; Pieterpol and Stewart, 2002; Ren *et al.*, 2002; Stark and Taylor, 2004). This provides a final opportunity for the cell to either repair the damage, or to abort cell proliferation and remove the damaged cell via apoptosis.

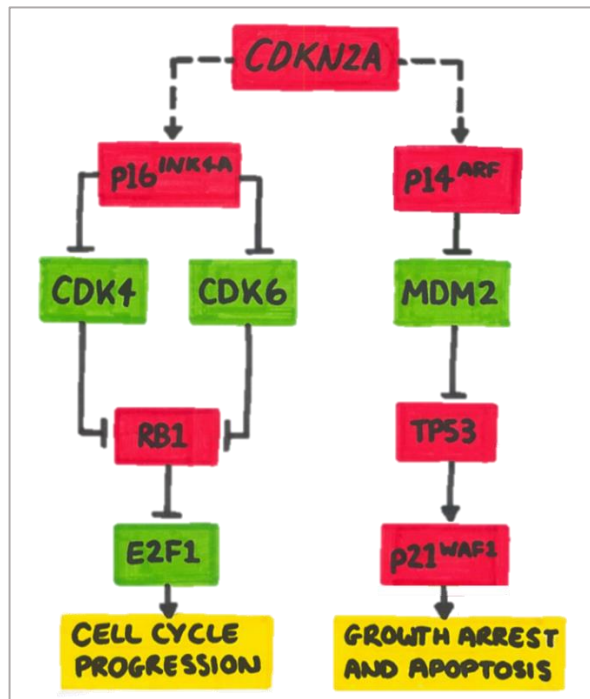


Figure 3 – Regulation of the restriction point by TP53 and RB1. Transcription of the *CDKN2A* locus results in synthesis of *p16^{INK4A}* and resultant inhibition of CDK4 and CDK6. This prevents hyper-phosphorylation of RB1. Active RB1 is then able to sequester the transcription factor E2F1. Alternatively, transcription of the *CDKN2A* locus can also lead to synthesis of *p14^{ARF}* and subsequent inhibition of the E3 ubiquitin-protein ligase Mdm2 (MDM2). This removes the inhibitory effect of MDM2 from TP53. As a result, TP53 can transactivate CDK inhibitor 1 (*p21^{WAF1}*), promoting inhibition of cyclin-CDK complexes and hypo-phosphorylation of RB1. Both pathways result in down-regulated expression of proteins required for cell cycle progression and finally growth arrest. Red proteins/genes are inhibitors of cell cycle advance; green proteins/genes are activators. Dotted arrows represent transcription and italicised labels are genes. Drawn by author (adapted from Soufir, 2000).

1.3.4 Mitosis & Cell Division

Prophase & Prometaphase

Upon successful G2-M transition, newly replicated DNA undergoes condensation, resulting in formation of distinct chromosomal units. These units consist of paired sister chromatids. During prometaphase, the mitotic spindle begins to form at opposing poles of the cell (Mitchison and Salmon, 2001; Schatten, 2013) and the nuclear envelope fragments (Schatten, 2013; Barnum and O'Connell, 2014).

Metaphase

The mitotic spindle is the macromolecular machinery that orchestrates separation of chromosomes. For this event to be successful, microtubules originating from the spindle must attach themselves to kinetochores, small structures formed at the centromeric region of each chromosome (Mitchison and Salmon, 2001). Microtubules will undergo a cycle of attachment-detachment until the correct connections can be made. This process is regulated by the Chromosomal Passenger Complex (CPC) (see 1.8). After successful formation of microtubule-kinetochore attachments, the dynamic activity of the bipolar spindle directs movement of the microtubule network to align the chromosomes along the equatorial plate, in preparation for anaphase (Schatten, 2013).

Anaphase

Once aligned at the metaphase plate, the chromosomes are primed for separation. At this point the microtubules begin to depolymerise, sliding past each other as they do so. This results in the subsequent segregation of sister chromatids to opposing poles, and formation of two distinct regions within the cell (Schatten, 2013).

Telophase & Cytokinesis

During telophase, the cell initiates reformation of the nuclear envelope around the two independent groups of chromosomes. This is shortly followed by cytokinesis, during which a contractile ring, composed mainly

of filamentous actin and myosin-2, forms under the surface of the plasma membrane (PM) (Barr and Gruneberg, 2007). As the ring constricts, due to movement of myosin-2 along actin filaments, it generates what is known as the 'cleavage furrow' at the cell periphery. This eventually results in abscission and release of resultant daughter cells (Barr and Gruneberg, 2007; Schatten, 2013).

Spindle-Assembly Checkpoint

As with interphase, cells passing through the various stages of mitosis are also subject to checkpoint control. The spindle assembly checkpoint (SAC) monitors the kinetochore-spindle attachments formed during prometaphase. This ensures the correct segregation of the chromosomes during anaphase by delaying this process in response to improper kinetochore-spindle attachment. This results in the equal sharing of chromosomes between the two poles of the cell upon initiation of cytokinesis (Walczak and Heald, 2008; Barnum and O'Connell, 2014; Petry, 2016).

1.4 Metabolism

Energy metabolism is described as the process of harvesting energy, generally stored in the form of adenosine triphosphate (ATP), from nutrients. Metabolism consists of a series of interconnected biochemical pathways that organise the breakdown of large molecules, known as catabolism, which can then be recycled (anabolism) in a process that generates both the molecular building blocks required by the cell and energy in the form of ATP. The balance between the activity of these opposing biochemical pathways alters depending upon the energy needs of the cell.

Mitochondria are the largest producers of energy within the cell. This energy is generated via a process known oxidative phosphorylation (OXPHOS) (Vakifahmetoglu-Norberg, Ouchida and Norberg, 2017; Spinelli and Haigis, 2018).

1.5 Mitochondria: The “powerhouses” of the cell

Mitochondria are theorised to have originated from an ancient, ancestral eubacterium that settled into the cellular landscape through a continued symbiosis with archaeobacterium, the eukaryotic forefather (Martin and Mentel, 2010). This long-term biological interaction has provided the eukaryotic cell with several evolutionary advantages, including more efficient metabolism and the ability to detoxify the cellular environment (Roger, Muñoz-Gómez and Kamikawa, 2017).

The mitochondrion also play a very distinct role in inter- and intra-cellular communication, making them responsible for regulating a vast range of processes, from PCD and gene transcription, to trafficking of signalling molecules and homeostatic control of metabolites (Gaude and Frezza, 2014; Kühlbrandt, 2015; Vakifahmetoglu-Norberg, Ouchida and Norberg, 2017; Spinelli and Haigis, 2018).

1.5.1 Structure & Function

Research into the morphology of the mitochondrion has been an area of continuous research since their discovery in the late 19th century. Since then much has been learnt about the various components and compartments that make up these unique organelles (O’Rourke, 2010).

Contrary to earlier belief, the mitochondria are actually remarkably plastic and flexible organelles which allows them to undergo fine structural alterations in order to cope with changes in physiological state and environmental conditions (Rosamond, 1987).

One of the most interesting features of the mitochondrion is the presence of an all-encompassing double membrane. The so-called inner and outer mitochondrial membranes serve, primarily, to separate the contents of the organelle from the surrounding cytosol, and, secondly, to define two individual compartments within the mitochondrion. These compartments are known as the matrix; region enclosed by the inner membrane, and the inter-membrane space; located between the inner and outer membranes (Figure 4) (Palade, 1953; Rosamond, 1987; Kühlbrandt, 2015).

The separation of these two compartments allows the mitochondrion to establish an electro-chemical gradient across the inner membrane. This gradient provides a source of energy that can then be utilised to generate ATP molecules via the mitochondria's major metabolic pathway – OXPHOS (Spinelli and Haigis, 2018).

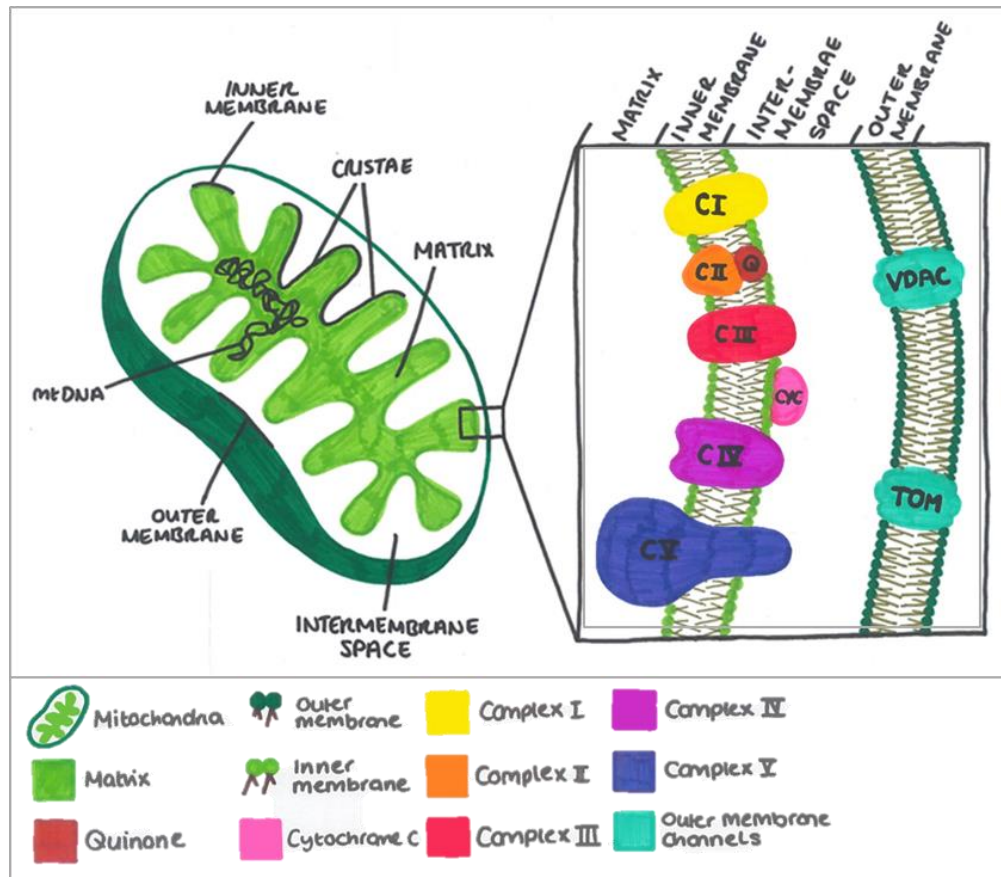


Figure 4 – Structure of the Mitochondrion. Mitochondrion consist of an all-encompassing double membrane. The outer membrane (dark green) separates the inter-membrane space from the cytoplasm and contains several proteinaceous pores that promote exchange of ions and small proteins. The inner membrane separates the inter-membrane space from the matrix and is impermeable. Protein components of the inner membrane consist of those involved in OXPHOS. Cristae are large invaginations in the inner membrane which house proteins of the electron transport chain. The matrix is the site of the tricarboxylic acid cycle and contains mitochondrial DNA. Drawn by author (adapted from Blanco, Rego and Ruiz-Romero, 2011).

In order to perform OXPHOS, the mitochondria require several specialised complexes, denoted as CI to IV, which make up a “processive” chain known as the electron transport chain (ETC). The irregular folding of the inner membrane generates multiple invaginations known as the cristae which extend into the matrix (Rosamond, 1987; Kühlbrandt, 2015). These folds provide a greater surface area for energy production by OXPHOS, to cope

with cellular ATP demand (Palade, 1953; Rosamond, 1987). During OXPHOS, reduced donor molecules, such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH), generated through iterative oxidative steps in the tricarboxylic acid (TCA) cycle, release electrons to these complexes which are then transferred to electron acceptor molecules and continue along the ETC until they reach complex IV, where they are combined with protons and molecular oxygen to generate water (Figure 5).

As electrons are transferred along the ETC, they trigger a change in conformation of the ETC proteins and, as a result, allow each complex to pump protons from the matrix into the intermembrane space, thereby generating the electro-chemical gradient (Osellame, Blacker and Duchen, 2012). The energy accrued by this gradient is utilised by an enzyme complex known as ATP synthase, also known as "complex V" or CV, to generate ATP. This process couples the movement of protons down their concentration gradient with the phosphorylation event that converts adenosine diphosphate (ADP), which is present in the matrix, to ATP (Figure 5) (Rosamond, 1987; Vakifahmetoglu-Norberg, Ouchida and Norberg, 2017; Spinelli and Haigis, 2018).

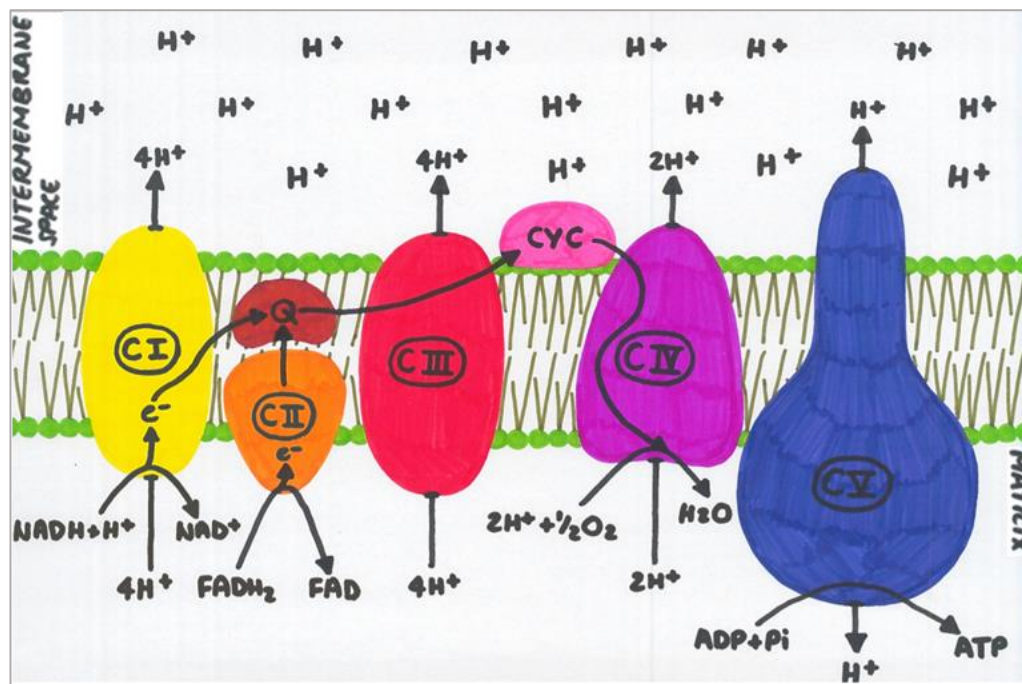


Figure 5 – OXPHOS and the Electron Transport Chain. Reduced NADH and FADH transfer electrons to CI (yellow) and CII (orange). Electrons are transported

via quinone (Q) to CIII (red) which then passes them to cytochrome c (CYC; pink). CIV (purple) then transfers electrons to molecular oxygen (O₂) to promote production of water (H₂O). The flow of electrons between complexes is accompanied by the pumping of protons from the matrix to the inter-membrane space. This generates an electrochemical gradient which is used by CV (dark blue) to convert ADP and Pi to ATP. Drawn by author (adapted from Blanco, Rego and Ruiz-Romero, 2011).

ATP molecules generated via OXPHOS are either released into the matrix, where they can be utilised by metabolic pathways present in the matrix, or partake in an ATP/ADP exchange event across the inner membrane, at which point ATP can passively diffuse across the outer membrane (Rosamond, 1987). To further increase the efficiency of the ETC, the inner membrane is also impermeable, therefore preventing leakage of protons back into the matrix.

1.5.2 Mitochondria and Cell Fate

Unlike the impermeable inner membrane, the function of the selectively permeable outer membrane is primarily to protect the contents of the mitochondria from exposure to the cytosolic environment. The outer membrane therefore acts as a barrier to cytosolic and mitochondrial proteins alike, carefully controlling their import and export in order to maintain cellular function (Kühlbrandt, 2015). An important example of this is the release of pro-apoptotic proteins from the mitochondria. Within healthy cells, these proteins are sequestered in the inter-membrane space (Dorn and Kitsis, 2012). However, upon release from the mitochondria, pro-apoptotic proteins play a crucial role in the activation of enzymes involved in PCD. Therefore, this spatial separation prevents inappropriate release of such proteins and unnecessary activation of PCD (Wang and Youle, 2009).

The mitochondria are highly adaptable organelles and can reorganise their structure in response to changes in the local environment. (Kühlbrandt, 2015). For example, the mitochondria of cells that have been exposed to stressors, such as exposure to ultra-violet (UV) radiation, appear punctate and dispersed (Rosamond, 1987). This observation is likely a result of excessive mitochondrial fission; an initial step in the intrinsic pathway of apoptosis (see section 1.6.1.2).

1.5.2.1 Mitochondrial Dynamics

Mitochondria are constantly being recycled to maintain their activity, structure and cellular distribution. This process is referred to as mitochondrial dynamics (Figure 6) and involves coordinated cycles of disassembly (fission) and reassembly (fusion) (Tilokani *et al.*, 2018). These swift and ephemeral changes in mitochondrial morphology are crucial for various biochemical processes that maintain cellular homeostasis, including the cell cycle (Horbay and Bilyy, 2016) and PCD (Wang and Youle, 2009; Westermann, 2010).

Fission

The term 'fission' refers to the multi-step division process that converts a single mitochondrion into two daughter mitochondria. This process is regulated by the activity of two major proteins referred to as Dynamin-related protein 1 (DRP1) and mitochondrial fission protein 1 (FIS1) (Figure 6). During fission, DRP1 is recruited to the outer mitochondrial membrane where it forms oligomeric spiral structures at different sites along the tubular mitochondrial network (Tamura, Iijima and Sesaki, 2010; Tilokani *et al.*, 2018). A small population of both DRP1 and FIS1 also localise to the endoplasmic reticulum, which, when wrapped around the mitochondria, marks these sites for fission by DRP1 and FIS1. It is hypothesised that FIS1 is also involved in inhibiting the opposing fusion machinery, therefore further promoting fission (Yu *et al.*, 2019). Interestingly, fission events lead to the production of two types of mitochondria, one of which has an increased membrane potential, and is therefore likely to survive and proceed into fusion, and another, less stable mitochondrion that is destined for mitochondrial-specific autophagy, or mitophagy (Zhu *et al.*, 2018).

Fusion

Mitochondrial fusion proceeds via two major events. The first of these involves the joining of mitochondrial outer membranes, from two independent mitochondria, followed by fusion of the associated inner membranes. The former is mediated by mitofusin 1 (MFN1) and 2 (MFN2) and the latter by optic atrophy 1 (OPA1) (Figure 6) (Tamura, Iijima and

Sesaki, 2010; Tilokani *et al.*, 2018). Prior to fusion of the two outer membranes, these opposing structures must first be physically aligned and tethered. The current hypothesis (Koshiba *et al.*, 2004) suggests that this process is performed by MFN1/2 via their carboxyl terminal heptad repeat (HR) and GTPase domains (Koshiba *et al.*, 2004). The HR region creates a bridge between the two mitochondria, at which point GTPases bind and hydrolyse their substrate, guanosine triphosphate. Activation of the GTPase induces a subsequent conformational change in the structure of MFN1/2 that results in increased contact between the two mitochondria, and eventually formation of a single outer membrane (Santel and Fuller, 2001). Following this, OPA1 is responsible for binding and merging the inner membranes via its GTPase activity (Koshiba *et al.*, 2004; Dorn, 2018; Tilokani *et al.*, 2018; Zhu *et al.*, 2018).

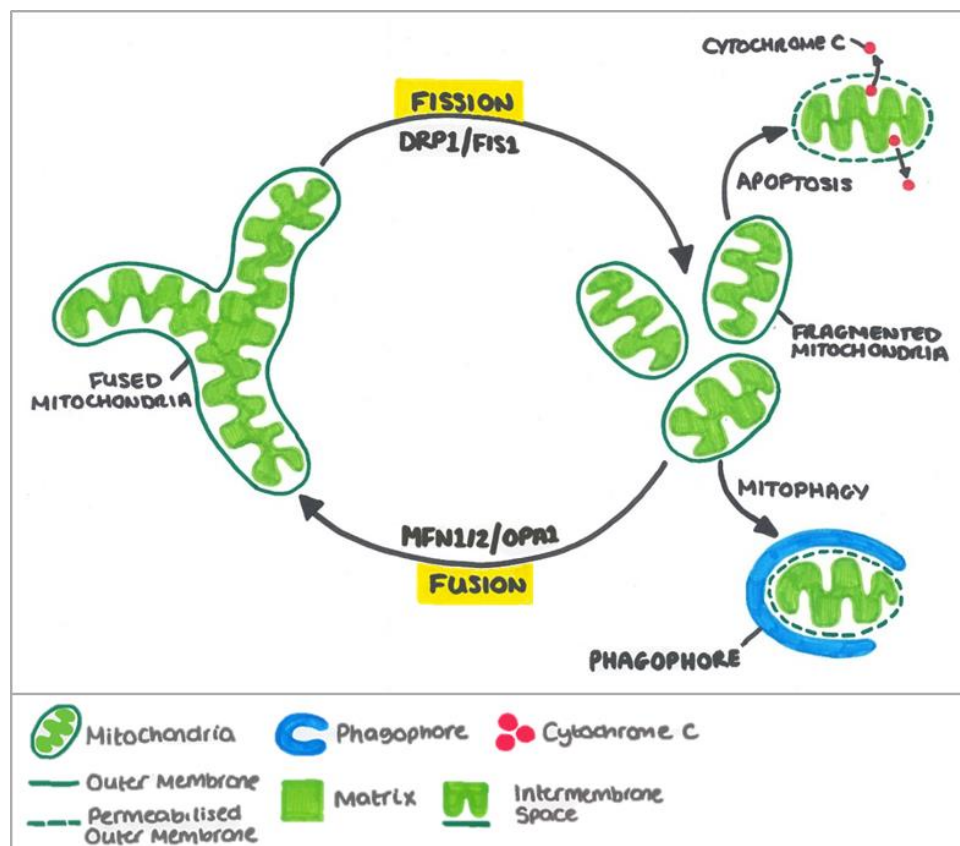


Figure 6 – Mitochondrial Dynamics and Cell Fate. The balance between fission and fusion determines mitochondrial morphology. Fission events are regulated by the activity of DRP1 and FIS1, whilst fusion is mediated by MFN1/2 and OPA1. Mitochondrial fission is a preparative step for mitochondrial recycling via mitophagy. Fragmented mitochondria are observed during apoptosis and permeabilisation of the mitochondrial outer membrane results in release of cytochrome c and cell death. Fusion results in formation of branched, tubular mitochondrial networks. Drawn by author (adapted from Seo *et al.*, 2010).

The exact mechanism by which OPA1 performs its fusion function is still unknown. It is believed that both the long (L-OPA1) and short (S-OPA1) forms of OPA1 are capable of affecting mitochondrial dynamics depending upon their abundance within the cell (MacVicar and Langer, 2016; Tilokani *et al.*, 2018). L- and S-OPA1 are generated by the protease activity of metalloendopeptidase OMA1 (OMA1) and ATP-dependent zinc metalloprotease YME1L1 (YME1L) (Käser *et al.*, 2003). In fact, it is postulated that cleavage events performed by OMA1 are a result of incurred stress and lead to subsequent mitochondrial fragmentation (Head *et al.*, 2009; Baker *et al.*, 2014), but that YME1L activity is actually stimulated by increased OXPHOS and results in mitochondrial fusion (Anand *et al.*, 2014; Mishra *et al.*, 2014).

Mitochondrial Dynamics & Cellular Function

An imbalance between fission and fusion causes significant changes in mitochondrial morphology, from excessive fragmentation and resultant punctate mitochondria (increased fission), to formation of a hyperfused network of long, tubular mitochondria (increased fusion). These dynamic transitions are essential for mitochondrial function, but it also ensures that the network can adapt in response to stress within the cellular environment (Wai and Langer, 2016; Tilokani *et al.*, 2018). For example, fragmented networks are commonly observed upon exposure to stress-inducing agents but can also be seen during the G2-M phase of the cell cycle as this morphology promotes mitochondrial motility and inheritance of mitochondrial DNA. Conversely, the presence of a hyperfused network is thought to result in increased OXPHOS, but can also promote cell survival by protecting the mitochondria from autophagy-related engulfment during nutrient starvation (Tilokani *et al.*, 2018).

1.6 Programmed Cell Death

When cells acquire damage or become dysfunctional, they can undergo a variety of processes that either promote removal of cellular components, such as defunct mitochondria, or activate cell-suicide programmes in the case of irreparable damage and dysfunction.

Although there is now a plethora of documented forms of PCD (Galluzzi *et al.*, 2018), there are three main mechanisms, which are of interest in this report, denoted as type I, II and III (Figure 7). PCD-I or “apoptosis”, is a carefully regulated method of cell suicide, characterised by cell shrinkage, chromatin condensation, and removal via phagocytosis (Festjens, Vanden Berghe and Vandenabeele, 2006). PCD-II is known as autophagic cell death and is caused by excessive cell matter recycling by autophagosomes. Finally, PCD-III, known as necroptosis (programmed necrotic cell death), is differentiated from type I by the rapid loss of PM integrity, and resultant leakage of cellular contents into the surrounding environment which often results in inflammation (Hotchkiss *et al.*, 2009; Portt *et al.*, 2011; Green and Llambi, 2015; Karch and Molkenin, 2015; Galluzzi *et al.*, 2018).

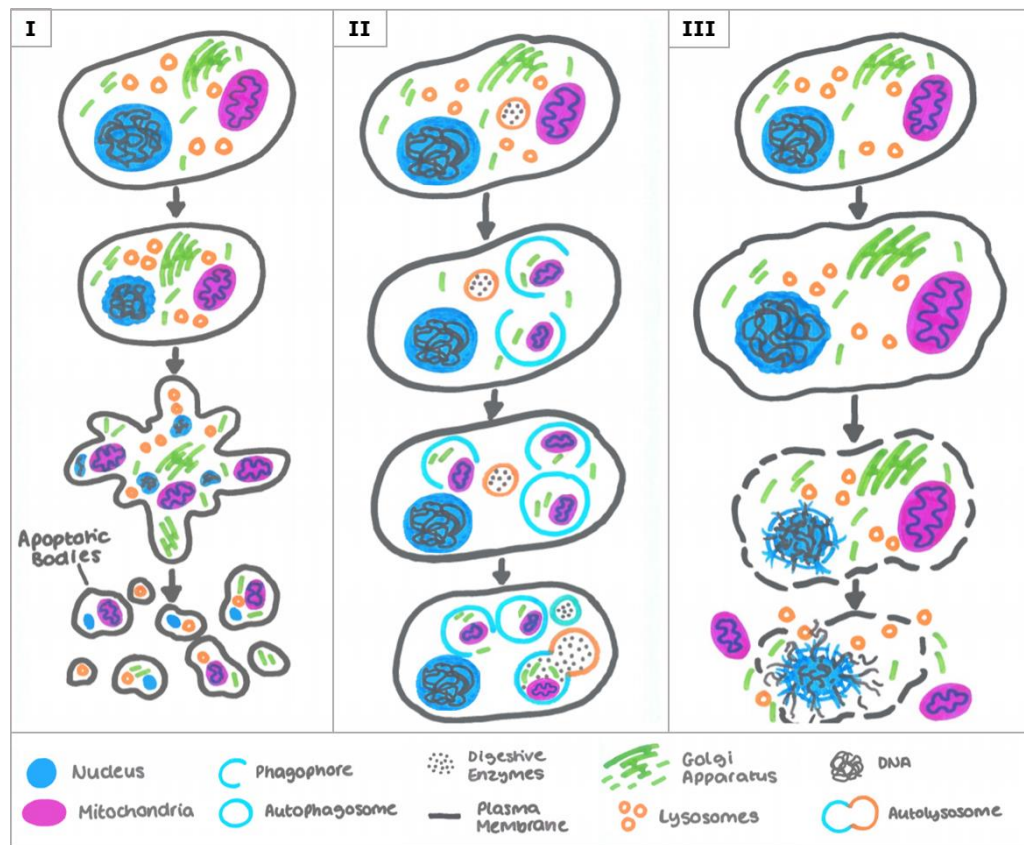


Figure 7 – Changes in cellular morphology during programmed cell death. Initiation of apoptosis (I) leads to cell shrinkage and chromatin condensation followed by fragmentation of the DNA and membrane blebbing. This eventually yield several apoptotic bodies which encapsulate the cellular contents ready for engulfment by macrophages. (II) Autophagic cell death is the result of excessive recycling of cellular contents, including organelles. (III) Necroptosis involves initial swelling of the cell, followed by bursting and leakage of cellular contents into the surrounding environment. This often results in inflammation.

1.6.1 Apoptosis

Apoptosis is initiated by a range of stimuli including exposure to overly stressful conditions within the surrounding environment, such as abnormally low oxygen levels (hypoxia), dysfunction of biochemical pathways within the cell, such as inefficient OXPHOS and resultant decrease in ATP production, or damage to cellular components, such as introduction of double-strand breaks (DSBs) in the DNA (Kerr, Wyllie and Currie, 1972).

Apoptosis involves a biochemical cascade that takes the form of two major pathways: the extrinsic pathway, initiated via stimulation of transmembrane death receptors found at the cell surface, and the intrinsic pathway (mitochondrial pathway), which is triggered by mitochondrial outer membrane permeabilisation (MOMP) followed by selective release of apoptogenic signalling factors (Wang and Youle, 2009; Portt *et al.*, 2011; Troy, Akpan and Jean, 2011; Green and Llambi, 2015). Both pathways provoke the activities of various cysteine-dependent aspartate-directed proteases, commonly referred to as caspases, that organise the dismantling of cellular components via a series of substrate activation-inactivation events (Nicholson and Thornberry, 1997; Duprez *et al.*, 2009; Galluzzi *et al.*, 2012; Galluzzi, Kepp and Kroemer, 2013; McIlwain, Berger and Mak, 2013; Green and Llambi, 2015).

Importantly, induction of apoptosis is determined not just by the activation of a single pathway, but ultimately by the balance between both pro- and anti-apoptotic signals present within the cell. If there is an excess of either, this will promote cell death or survival, respectively. An example of this is the BCL2 family of proteins which comprises both pro- (e.g. apoptosis regulator BAX or BAX) and anti-apoptotic (e.g. apoptosis regulator Bcl-2 or BCL2) proteins (Reed, 1996; Fulda *et al.*, 2010). Reduced expression of anti-apoptotic genes has been shown to sensitise cells to sublethal dosages of apoptosis-inducing agents. Conversely, increasing the expression of anti-apoptotic genes promotes resistance to such agents (Portt *et al.*, 2011).

To ensure that apoptosis is only triggered when necessary, caspases are generated as inactive zymogens which gain their catalytic activity following proteolytic cleavage events that yield mature, active caspases. Another level of control is introduced by the presence of two different types of caspases. Initiator caspases, which are the primary caspases in the apoptotic pathway, act as an amplification point for the downstream activation of executioner caspases, which themselves are required for coordinated demolition of key structural proteins (McIlwain, Berger and Mak, 2013). This ultimately results in a number of morphological changes that are characteristic of apoptotic cells, including: cell shrinkage and blebbing, karyorrhexis (nuclear fragmentation), pyknosis (chromatin condensation) and fragmentation of genetic materials, and finally formation of small vesicles known as apoptotic bodies (Xu, Lai and Hua, 2019).

1.6.1.1 Extrinsic Pathway of Apoptosis

As the name suggests, the extrinsic pathway is the result of external stimuli that are detected by receptors at the cell surface (Figure 8). The tumour necrosis factor receptor (TNFR) family is the most widely studied extrinsic pathway. Interaction with an extracellular ligand results in subsequent TNFR oligomerisation, stimulating the recruitment of Fas-associated death domain (FADD) and TNFR1-associated death domain (TRADD). Association of these adaptor proteins with initiator caspase 8 (CASP8) results in complete assembly of the death-inducing signalling complex (DISC) and subsequent activation of executioner caspase 3 (CASP3) and 7 (CASP7) (Budihardjo *et al.*, 2002). Active caspases then proceed by cleaving a range of substrates within the cells that result in complete cell demise (Duprez *et al.*, 2009; Portt *et al.*, 2011; Fernald and Kurokawa, 2013; Xu, Lai and Hua, 2019). However, the exact mechanism of extrinsic apoptosis, including the adaptor proteins that relay the external death signal, is ultimately determined by the nature of the ligand that binds to the TNFR (Xu, Lai and Hua, 2019).

1.6.1.2 Intrinsic Pathway of Apoptosis

The intrinsic pathway of apoptosis is triggered by MOMP, which is regulated by the activity of both pro- and anti-apoptotic proteins. MOMP results in depolarisation of the outer mitochondrial membrane, but the exact mechanism by which this process occurs is unknown (Green and Kroemer, 2004; Liu, Perfettini and Brenner, 2018). There are three generally accepted models for MOMP which include: rupture of the mitochondrial outer membrane as a result of mitochondrial permeability transition pore (MPTP) opening and subsequent mitochondrial swelling (Leung and Halestrap, 2008), formation of pores in the outer mitochondrial membrane mediated by pro-apoptotic BAX and Bcl-2 homologous antagonist/killer (BAK) (Wei *et al.*, 2001; Kuwana, 2018), or mitochondrial fragmentation (Karbowski *et al.*, 2002; Youle and Karbowski, 2005; Estaquier and Arnoult, 2007; Suhaili *et al.*, 2017). Although it has not been verified, MOMP as a result of MPTP opening has been shown to be more closely linked with late apoptosis and necrosis (Lalier *et al.*, 2007; Kinnally *et al.*, 2011; Galluzzi, Kepp and Kroemer, 2013; Kalkavan and Green, 2018). As a result of MOMP, pro-apoptotic proteins that would normally reside in the intermembrane space, namely cytochrome c (CYC), apoptosis-inducing factor-1 (AIF1), the second mitochondrial derived activator of caspases (SMAC), and high temperature requirement protein A2 (HTRA2 or OMI) are released into the cytosol (Figure 8) where they promote activation of initiator caspase 9 (CASP9) and downstream executioner caspases (Duprez *et al.*, 2009; Nowsheen and Yang, 2012; Galluzzi, Kepp and Kroemer, 2013; Loane, Stoica and Faden, 2015; Cosentino and García-Sáez, 2017).

BAX/BAK-Mediated MOMP

BAX and BAK are both members of the BCL2 family of proteins and as such contain several BCL2 Homology (BH) domains, as well as a transmembrane domain, which is responsible for their pore-forming activity (Lalier *et al.*, 2007; Brunelle and Letai, 2009; Cosentino and García-Sáez, 2017). BAX/BAK-mediated MOMP is negatively regulated upstream by other members of the BCL2 family, including BCL2, Bcl-2-like protein 1 (BCLX), and myeloid leukaemia cell differentiation protein (MCL1), which sequester

BAX and BAK, therefore preventing their insertion into the outer mitochondrial membrane (Brunelle and Letai, 2009; Westphal *et al.*, 2011; Galluzzi, Kepp and Kroemer, 2013; Singh, Letai and Sarosiek, 2019). Furthermore, it has also been shown that BAX is capable of self-inhibition through dimerization in the cytosol (Garner *et al.*, 2016). Conversely, BAX and BAK activity can be propagated by BCL2 Homology domain 3 (BH3)-only proteins, such as BH3-interacting domain death agonist (BID) (Desagher *et al.*, 1999), Bcl-2-binding component 3 (PUMA), and phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1 or NOXA), which aid MOMP by inhibiting anti-apoptotic rivals (Reed, 1996; Kuwana and Newmeyer, 2003; Youle and Strasser, 2008; Letai, 2011; Fernald and Kurokawa, 2013; Galluzzi, Kepp and Kroemer, 2013; Marie Hardwick and Soane, 2013; Czabotar *et al.*, 2014; Kalkavan and Green, 2018; Singh, Letai and Sarosiek, 2019).

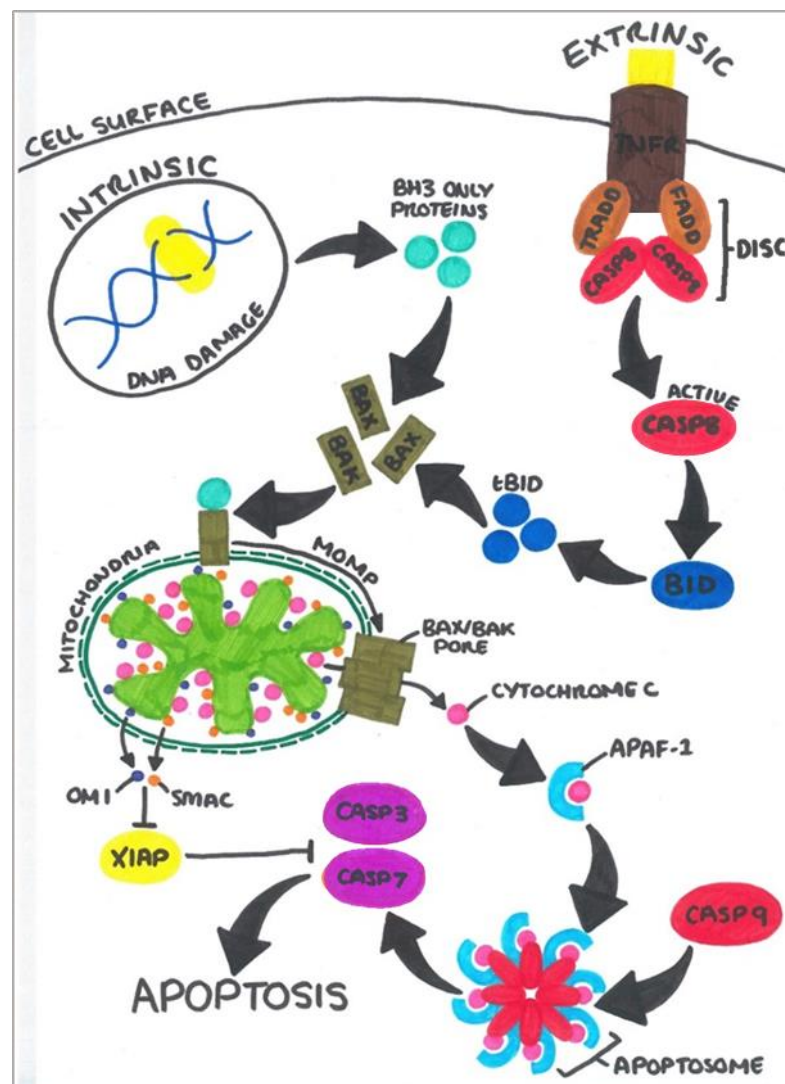


Figure 8 – Pathways of Apoptotic Cell Death. *The extrinsic pathway is triggered upon binding of death inducing ligands to TNFRs and subsequent recruitment of intracellular adaptor proteins (FADD or TRADD) and CASP8 to form the DISC. Activation of CASP8, results in cleavage of BID to pro-apoptotic, truncated BID (tBID). Alternatively, cellular stress can induce the intrinsic pathway via activation of BH3-only proteins. At this point, the two pathways converge upon induction of MOMP, following oligomerisation of BAX, and subsequent release of inter-membrane space proteins (CYC, SMAC and OMI). Cytoplasmic CYC promotes formation of the apoptosome (APAF1 and CASP9) and activation of CASP3 and CASP7. Drawn by author (adapted from Ramaswamy, Deng and Siegel, 2011).*

Mitochondrial Fragmentation and MOMP

During early apoptosis the mitochondria experience rapid and extensive fragmentation, but the exact role that this event plays in the cell death program remains controversial. However, research has shown that although mitochondrial fragmentation cannot be prevented following inhibition of caspases, it can in fact be inhibited as a result of DRP1 inactivation (Estaquier and Arnoult, 2007). In turn, loss of DRP1 activity prevents CYC release from the intermembrane space. This not only indicates that mitochondrial fragmentation occurs prior to caspase activity, and is therefore an early step in the apoptosis pathway, but that it is DRP1 dependent (Frank *et al.*, 2001; Youle and Karbowski, 2005). Unfortunately, this observation requires additional verification as some reports still suggested that CYC release precedes mitochondrial fragmentation (Gao *et al.*, 2001; Arnoult *et al.*, 2005).

Further research has also shown that the inhibition of CYC release observed following down-regulation of DRP1 only results in a delay in apoptosis, and does not completely prevent the pathway from proceeding (Estaquier and Arnoult, 2007). It was noted, however, that CYC release was selectively inhibited by DRP1-depleted cells and that release of other intermembrane space proteins is not affected. This suggests that MOMP has still occurred, likely via BAX/BAK pore formation, but that the role of DRP1 and mitochondrial fragmentation may be additional or complementary to the MOMP, and specific to CYC release (Parone *et al.*, 2006; Estaquier and Arnoult, 2007).

Although fragmentation involves fission of the mitochondria, there is evidence that suggests a role for the fusion protein OPA1 in this process. It has been reported that OPA1 mediates cristae reorganisation, and that

this is essential for facilitating release of CYC from the mitochondria where it is normally stored in the folds of the cristae (Olichon *et al.*, 2003; Arnoult *et al.*, 2005; Frezza *et al.*, 2006). Alongside this, OPA1 is cleaved by a range of protease enzymes which yield S-OPA1 and L-OPA1. It is the S-OPA1 form that is involved in regulating cristae morphology (Cipolat *et al.*, 2006), and it does this by forming oligomeric structures with the L-OPA1 forms. These structures control how tight the junctions of the cristae are. During apoptosis, MOMP results in opening of these junctions and release of CYC (Frezza *et al.*, 2006).

The structure of the mitochondrial network is determined by the balance of fission and fusion. It is therefore assumed that changes in one of these pathways can affect the other and vice versa. I.e. if DRP1 deficiency results in delays in CYC release, then it would be reasonable to speculate that this could affect the activity of fusion proteins such as OPA1. In fact, it has been observed that the inhibition of DRP1 leads to an increase and decrease in S-OPA1 and L-OPA1, respectively (Arnoult *et al.*, 2005; Estaquier and Arnoult, 2007; Burke, 2017). Due to the role that S-OPA1 plays in regulating cristae junctions (Frezza *et al.*, 2006), the increase in S-OPA1, as a result of DRP1 knockdown, could also promote inhibition of CYC release by further tightening the cristae junctions.

CYC and the Apoptosome

CYC release has been identified as a pivotal point in the intrinsic pathway of apoptosis and is therefore the focus of much research with regards to anti-cancer treatments. This vital molecule is required to bridge the gap between recognition of severe cellular stress, and activation of the caspases that perform the cell death protocol. However, in order to activate the caspase cascade, two events must take place which result in the release of CYC. These events include solubilisation of CYC, which is achieved via disruption of its association with cardiolipin, followed by MOMP and subsequent membrane depolarisation (Ott *et al.*, 2002).

Once in the cytosol, CYC co-forms a structure known as the apoptosome via its association with apoptosis-protease activating factor-1 (APAF1), ATP, and inactive CASP9. Once incorporated into the apoptosome, CASP9

can cleave and activate downstream executioner caspases (Li *et al.*, 1997; Jiang and Wang, 2000; Adrain, Creagh and Martin, 2001; Budihardjo *et al.*, 2002; Ott *et al.*, 2002; McIlwain, Berger and Mak, 2013).

Synchronous with the expulsion of CYC is the release of other pro-apoptotic proteins, SMAC and OMI, which promote CYC-dependent caspase activation. They achieve this by binding to and incapacitating Inhibitor of Apoptosis (IAP) family members, such as X-linked IAP (XIAP) (Green and Evan, 2002). This prevents their caspase-inhibitory function, therefore promoting further progression through the apoptosis pathway (Adrain, Creagh and Martin, 2001; Fernald and Kurokawa, 2013).

1.6.1.3 UV-Induced Apoptosis

Apoptosis can be induced by a range of stimuli, including hypoxia, nutrient starvation, exposure to ionising radiation such as UV and X-ray, and treatment with certain cytotoxic drugs (Roos and Kaina, 2006, 2013; Lee *et al.*, 2015). However, in all cases the tumour-suppressor protein TP53 is a common point of apoptosis initiation, and its activation involves three key steps: stabilisation, DNA-binding, and transcriptional activation (Vousden, 2002).

The activity of TP53 is dependent upon trafficking between the nucleus and cytoplasm, which is determined by the interaction of TP53 with both TP53-associated parkin-like cytoplasmic (PARC; cytoplasm) protein and MDM2 (nucleus), both of which are E3-ubiquitin ligases (Nikolaev *et al.*, 2003). In the absence of stress, MDM2 maintains very low TP53 levels by targeting it for proteasomal degradation. PARC aids this process by sequestering TP53 in the cytoplasm, thereby preventing its entry into the nucleus. However, when exposed to stress-inducing stimuli, cells can activate a range of biochemical pathways, some of which converge upon inhibition of MDM2 and possibly PARC. This results in nuclear localisation of TP53 and subsequent downstream transcriptional activation of a variety of genes. Initial activation of TP53 generally results in cell cycle arrest, giving the cell time to repair any damage incurred. However, if the damage is irreparable, TP53 can activate the transcription of genes required to trigger apoptosis (Vousden, 2002).

DNA-Damage Response

Upon exposure to UV light, cells incur damage to their DNA. This can be a result of both DNA damaged caused by UV-induced activation of reactive oxygen species (ROS) or through introduction of DSBs following replication of unrepaired UV-induced DNA lesions (Roos and Kaina, 2006, 2013; Fulda *et al.*, 2010). The most common lesions formed as a result of UV exposure are pyrimidine dimers and (6-4) photoproducts (Kiefer, 2007; Rastogi *et al.*, 2010). Following recognition of a DSB in the DNA, the DNA damage response pathway is triggered. At this point the MRE11–RAD50–NBS1 (MRN) complex, consisting of meiotic recombination 11 homolog 1 (MRE11), ATP-binding cassette–ATPase (RAD50), and phosphopeptide-binding Nijmegen breakage syndrome protein 1 (NBS1), is activated, leading to recruitment of ataxia telangiectasia mutated (ATM) (Syed and Tainer, 2018).

At sites of DNA damage, ATM is responsible for phosphorylating a range of substrates (Figure 9), including the serine/threonine-protein kinase Chk2 (CHK2) which promotes downstream phosphorylation, and subsequent activation, of TP53 (Christmann *et al.*, 2003; Harper and Elledge, 2007). Active TP53 can then induce transcription for a range of genes that promote cell survival, such as p21, which triggers cell cycle arrest. This gives the cell time to initiate DNA repair via either homologous recombination or non-homologous end-joining (Jackson, 2002; Valerie and Povirk, 2003). In the case of severe and irreparable damage, TP53 can propagate the cell death signal by activating transcription of pro-apoptotic target genes, including PUMA, NOXA and BAX (Roos and Kaina, 2006, 2013; Vousden and Lane, 2007; Fulda *et al.*, 2010). PUMA and NOXA are both BCL2 inhibitors and therefore aid apoptosis progression by inhibiting the anti-apoptotic activity of BCL2. BAX on the other hand will promote MOMP upon association with BAK, as described previously.

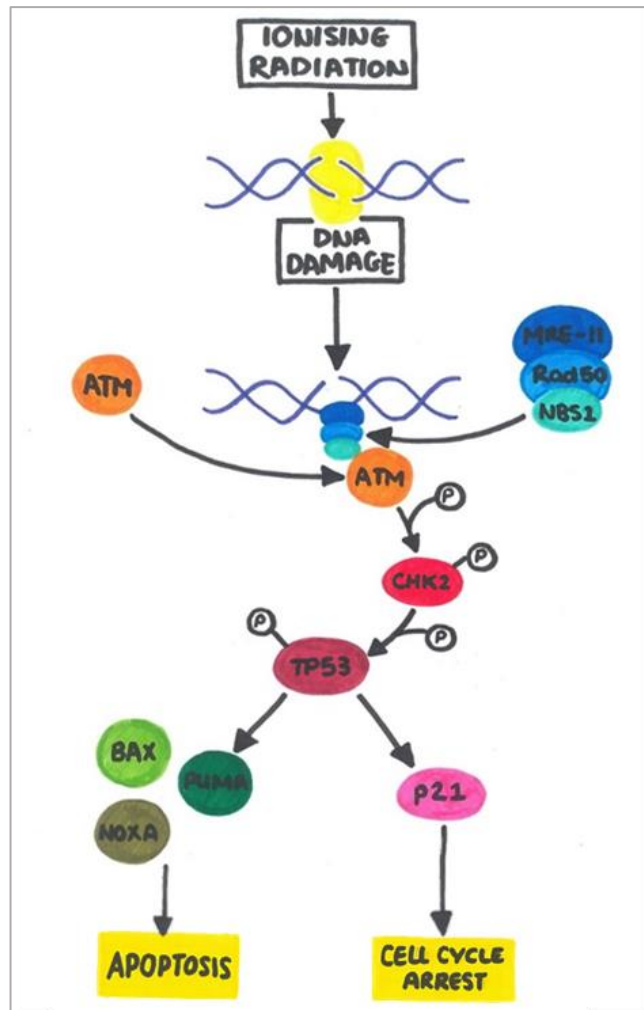


Figure 9 – DNA Damage Response to Irradiation. Exposure to ionising radiation introduces DSBs into the DNA and results in activation of the damage response pathway. The MRN complex promotes recruitment of ATM to the damage site where it initiates a downstream phosphorylation cascade that leads to activation of CHK2 and TP53. Transcription of BH3-only proteins and p21 is instigated by TP53 and causes cell cycle arrest and, in the case of irreparable damage, apoptosis. Drawn by author (adapted from Sulli, Di Micco and Di Fagagna, 2012).

Mutations in TP53

Mutations that perturb TP53 activation, and therefore allow for uncontrolled progression through the cell cycle, have been identified in more than half of all cancer cases (Vousden and Lu, 2002; Vousden and Lane, 2007; Kruse and Gu, 2009). TP53 plays a central role during the cell cycle, coordinating a range of cellular responses in the presence of stress-inducing factors, and ultimately deciding if a cell should survive or die (Lane, 1992; Fulda *et al.*, 2010). Therefore, mutations in the gene for TP53 that render it inactive have catastrophic consequences for the cell, by

allowing it to accumulate an excessive number of other mutations as a result of errors in DNA replication that are left unrepaired (Nowsheen and Yang, 2012).

1.6.2 Autophagy

Autophagy is a recycling mechanism used by the cell to target specific cellular components, mainly organelles, for removal. This requires the initial “labelling” of the target component, followed by recognition and subsequent engulfment of said component by a large autophagy-associated vacuole known as an autophagosome. Although the autophagosome is not responsible for removal of the entire cell, it is often involved in actively promoting cell death (Figure 7) (Green and Llambi, 2015).

Mitophagy is a specific sub-type of autophagy concerned with the removal of dysfunctional mitochondria. Recognition of damaged mitochondria results in binding and stabilisation of PTEN-induced kinase 1 (PINK1) at the outer mitochondrial membrane. Parkin is then recruited by PINK1, resulting in activation of its E3-ligase activity and subsequent ubiquitination of various proteins at the outer membrane. These proteins are then degraded by the proteasome and the remaining mitochondrial components removed via autophagy (Sato and Furuya, 2017).

1.6.3 Necroptosis

Necrosis is described as the uncontrolled destruction of cells due to disease or injury. However, research has revealed a form of programmed necrotic cell death which is referred to as “necroptosis”. This seemingly chaotic pathway is actually the result of widespread cooperation, at multiple cellular levels, between several biochemical and molecular events. This promotes what is often observed as a sort of cellular “explosion” (Figure 7) (Festjens, Vanden Berghe and Vandenabeele, 2006). In fact, many of the pro- and anti-death signalling molecules which are active during apoptosis are also involved in necroptosis (Galluzzi *et al.*, 2018). For example, activation of this pathway requires association of FADD with two other protein, receptor-interacting protein 1 and receptor-interacting

protein 3 kinase. Together, these proteins form the necrosome (Seifert and Miller, 2017). Activation of this complex and subsequent downstream signalling eventually results in the classic morphological changes associated with necrosis – cellular swelling, rupture of the PM and leakage of cellular contents (Dunai, Bauer and Mihalik, 2011).

1.7 Cancer

Carcinogenesis is a complex process that occurs as a result of numerous, dynamic changes within the cell. Cancerous tumours differ from their benign counterparts in that they are capable of aggressively proliferating and invading secondary locations around the body, whereas non-malignant tumours remain relatively static. For healthy cells to progressively evolve and form neoplastic structures, they must first acquire several sequential mutations, at a variety of sites, that promote procurement of certain biological capabilities, termed hallmarks (Foulds, 1954). Such mutations are generally attained following mis-repair of damaged DNA and can result in either loss of function/inactivation of genes which inhibit proliferation (tumour suppressor genes) or gain of function/activation of genes that promote cell survival or stimulate proliferation (oncogene) (Bishop and Weinberg, 1996). These types of mutations are often classified as driver mutations because they confer an advantage onto cells that “drive” progression to cancer. In contrast, passenger mutations are often referred to as those that have little or no effect on cancer progression, and these account for the majority of mutations identified in various cancer genomes (Pon and Marra, 2015; Poulos and Wong, 2019). Therefore, increased acquisition of epigenetic changes within the cell promotes genomic instability, and vice versa, ultimately resulting in formation and development of cancer.

1.7.1 Hallmarks of Cancer

Each successive insult has the potential to cause transformative alterations. If these changes confer an advantage to a particular cell, then that cell will undergo clonal expansion, in the presence of selective pressures, thereby providing the resultant cell population with the ability

to survive and thrive (Nowell, 1976). Consequently, tumorigenic cells acquire numerous traits that enable them to endure the harsh environments of the body. The most recent discussions of these hallmarks (Fouad and Aanei, 2017) concluded that although the previously described traits, put forward by Hanahan and Weinberg, encompassed many of the characteristics of cancer, they were not specific to the disease (Hanahan, D and Weinberg, RA, 2000; Hanahan and Weinberg, 2011). Therefore, the newly defined hallmarks of cancer include: an abetting microenvironment, vascularisation, invasion and metastasis, immune modulation, selective growth and proliferative advantage, metabolic rewiring, and finally altered stress response favouring survival (Figure 10) (Fouad and Aanei, 2017).

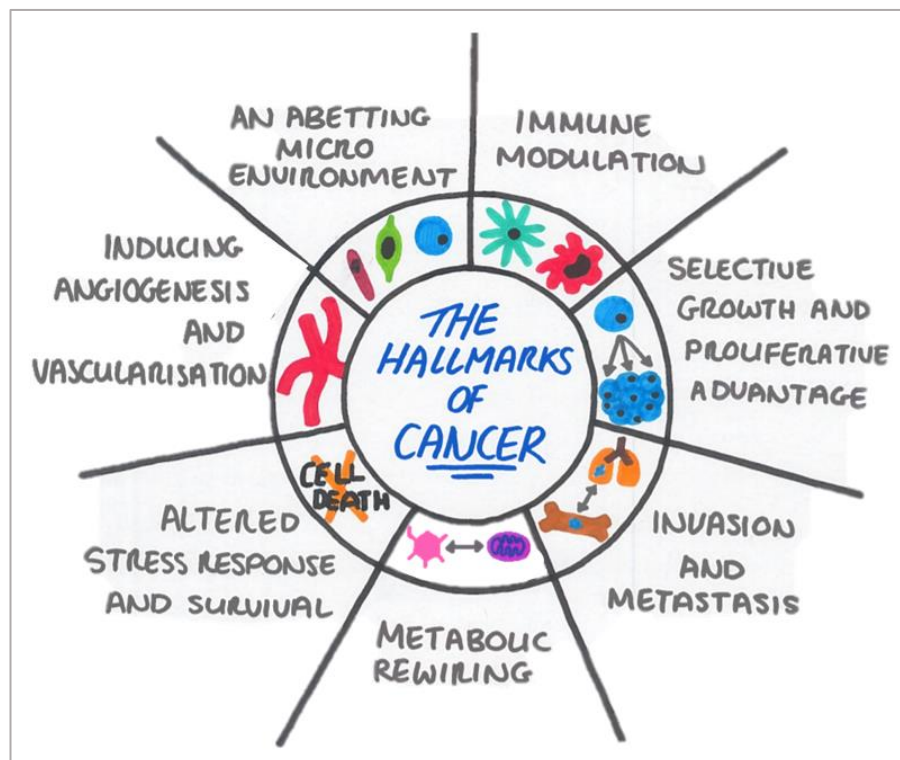


Figure 10 – Hallmarks of Cancer. At the core of cancer biology and behaviour are several hallmarks which are thought to promote tumorigenesis. As cancerous cells acquire a variety of hallmarks, they gain characteristics that enable them to overcome certain obstacles which prevent their development into metastasis. Drawn by author (adapted from Hanahan and Weinberg, 2011 and Fouad and Aanei, 2017)

An Abetting Microenvironment

Due to the heterotypic nature of cancer cells, it is unsurprising that they form part of a complex tumour microenvironment (TME). The TME contains

a diverse range of other cell types which are essential for successful tumour development. This spatial and temporal heterogeneity not only provides a suitable environment for metastatic cells to grow within, but also promotes integration between the various hallmarks. Therefore, it is very apparent that the composition of the TME is vital for cancer cell survival and expansion.

Vascularisation

For cells within a tissue to grow they must be able to access nutrients and oxygen. This function is performed by blood vessels which transport macromolecules to all areas of the body whilst also providing a platform for gas exchange and removal of cellular waste products. Cancer cells not only require excessive amounts of nutrients to fuel their over-proliferative activity, but they also grow in very close proximity, with cells at the centre of the tumour having little or no access to local vasculature. Therefore, cancer cells, along with other cell types in the TME, continuously release factors to promote angiogenesis at the site of the tumour. (Carmeliet and Jain, 2000; Hanahan, D and Weinberg, RA, 2000; Fouad and Aanei, 2017).

Despite this attempt to develop a diverse vascular network, some cells within a tumour are still subject to sub-optimal oxygen levels – Hypoxia. Although these conditions would normally result in extreme stress and PCD, hypoxia has, in fact, been shown to promote the metabolic rewiring (see "*Metabolic Rewiring*") of cancer cells by stabilising hypoxia-inducible factor 1-alpha (HIF1A); a transcription factor which can activate expression of proteins involved in glycolysis (Semenza, 2011; Vara-Perez, Felipe-Abrio and Agostinis, 2019).

Invasion & Metastasis

The ability to invade and form secondary metastases is often seen as a gateway between benign tumours and the pathway to cancer. Prior to initiation of the invasion-metastasis cascade (IMC), cells undergo a process known as epithelial-mesenchymal transition. This transition is triggered during exposure to stressful conditions and, as a result, cells adopt certain phenotypes and capabilities that will assist them during IMC (Ye and

Weinberg, 2015). The IMC is a complex, multistep process which involves degradation of the extracellular matrix, intravasation and transportation to a secondary site, manipulation of the new microenvironment, development of micro-metastases and eventually colonisation (Valastyan and Weinberg, 2011; Massagué and Obenauf, 2016; Fouad and Aanei, 2017).

Immune Modulation

The immune system is normally the best defence against disease and infection. This is due to the concept of self/non-self dichotomy whereby cells of the immune system are equipped to detect foreign proteins and materials, allowing them to identify and destroy cells of foreign origin. However, as cancer cells originate from the host's own cells they are not recognised as foreign by the immune system (Burgio, 1990; Soares, Teixeira and Moita, 2017). Cancer immunoediting is a process utilised by the immune system to target tumours (Dunn *et al.*, 2002). Unfortunately, this process also inadvertently promotes progression of the disease by selecting for those cells that are resistant to immune attack (Schreiber, 2016; Fouad and Aanei, 2017). Such genetic alterations commonly result in resistance to PCD. Immune modulation can also result from metabolic rewiring, interlinking the two hallmarks and further emphasizes the importance of mitochondria in cancer biology (Fouad and Aanei, 2017).

Selective Growth & Proliferative Advantage

During normal cell growth and maintenance of tissue homeostasis, cells depend on signalling factors that regulate their progression through the cell cycle. However, these processes are often disrupted in cancer, with mutations in proteins required to regulate various points along each pathway. Deregulation of the cell cycle and its affiliated checkpoints further promotes cancer progression (Schönthal, 2004). Inactivating mutations in both RB1 and TP53 occur in a multitude of malignancies, and the consequences of such alterations is not limited to just cell growth but also affects genomic stability, metabolism and apoptosis (Indovina *et al.*, 2013; Kruiswijk, Labuschagne and Vousden, 2015; Fouad and Aanei, 2017).

Metabolic Rewiring

In parallel to the previously discussed hallmarks is rewiring of metabolic pathways. Alterations in metabolism result in a multitude of transient changes within the cell and its surrounding environment, predominantly because of the role of the mitochondria in this process. Acquisition of this hallmark fulfils the energy demands of rapidly proliferating cancer cells (Cairns, Harris and Mak, 2011), thereby promoting their survival in an environment of impoverished nutrient and oxygen availability (Costa and Frezza, 2017). Metabolic reprogramming has previously been categorised into six further “sub-hallmarks” which include: (1) Alterations in metabolite-driven gene regulation, (2) utilisation of glycolytic and TCA cycle intermediates for biosynthesis, (3) interaction between metabolic pathways and the microenvironment, (4) deregulated uptake of glucose and amino acids, (5) employment of opportunistic methods for acquisition of nutrients, and finally (6) increased demand for nitrogen (Pavlova and Thompson, 2016; Fouad and Aanei, 2017).

One aspect of metabolic remodelling that is of current interest relates to hallmark (2) and involves reduced activity of the OXPHOS pathway, and a subsequent, compensatory increase in glycolysis to maintain ATP, even when oxygen is not the limiting factor. This observed imbalance is coined the “Warburg Effect” (Otto Warburg, Wind and Negelein, 1923; Schwartz, Supuran and Alfarouk, 2017) and a major driver behind this metabolic shift is activation of HIF1A (see “*Vascularisation*”) (Semenza, 2011; Vara-Perez, Felipe-Abrio and Agostinis, 2019; Vernucci *et al.*, 2019). The Warburg Effect has been linked to various other changes in cancer cell activity such as resistance to cell death.

Altered Stress Response & Survival

Alterations in the stress response is of particular relevance here due to its role in enabling metastatic cells to avoid cell death. Cells are continuously exposed to a range of stresses throughout their lifetime, but multiple mechanisms have been developed by the cell that can respond to these stress stimuli, preventing them from causing permanent damage. For example, TP53 can not only halt the cell cycle upon recognition of DNA

damage, and initiate DNA repair machinery, but can also trigger induction of apoptosis to promote removal of overly damaged cells, thereby avoiding further damage to the neighbouring population. In other words, one cell is eliminated for the sake of the whole tissue. Therefore, it is unsurprising that such mechanisms are overthrown in cancer to promote survival of genetically altered cells (Fouad and Aanei, 2017). Cancerous cells are able to evade apoptosis via an array of different mechanisms (Green and Evan, 2002; Lopez and Tait, 2015), including inactivating mutations in stress sensors, such as TP53, and other pro-apoptotic proteins, including those in the BCL2 family (Gerl and Vaux, 2005; Fernald and Kurokawa, 2013). On the other hand, proteins that suppress cell death progression can also be over-expressed to promote inhibition of apoptosis. Such proteins include members of the IAP family, such as survivin (Chen *et al.*, 2016; Li, Hu and Li, 2018).

1.7.2 Evading Apoptosis in Cancer

Exposure of cells to stressful conditions can lead to induction of apoptosis via the extrinsic and/or intrinsic pathways (see 1.6.1). However, unlike healthy cells, cancer cells are under constant stress due to genomic instability, excessive proliferation (hypoxia, reduced nutrient availability, limited space, inefficient waste removal etc.), and activation of genes promoting tumorigenesis (oncogenic stress) (Fernald and Kurokawa, 2013). However, many cancer cells appear to be unaffected by such stresses. This is because cancer cells do not necessarily respond to stress stimuli by activating apoptosis, but instead inhibit this PCD programme to promote survival.

Research has shown that cancer cells can in fact inhibit apoptosis by altering the expression (transcription), stability (translation) and function (post-translation) of proteins that modulate its progression (Fernald and Kurokawa, 2013). Several recent publications have identified such changes in a number of apoptosis-associated proteins (Vazanov *et al.*, 2018), including both pro- and anti-apoptotic members of the BCL2 family of proteins (Onyeagucha *et al.*, 2017; Campbell and Tait, 2018; Kønig *et al.*, 2019). Of particular interest is the postulated role of mitochondrial

metabolism in these protective mechanisms (Márquez-Jurado *et al.*, 2018; Fakouri *et al.*, 2019). Furthermore, it has been hypothesised that processes such as mitochondrial fission/fusion, MOMP and ROS production, if incorrectly modulated by the mitochondria, promote evasion from cell death (Porporato *et al.*, 2018). Therefore, the role of the mitochondria in oncogenesis and cancer cell survival makes them a promising target for the development of novel anti-cancer agents.

1.7.2.1 Mitochondrial dysfunction in cancer

As described previously (see 1.5), the mitochondria are involved in modulating a range of cellular processes. Therefore, changes in their activity have a significant impact on many processes that have been linked to the development of cancer, from increased metabolic flexibility to reduced sensitivity to PCD induction (Gaude and Frezza, 2014; Porporato *et al.*, 2018). Furthermore, research has highlighted a recondite link between mitochondrial metabolism and cell death, with many proteins associated with one pathway also having roles in the other and vice versa (Green, Galluzzi and Kroemer, 2014). For example, CYC is essential for downstream activation of the caspase cascade but, whilst retained in the mitochondria, is also required to shuttle electrons between complexes in the ETC. Therefore, it has been suggested that cell fate is determined by metabolic checkpoints. That is, there exists molecular mechanisms that, upon changes in metabolism, determine whether a cell will enter apoptosis or continue to survive (Green, Galluzzi and Kroemer, 2014; Márquez-Jurado *et al.*, 2018).

Altered Mitochondrial Metabolism & Dynamics

Mitochondria are remarkably plastic in terms of their bioenergetic capabilities. Transformed cells utilise this characteristic to promote reconfiguration of the metabolic landscape. This is achieved through changes in both the genetic makeup and dynamic activity (fission and fusion) of the mitochondria. It is therefore hypothesised that accumulation of defunct mitochondria not only supports modification of the metabolic network, but also promotes changes in the (epi)genetic framework of cells,

thereby facilitating their pathogenic transformation (Gaude and Frezza, 2014).

The dynamic nature of the mitochondria allows cells to respond to various intra and extracellular changes. This is made possible by the action of fission and fusion events which modulate the ever-changing behaviour of the mitochondrial network. The ability to remodel in response to stress is essential for manipulation and coordination of apoptosis (Westermann, 2010). An imbalance between fission and fusion events has been documented in a number of cancers, and it has been hypothesised that the resultant change in structure and function of the mitochondrial network could be involved in the acquired resistance of cancer cells to cell death (Porporato *et al.*, 2018).

Previous research has suggested a role for both decreased fission and/or increased fusion in resistance to cell death. For example, increased fusion is believed to aid cell survival by promoting more efficient production of ATP as a result of an extensively protruding mitochondrial network (Cocetta, Ragazzi and Montopoli, 2019). Additionally, inhibition of mitochondrial fragmentation has been shown to delay the onset of apoptosis by causing, amongst other things, extensive remodelling of the cristae and subsequently reduced expulsion of CYC into the cytoplasm (Cocetta, Ragazzi and Montopoli, 2019). Further to this, over-expression of OPA1, a key regulatory protein involved in fusion, has been linked to increased resistance of cancerous cells to cell death through the previously suggested mechanism (Frezza *et al.*, 2006; Fang *et al.*, 2012). Furthermore, downregulation of DRP1, a protein involved in the OPA1-opposing fission machinery, has also been shown to prevent release of CYC by modifying the pattern of OPA1 isoforms present in the cell; increased production of S-OPA1 results in tightening of the cristae junctions and subsequent confinement of CYC to these regions (Arnoult *et al.*, 2005; Estaquier and Arnoult, 2007). However, over-expression of DRP1 has also been shown to reduce the sensitivity of some cancers to cell death. Therefore, DRP1 has an alternating role in resistance of cancer cells to certain stressors, although the reason for this is unknown (Cocetta, Ragazzi and Montopoli, 2019).

Altered Sensitivity to MOMP Induction

Another possible explanation for the increased resistance of cancer cells to apoptosis is that the mitochondria of malignant cells have altered sensitivity towards signals that promote MOMP or MPTP opening (Suh *et al.*, 2013; Green, Galluzzi and Kroemer, 2014). Therefore, these cells require a much greater level of outer membrane permeabilization in order to reach the threshold, at which point cell death would be induced (Bonnet *et al.*, 2007; Czabotar *et al.*, 2014; Porporato *et al.*, 2018). In other words, if the threshold cannot be reached, then the cell death pathway cannot ensue. One possible reason for this is the archetypal loss of TP53. In non-tumorigenic cells, TP53 regulates expression of genes encoding a variety of proteins, including BAX, PUMA and NOXA (Matsuura *et al.*, 2016). BAX is required for pore formation at the mitochondrial outer membrane during the initial stages of apoptosis. However, loss of BAX, as a result of TP53 inactivity, could raise the threshold for MOMP, at which point malignant cells will have successfully evaded cell death.

Increased mitochondrial transmembrane potential has also been linked to increased utilisation of the glycolytic pathway (Bonnet *et al.*, 2007). The glucose-processing hexokinase type-II enzyme, which is normally downregulated by TP53, has been shown to promote closure of voltage-dependent anion-selective channel (VDAC) proteins on the outer mitochondrial membrane (Pedersen *et al.*, 2002; Matsuura *et al.*, 2016). Previous research indicates that when functional, VDAC1 and VDAC2 can associate with pro-apoptotic BAX to initiate pore formation and subsequent CYC release (Chin *et al.*, 2018; Magrì, Reina and De Pinto, 2018). Therefore, it appears that binding of hexokinase to VDAC isoforms hinders this pro-apoptotic function (Pastorino, Shulga and Hoek, 2002; Mathupala, Ko and Pedersen, 2006).

Overexpression of anti-apoptotic members of the BCL2 family, such as BCL2, BCLX and MCL1, promotes altered sensitivity to apoptotic cues and acquired resistance to therapeutic intervention (Fernald and Kurokawa, 2013; Czabotar *et al.*, 2014; Green, Galluzzi and Kroemer, 2014). This could be a result of BCL2-mediated BAX inhibition which increases the

threshold for MOMP induction as described previously (Fernald and Kurokawa, 2013; Kuwana, 2018; Vazanov *et al.*, 2018). Alternatively, recent research has suggested that upregulation of XIAP, a member of the IAP family, in some cancers has been associated with delayed and incomplete MOMP. This is due to changes in the ratio of anti-apoptotic BCL2 proteins (BCL2 and BCLX) to pro-apoptotic BAX, resulting in greater antagonism of BAX and decelerated pore formation (C. Chen *et al.*, 2018).

Elevated ROS Production & Desensitisation

ROS are involved in the control of a number of processes which affect cell physiology and pathophysiology (Diebold and Chandel, 2016; Redza-Dutordoir and Averill-Bates, 2016). For example, physiological levels of ROS are required for careful regulation of mitochondrial dynamics, as their role in redox signalling maintains homeostatic control of mitochondrial morphology and function (Willems *et al.*, 2015). However, during oncogenesis cancer cells generate supraphysiological amounts of ROS, due to uncontrolled glucose metabolism and resultant mitochondrial fragmentation (Yu, Robotham and Yoon, 2006). Slightly elevated ROS levels are also associated with an increased resistance to cell death.

One current theory suggests that by increasing ROS production by small amounts, cancer cells trigger an adaptive, hormetic response that resembles aspects of ischemic preconditioning (Ristow, 2014; Hausenloy and Yellon, 2016; Porporato *et al.*, 2018). In other words, by constantly exposing cancerous cells to low levels of genotoxic ROS, these cells no longer respond appropriately to such apoptotic cues. An example was observed in cells exposed to hypoxia as accumulation of the transcription factor HIF1A (see 1.7.1) is induced by elevated ROS production. HIF1A is involved in upregulating glucose metabolism during hypoxia, and could therefore promote preconditioning by inhibiting mitochondrial metabolism (Semenza, 2011), although the exact mechanism by which this occurs is yet unknown.

Increased generation of ROS results in oxidative stress; upregulation of antioxidants, such as glutathione peroxidase (GPX), is a natural countereffect which aims to protect the cell from ROS-induced damage (He

et al., 2017). This feedback mechanism is advantageous to cancer cells when faced with chemotherapeutic agents that promote ROS production (Cocetta, Ragazzi and Montopoli, 2019). Maintenance of optimal antioxidant levels can help prevent changes in mitochondrial membrane potential and subsequent initiation of apoptosis (Okon and Zou, 2015; Cocetta, Ragazzi and Montopoli, 2019). Furthermore, should the mitochondria become dysfunctional, as a result of elevated ROS production, proteins such as PINK1 and Parkin are recruited to the mitochondria to promote their removal via mitophagy (see 1.6.2) (Fan *et al.*, 2019).

As aforementioned, sensitivity of cancer cells to apoptosis can also be regulated by mechanisms that depend on glucose metabolism. In fact, previous reports have shown that the pro-apoptotic activity of CYC is controlled by its redox state (Vaughn and Deshmukh, 2008). Under normal circumstances, an increased production of ROS, as a result of an apoptotic insult, results in oxidation on CYC, thereby activating its pro-apoptotic function. However, upregulated glucose metabolism can result in redox inactivation and sequestration of CYC by glutathione, a cofactor for GPX, and therefore inhibition of cell death (Li, Wang and Xu, 2008; Vaughn and Deshmukh, 2008; Tait and Green, 2013; Matsuura *et al.*, 2016). This increased utilisation of the glycolytic pathway is often caused by an increased production of ROS, synergistic to accumulation of damaged mitochondria (Vaughn and Deshmukh, 2008; Liemburg-Apers *et al.*, 2015).

1.8 Survivin

Survivin (Svn) is a multifunctional protein with roles in both cell proliferation and cell death. During the cell cycle, survivin forms an integral part of a large protein complex, known as the CPC (Vader *et al.*, 2006). This complex ensures correct alignment and segregation of the chromosomes during (pro)metaphase and anaphase, respectively, as well as partitioning of the cytoplasm during cytokinesis (see 1.3.4) (Cooke, Heck and Earnshaw, 1987; Ruchaud, Carmena and Earnshaw, 2007). Conversely, the presence of a baculovirus IAP protein repeat (BIR) domain

identifies survivin as a member of the IAP family and, as such, it has an important role in regulating cell death, although the exact mechanisms underpinning this function are yet unknown (Li *et al.*, 1998).

Due to its varying roles in a range of cellular pathways, it is unsurprising that over-expression of Svn in cancer is associated with excessive proliferation and resistance to cell death stimuli. Furthermore, these characteristics make Svn a desirable target for cancer therapy as inhibiting its activity would ideally down-regulate proliferation whilst sensitising cancerous cells to apoptotic agents (Ansell *et al.*, 2004; Plescia *et al.*, 2005; Chang *et al.*, 2015). However, despite much research and testing, a suitable anti-cancer agent which targets Svn has not yet reached the clinic. This is likely because Svn, although involved in regulating several pathways, has itself no enzymatic activity and instead relies on interactions with other proteins to perform its functions (Li, Aljahdali and Ling, 2019). As a result, efficient and selective targeting of Svn, though attractive, is a major challenge.

1.8.1 Structure & Domains

At just 16.5 kDa, and a length of 142 amino acids (aa), survivin is the smallest member of the IAP family (Ambrosini, Adida and Altieri, 1997). It contains just one BIR domain at its amino-terminal region which is linked closely to the opposing carboxyl-terminus via an amphipathic helical structure (Li *et al.*, 1999; Verdecia *et al.*, 2000). The gene encoding Svn is located on chromosome 17q25 (Ambrosini, Adida and Altieri, 1997). Transcription of this gene is controlled by a TATA-less promoter, three cell cycle-dependent elements (CDEs) and a single cell cycle homology region (CHR) (Li and Altieri, 1999). This level of control ensures the correct and timely expression of Svn such that minimal amounts are present during G1 phase, but that plenty is available for formation of the CPC during mitosis (Li and Altieri, 1999).

1.8.2 Expression & Regulation

In non-tumorigenic cells, Svn is only expressed during development (Uren *et al.*, 2000) and in mature proliferating cells (Li *et al.*, 1998). However, in

metastatic cells, Svn expression is upregulated (Velculescu *et al.*, 1999). At the transcriptional level, Svn expression is controlled by several factors, including HIF1A, nuclear factor κ B (NF κ B), signal transducer and activator of transcription (STAT) proteins and β -catenin (Boidot, Végran and Lizard-Nacol, 2014). Further to this, Svn is transcriptionally repressed by the activity of a plethora of other proteins, such as the tumour suppressors, TP53 and RB1. Previous data suggests that repression of Svn by TP53 is dependent upon both direct interaction between the transactivation domain of TP53 and the CDE/CHR element on the Svn promoter, as well as the downstream activity of p21 (Hoffman *et al.*, 2002). Active p21 inhibits cyclin-CDK complexes, preventing hyperphosphorylation of RB1. This results in sequestration of the transcription factor E2F1 and ultimately down-regulation of Svn transcription (Jiang *et al.*, 2004; Raj *et al.*, 2008).

1.8.3 Localisation of Survivin

During the cell cycle Svn expression is at its lowest in G1 and increases throughout the cycle, peaking in G2 and mitosis where it regulates the SAC (see 1.8.4.1) (Beardmore *et al.*, 2004). Svn performs this function by interacting with members of the CPC (see 1.3.4) which initially localise to the centromeres (nucleus) before settling at the spindle mid-zone during anaphase (Ruchaud, Carmena and Earnshaw, 2007).

In cancerous cells, Svn has also been observed in the cytoplasm and even the mitochondria (Dohi, Beltrami, *et al.*, 2004; Stauber, Mann and Knauer, 2007). Research indicates that Svn found in the latter two locations has cytoprotective capabilities and therefore promotes survival of cancer cells. Furthermore, shuttling of Svn between the nucleus and cytoplasm appears to be essential for its anti-apoptotic function as Svn restricted to the nuclear compartment cannot inhibit cell death (Colnaghi *et al.*, 2006; Stauber *et al.*, 2006). On the other hand, localisation of Svn to the mitochondria is thought to involve the molecular chaperone heat-shock protein (HSP) 90 (Fortugno *et al.*, 2003; Dunajová *et al.*, 2016). Therefore, the localisation of Svn to several compartments further highlights its role in a variety of complex signalling pathways.

1.8.4 Multifunctional Role of Survivin

1.8.4.1 Survivin in Mitosis

Genomic instability is a key factor in the development of cancer and often results from mis-segregation of the chromosomes. Therefore, during mitosis, accurate division of the chromosomes is carefully monitored by the SAC (Vader *et al.*, 2007). Recognition of improper kinetochore-spindle attachments results in halted progression from metaphase to anaphase. Therefore, the cell requires a sophisticated mechanism that can not only detect such attachments, but can also correct them, so that cell cycle may continue (Haase *et al.*, 2017).

The CPC is a major regulatory complex that ensures formation of correctly orientated kinetochore-spindle attachments, in order to satisfy the requirements of the SAC (Lampson *et al.*, 2004; Ruchaud, Carmena and Earnshaw, 2007). The CPC consists of Aurora-B kinase (ABK), inner centromere protein (INCENP), Svn and Borealin/Dasra-B (BDB) (Jeyapragash *et al.*, 2007). The role of Svn in this complex lies with its interaction with INCENP and BDB (Wheatley *et al.*, 2001; Vader *et al.*, 2006). This regulatory triplet controls the centromere-targeting module of the CPC, therefore allowing for its localisation to the centromere and subsequent activation of ABK. ABK-associated phosphorylation of proteins within the kinetochore promotes weakening of erroneous attachments (Lampson *et al.*, 2004; Cimini *et al.*, 2006). The chromosomes are then free to form new attachments with the spindle.

1.8.4.2 Survivin in Apoptosis

Previous research has shown that over-expression of Svn in cancer cells promotes evasion from apoptosis and aids tumorigenesis. Although the exact mechanism by which Svn inhibits apoptosis is unknown, research has identified several proteins that promote Svn up-regulation (Gritsko *et al.*, 2006; Li *et al.*, 2012; Cui *et al.*, 2017) as well as those that may be the target of Svn activity. For example, Svn appears to interact with XIAP, leading to stabilisation and caspase inhibition, SMAC, relieving its inhibitory effect on XIAP, and finally hepatitis B X-interacting protein (HBXIP),

hindering apoptosome formation (Marusawa *et al.*, 2003; Song, Yao and Wu, 2003; Dohi, Okada, *et al.*, 2004). Svn also appears to be the target of post-translational modifications that can alter its molecular functions. An example of this is phosphorylation of Svn on Thr34 by CDK1 which has been shown to promote the anti-apoptotic activity of Svn (O'Connor *et al.*, 2000; Barrett, Osborne and Wheatley, 2009). To further add to this complex and mysterious role in cell survival, there are several documented cancer-specific pools of Svn that have also been shown to promote its anti-apoptotic function (Dohi, Beltrami, *et al.*, 2004; Stauber, Mann and Knauer, 2007; Kreger *et al.*, 2016). Therefore, although Svn appears to inhibit aspects of the extrinsic and intrinsic pathways of apoptosis, a much greater understanding of the roles for its cytoplasmic and mitochondrial pools in either of these processes is required.

1.8.5 Survivin and Cancer

Diseases such as cancer (see 1.7) are caused by an imbalance between cell survival and cell death (see 1.2). Overexpression of Svn has been documented in numerous types of cancer, including lung, breast and ovarian (Altieri, 2003), and is associated with excessive proliferation, resistance to therapy, development of an invasive phenotype, and ultimately poor prognosis (Li, Hu and Li, 2018).

1.8.5.1 Rewiring the Metabolic Landscape

Localisation of Svn to the mitochondria appears to be essential for several roles within tumorigenesis. For example, the ability to rewire the metabolic landscape of the cell is a major hallmark of cancer (see 1.7) and is often due to altered utilisation of the OXPHOS pathway. Previous research has identified Svn as a key player in such metabolic remodelling, although it is unclear as to whether it promotes (Hagenbuchner *et al.*, 2013) or represses (Rivadeneira *et al.*, 2015) use of the glycolytic pathway as an alternate means of ATP production.

1.8.5.2 Altering Responses to Apoptotic Stimuli

Cytoplasmic Svn Promotes Cell Survival

Previous research has suggested that following exposure to apoptotic stimuli, Svn is rapidly released from the mitochondria in order to prevent caspase activation (Dohi, Beltrami, *et al.*, 2004). Therefore, its presence in the cytoplasm is also important for its anti-apoptotic function. Whilst in the cytoplasm, Svn can complex with XIAP (Dohi, Okada, *et al.*, 2004). This not only stabilises Svn by preventing its degradation, but also allows it to utilise the caspase-inhibitory activity of XIAP to prevent activation of the caspase cascade. Furthermore, during MOMP, several inter-membrane space proteins are released from the mitochondria, including SMAC (see 1.6.1.2). Direct interaction of Svn with SMAC is thought to be essential for its anti-apoptotic function (Song, Yao and Wu, 2003). SMAC is a potent inhibitor of IAP proteins, especially XIAP (Wang, 2001), and therefore, by binding to and inhibiting SMAC, Svn can promote their activity. Finally, Svn has also been shown to inhibit the activity of both initiator and executioner caspases (Tamm *et al.*, 1998; O'Connor *et al.*, 2000; Marusawa *et al.*, 2003; Song, Yao and Wu, 2003). For example, when complexed with HBXIP, Svn prevents recruitment of inactive CASP9 to APAF1 at the apoptosome. Interestingly, whilst HBXIP was found to associate with both wild type and phospho-mimetic T34E mutant Svn, there was no evidence of an interaction with the T34A mutant which lacked resembled un-phosphorylated Svn (Marusawa *et al.*, 2003). Therefore, suggesting that phosphorylation of Svn optimises its interaction with HBXIP.

Role for Mitochondrial Svn in Apoptosis Inhibition

Although most of the observed anti-apoptotic effects of Svn occur in the cytoplasm, its localisation to the mitochondria may be important for inhibiting the early stages of apoptosis, such as MOMP and release of CYC. Moreover, removal of its bona fide mitochondrial-targeting sequence (MTS; aa-10) appears to depress the anti-apoptotic function of Svn (Wheatley, 2015; Dunajová *et al.*, 2016), further suggesting that its presence in the mitochondria is essential for its ability to inhibit apoptosis. Several

hypotheses have been put forward which propose a variety of roles for Svn in the mitochondria, including altering the activity of fission-fusion proteins and preventing release of pro-apoptotic proteins from the mitochondrial inter-membrane space.

Research has suggested that localisation of Svn to the mitochondria alters their dynamic nature due to recruitment of DRP1 and resultant fragmentation (Hagenbuchner *et al.*, 2013; L. Chen *et al.*, 2018). Over-expression of Svn has been shown to increase the levels of DRP1 in neuroblastoma cells. Interestingly, this also resulted in reduced phosphorylation of DRP1 (unpublished data) (Hagenbuchner *et al.*, 2013). It has been suggested previously that the phosphorylation state of DRP1 can be a key factor in determining cell fate (Jahani-Asl and Slack, 2007). For example, phosphorylation of DRP1 on Ser637 or Ser693 by protein kinase A (PKA) and glycogen synthase kinase 3 β (GSK3 β), respectively, results in inhibition of DRP1 GTPase activity, mitochondrial alterations that prevent fragmentation and reduced sensitivity to apoptotic stimuli (Chang and Blackstone, 2007; Cribbs and Strack, 2007; Chou *et al.*, 2012). Elevated levels of DRP1 have also been shown to increase the expression of Svn in triple-negative breast cancer cells through a positive feedback loop between fission and the Notch signalling pathway (L. Chen *et al.*, 2018). Neurogenic locus notch homolog protein 1 (NOTCH1)-associated up-regulation of Svn expression has also been observed in both estrogen receptor-negative breast cancer cells (Lee *et al.*, 2008) and non-small cell lung cancer (NSCLC) cells (Chen *et al.*, 2011). Interesting, this observation was made following exposure of NSCLC cells to hypoxic conditions, and the resultant up-regulation of Svn by NOTCH1 was further synergized by the activity of HIF1A (Chen *et al.*, 2011).

Over-expression of Svn has also been shown to block the release of certain pro-apoptotic proteins found in the mitochondrial inter-membrane space. These proteins include SMAC and CYC which are both normally released following MOMP. Several papers have reported that Svn directly binds to and blocks release of SMAC from the mitochondria, resulting in decreased sensitivity to apoptotic stimuli (Song, Yao and Wu, 2003; Dohi, Beltrami, *et al.*, 2004; Ceballos-Cancino *et al.*, 2007). Although no evidence has yet

been found to show a direct inhibitory effect for Svn on CYC release, research indicates that Svn is able to inhibit this event by altering the activity of proteins upstream. For example, over-expression of Svn was shown to repress the activity of both Bcl-2-like protein 11 (BCL2L1 or BIM) and BAX (Obexer *et al.*, 2009; Hagenbuchner *et al.*, 2013). During apoptosis, BH3-only protein BIM inhibits the activity of BCL2, whilst BAX promotes pore formation in the outer mitochondrial membrane. Therefore, by repressing the activity of both BIM and BAX, Svn can prevent MOMP and ultimately CYC release.

1.9 Hypothesis to be Tested

Previous research has identified a role for Svn in promoting resistance to cell death and it has been observed that removal of the MTS of Svn affects its ability to inhibit cell death processes. Therefore, the aim of this research is to investigate the response of cancer cells to UVC treatment in the presence and absence of ectopically expressed Svn. It is hoped that the results of this investigation will aid in producing a theoretical model for the molecular mechanism behind Svn-dependent apoptosis inhibition.

CHAPTER 2: Materials & Methods

All materials were sourced from Sigma unless stated otherwise.

All eppendorf and falcon tubes were sourced from Sarstedt.

2.1 Molecular Biology

Table 1 – Plasmid construct information

CONSTRUCT	VECTOR	REFERENCE
Green Fluorescent Protein (GFP)	pcDNA3.1(+)	Carvalho, <i>et al.</i> (2003)
Svn-GFP	pcDNA3.1(+)	Carvalho, <i>et al.</i> (2003)
Svn ¹⁻¹⁰ -GFP	pcDNA3.1(+)	Dunajová, <i>et al.</i> (2016)

2.2 Mammalian Cell Lines and Tissue Culture

'Henrietta Lacks' (HeLa) immortalised cervical cancer cell lines were provided by Dr Wheatley's laboratory (derived from original ATCC stock). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; D6429) supplemented with 10 % Fetal Calf Serum (HyClone) and 1 % Antibiotic Antimycotic (A5955). HeLa cells expressing GFP, SvnGFP and Svn¹⁻¹⁰GFP were maintained under selective pressure with 1 mM G418 sulphate (ACROS organics, #10165633) antibiotic. Cells were incubated in a humidified atmosphere with 5 % CO₂ at 37 °C (MCO-17AIC CO₂ Incubator; Sanyo) until 70-90 % confluent at which point they were passaged; trypsinised with Trypsin-Ethylenediaminetetraacetic acid (EDTA) solution (T4174) and seeded down in tissue culture treated plates (Corning Incorporation).

2.3 UVC Dose Response

Cells were seeded down into individual wells of a CELLSTAR[®] black 96-well polystyrene μ Clear[®] plate (Greiner, #655090) and incubated overnight in a humidified atmosphere with 5 % CO₂ at 37 °C. Triplicate wells were treated with increasing dosages (0.001 – 5.0 J·cm²) of 254 nm UVC (Vilber Lourmat; BLX 254). Cells were incubated for desired time, at which point the media in each well was discarded and replaced with 50-100 μ l CO₂-

independent phenol red free media. Resazurin (R7017) were aliquoted into each well at a final concentration of 20 μ M. Samples were placed back in an incubator for 1 hour before reading the absorbance of resorufin, generated following reduction of resazurin at the mitochondria, at 595 nm using a spectrofluorometer (FLUOstar Galaxy, BMG Labtech).

2.4 Guava® Muse® Cell Analyser

0.4×10^6 cells were seeded down into CELLSTAR® 12-well plates (Greiner, #665180) and left to culture in a humidified atmosphere with 5 % CO₂ at 37 °C. Once cells had reached 70-90 % confluency, cells were washed with 1X phosphate buffered saline (PBS; Gibco, Invitrogen) before being exposed to 180 mJ·cm² of UVC (254 nm) radiation. Plates were incubated and cell harvested at 3, 6, and 9 hours post-treatment. Media and subsequent 1X PBS wash from each plate were transferred into fresh 15 ml Falcon tubes before cells were incubated with Trypsin-EDTA (T4174) for 4 minutes. The cell suspension was transferred to falcon tubes. Samples were centrifuged at 300 x g for 3 minutes to pellet cells, the supernatant discarded, and the cells resuspended to a final concentration of 0.1×10^6 - 5.0×10^6 in desired buffer (see 2.4.1 and 2.4.2). Aliquots containing $\sim 0.1 \times 10^5$ - 5.0×10^5 cells were transferred to Eppendorf tubes and incubated with equal volumes of required assay reagent(s) (see 2.4.1 and 2.4.2). Samples were then diluted into a final volume of 1 ml to achieve a final concentration of 10-500 cells· μ l.

2.4.1 Muse™ Annexin V & Dead Cell Assay Kit (MCH100105)

As per the manufacturer's instructions, pelleted cells were resuspended in 1X PBS (Gibco, Invitrogen), supplemented with 1 % bovine serum albumin (BSA; A9418), before being aliquoted and incubated with Muse™ Annexin V & Dead Cell Reagent (Merck, #4700-1458) for 20 minutes at room temperature (RT). Each sample was resuspended thoroughly prior to analysis using the Guava® Muse® Cell Analyzer (Merck, #0500-3115).

2.4.2 Muse™ Caspase-3/7 Assay Kit (MCH100108)

As per the manufacturer's instructions, pelleted cells were resuspended in 1x Assay Buffer BA (Merck, #4700-1360). Muse™ Caspase-3/7 Reagent (contains NucView® caspase substrate) (Merck, #4700-1505) was diluted 1:8 with 1X PBS (Merck, #4700-1515) before being added to the cell suspension. Cell suspensions were then placed in a water bath (VWR) for 30 minutes at 37 °C. A working solution of Muse™ 7-AAD Reagent (Merck, #4700-1510) was prepared by adding 2 µl of the reagent to 148 µl of 1x Assay Buffer BA. Aliquots of 7-AAD working solution were then added to each sample before mixing thoroughly and analysing using the Guava® Muse® Cell Analyzer (Merck, #0500-3115).

2.5 Microscopy Techniques

2.5.1 Live Sample Imaging

2.5.1.1 Cell Preparation & Fluorescent Staining

0.1×10^5 cells were seeded down into individual wells of a µ-Slide 8-well (Ibidi, #80826) live imaging chamber and left to culture overnight in a humidified atmosphere with 5 % CO₂ at 37 °C. Once cells had reached 70-80 % confluency they were treated with 180 mJ·cm² UVC (254 nm) radiation and returned to the incubator for 5 hours. Prior to imaging, cells were incubated with MitoTracker™ Red (MTR; Invitrogen) and NucBlue™ Live ReadyProbes™ Reagent (2 drops·ml⁻¹ medium; Invitrogen) stains for 15 minutes before gently washing with 1X PBS and replacing with CO₂-independent, phenol-red free, antibiotic free DMEM.

2.5.1.2 Live Cell Fluorescence Imaging

Samples were imaged individually using a DeltaVision Elite microscope. For each sample, including controls, 3-5 randomly selected fields of view were acquired at 60x magnification. Images were taken of the following channels at the excitation/emission (nm) values provided: TRITC (542/594), FITC (475/523) and DAPI (390/435). Exposures were set to suit the highest level of fluorescence in each channel. Images were deconvolved using an iterative refinement method built into the microscope software. Restored

images were saved in .dv or .tif format before being prepared in Fiji (see 2.5.3.2) image processing software.

2.5.2 Fixed Sample Imaging

2.5.2.1 Coverslip Preparation – Poly-L-Lysine Coating

Coverslips (VWR, #631-0125) were sterilised in 70 % ethanol (Fisher Scientific, #10000652) before being placed onto a Dri-Block® heater (Techne, #FDB02A) at 60 °C to dry. Each coverslip was then covered with a thin layer of 10 % Poly-L-Lysine before being placed back on the Dri-Block® heater. Once dry, coverslips were washed three times in 1X PBS (Gibco, Invitrogen), each time being placed back on the Dri-Block® heater to dry before repeating. Coverslips were placed into individual wells in a CELLSTAR® 6-well plate (Greiner, #657160) before being sterilised with 10 J·cm² of 254 nm UVC radiation (Vilber Lourmat; BLX 254).

2.5.2.2 Cell Fixing & Immunofluorescent Staining

1.0×10^6 cells were seeded down onto Poly-L-Lysine (P8920) coated coverslips (see 2.5.2.1) in each well of a CELLSTAR® 6-well plate (Greiner, #657160), and left to culture overnight in a humidified atmosphere with 5 % CO₂ at 37 °C. Once cells had reached 70-90 % confluency, they were exposed to 180 mJ·cm² UVC (254 nm) radiation before being returned to an incubator. At desired timepoints, cells were incubated with MitoTracker™ Deep Red (MTDR; 1:3000) stain, diluted in DMEM, for 15 minutes before fixing with a methanol fixing mixture, containing 95 % methanol (VWR BDH, #20847.307) and 5 mM EDTA (ACROS organics, #409975000), for 15 minutes at -20 °C. Coverslips were washed three times for 5 minutes with 1X PBS (Gibco, Invitrogen). Background signal was blocked through incubation with PBA buffer (1X PBS supplemented with 1 % BSA (A9418) and 0.1 % Azide (S2002)), for 1 hour at RT. Coverslips were inverted onto relevant primary antibody (see 2.12), diluted appropriately in blocking buffer, for 1-2 hours at RT in the dark. Coverslips were then washed with 1X PBS for 15 minutes, before inversion upon relevant secondary antibody (see 2.12), diluted appropriately in blocking buffer, for 1 hour at RT in the dark. Cells were again washed with

1X PBS for 15 minutes before coverslips were inverted onto 4',6-diamidino-2-phenylindole (DAPI) stain (Thermo Fisher), diluted 1:500 in blocking buffer, and incubated for 10 minutes at RT in the dark. Cells were washed a final time with 1X PBS for 15 minutes before being mounted onto SuperFrost® microscope slides (VWR, #631-0114) using Mowiol® 40-88 mounting medium.

2.5.2.3 Fixed Cell Fluorescence Imaging

Samples were imaged individually using a Zeiss Exciter Widefield microscope. For each sample, including controls, 3-5 randomly selected fields of view were acquired at 40x magnification. Z-stacks were obtained by capturing images at different points along the z-axis. The following channels were used at the excitation/emission (nm) values provided: Cy5 (649/666), TRITC (542/594), FITC (475/523) and DAPI (390/435). Exposures were set to suit the highest level of fluorescence in each channel. Images were deconvolved using Huygens Professional (see 2.5.3.1). Restored images were saved in .r3d format before being prepared in Fiji (see 2.5.3.2) image processing software.

2.5.3 Image Processing

2.5.3.1 Huygens Professional

Z-stack images were restored using Huygens Deconvolution software. Parameters such as numerical aperture (NA) of the lens (40x = 1.3 NA), emission values (nm) for fluorescent channels (Cy5 = 666, TRITC = 576, FITC = 525 and DAPI = 470) and refractive index (RI) for the lens oil immersion (RI = 1.522) and Mowiol® mounting medium (RI = 1.42) were set. The Deconvolution Wizard was then used to batch process images using the parameters set previously.

2.5.3.2 Fiji Image Processing Software

Deconvolved Z-stacks were processed in Fiji by firstly generating a Maximum Intensity projection from each stack. This converts each Z-stack into a single image of whose pixels contain the maximum intensity value across all slices within the original stack. Threshold values for each channel

were optimised for the control images. These values were then used to threshold all other images. Images were then saved in a .tif or .jpg format. For co-localisation analysis, processed images were analysed using a macro (see 7.1) which generates plots to show the degree of co-localisation between pixels in two different channels within the same image.

2.6 Harvesting Cellular Extracts

2.6.1 Preparation of Whole Cell Extracts

Cells grown to 70-90 % confluency in CELLSTAR® 12-well plates (Greiner, #665180) were exposed to 180 mJ·cm² of 254 nm UVC radiation (Vilber Lourmat; BLX 254) before being returned to an incubator for 6 hours (humidified atmosphere, 5 % CO₂ and 37 °C). After 6 hours, the media in each well was discarded and the cells were gently washed with 1X PBS (Gibco, Invitrogen). The wash volume was removed, and cells scraped into ice-cold 1X PBS. Cells were pelleted at 500 x g for 5 minutes at 4 °C, supernatant removed, and cell pellet re-suspended in 10-50 µl NP40 buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl (Fisher), 0.5 mM EDTA, 0.5 % NP-40 (Roche)), supplemented with 100 µM Phenylmethylsulfonyl fluoride (PMSF), 1 µg·ml⁻¹ 1:1:1:1 Chymostatin-Leupeptin-Antipain-Pepstatin A (CLAP), 2 mM β-glycerophosphate, 20 mM NaV, 2 mM MgCl₂ (BDH), and 4 units·ml⁻¹ DNase (Quanta Biosciences). Samples were left to lyse for 30 minutes with interim re-suspension, before performing a Bradford assay (see 2.7) to determine Protein concentration.

2.6.2 Mitochondrial Isolation

Cells grown to 80-90 % confluency in 150 mm² petri dishes (Sarstedt, #83.3903) were washed and scraped into ice cold 1X PBS (Gibco, Invitrogen). Cells were pelleted at 300 x g for 3 minutes at 4 °C, supernatant removed, and cells re-suspended in 1 ml extraction buffer (1.5 mM Mannitol, 0.5 mM mM Sucrose, 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5) supplemented with 100 µM PMSF, 1 µg·ml⁻¹ CLAP cocktail, 20mM NaV, 2 mM β-glycerophosphate, 2 mM MgCl₂, and 4 units·ml⁻¹ DNase (Quanta Biosciences). Suspension

was homogenised in a glass homogeniser (Teflon) and transferred to a fresh eppendorf tube. Lysed cells were centrifuged at 1000 x g, for 5 minutes at 4 °C. The supernatant was transferred to a fresh tube and the remaining nuclear pellet discarded. The supernatant was then centrifuged at 1000 x g, for 5 minutes at 4 °C, removing any residual nuclear pellet. The supernatant was transferred to a fresh tube before undergoing centrifugation at 10,000 x g, for 15 minutes at 4 °C to pellet the mitochondria. The supernatant was discarded. The mitochondrial pellet was re-suspended in 500 µl extraction buffer and spun again to increase mitochondrial purity. This step was repeated up to three times before the pellet was resuspended in 20-200 µl Locke's buffer (154 mM NaCl, 5.6 mM KCl (BDH), 2.3 mM CaCl₂ (Fischer), 1 mM MgCl₂, 3.6 mM NaHCO₃, 5 mM Glucose and 5 mM HEPES pH 7.5 (BDH)) for analysis via metabolism assays (see 2.9). Protein concentration was established using a Bradford assay (see 2.7).

2.7 Bradford Assay

Aliquots of 1-5 µl of harvested cell extracts were transferred to eppendorf tubes before being mixed with 0.5 ml sterile distilled water (SDW) and incubated for 10 minutes at RT. 0.5 ml Bradford reagent (B6916) was then added and the assay mixture left to incubate for a further 10 minutes at RT. Protein concentration of each sample was determined by measuring their absorbance at 595 nm. These values were then compared to a standard curve previously determined using 0, 1, 2.5, 5, 7.5, 10, 15, 20 and 25 µg·ul⁻¹ of BSA (A9418).

2.8 SDS-PAGE & Western Blotting

2.8.1 SDS-PAGE

Protein samples were prepared with 5X SDS sample buffer (250 mM Tris pH 6.8, 10 % Sodium Dodecyl Sulphate (SDS), 0.5 % Bromophenol blue, 50 % glycerol, 500 mM β-mercaptoethanol) and boiled on a Dri-Block® heater (Techne, #FDB02A) for 10 minutes at 90 °C. Samples were loaded onto 12 % acrylamide SDS-PAGE gels. Gels were run at 120 V for 20

minutes followed by 200 V for 40 minutes, in 1X SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS).

2.8.2 Western Blotting

SDS-page gels were transferred onto a 0.22 µM nitrocellulose membrane (BIOTRACE, PALL life sciences) by a wet transfer system in 1X transfer buffer (24 mM Tris, 195 mM glycine, 0.1% SDS, 10% methanol (VWR BDH, #20847.307) at 350 mA for 1.5 hours. Membranes were then blocked with 5% non-fat milk (Marvel, in 1X PBS + 0.1% Tween 20) for 1 hour at RT. Membranes were incubated with appropriate primary antibody (see 2.12) overnight at 4°C, then washed three times with 1X TBST (Tris Buffered Saline (TBS; 0.5 M Tris-HCl + 1.5 M NaCl) + 0.1 % Tween 20). Membranes were then incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody (see 2.12) in 5% non-fat milk for 1 hour at RT. Amersham ECL Western Blotting Detection Reagent (GE Healthcare, #RPN2106) was then added before exposure to detection film (Roche).

2.8.2.1 Semi-quantitative Analysis

Western blots were quantified using Fiji image processing software. The pixel intensity values for each protein band were measured, and values for control and treated samples were grouped together. The intensity values for each sample and protein within each group were then expressed as a percentage of the loading control for that group. Calculated values were then expressed as a percentage of the average of the same protein in the control samples. The final values were then determined by expressing all percentages as decimals and transforming those values as a function of log2. This process is summarised below.

$$\text{normalised abundance} = \frac{\text{sample intensity}}{\text{sample group loading control average intensity}}$$

$$\text{normalised relative abundance} = \frac{\text{normalised abundance}}{\text{ctrl sample group average normalised abundance}}$$

$$\text{fold change} = \log_2 (\text{normalised relative abundance})$$

2.9 Mitochondrial Metabolism Assay

20 µg of isolated mitochondria from HeLa cell lines over-expressing GFP or SvnGFP were re-suspended in Locke's buffer (154 mM NaCl, 5.6 mM KCl (BDH), 2.3 mM CaCl₂ (Fischer), 1 mM MgCl₂, 3.6 mM NaHCO₃, 5 mM Glucose and 5 mM HEPES pH 7.5 (BDH)), following extraction via mitochondrial isolation (see 2.6.2) and plated into triplicate wells of a CELLSTAR® black 96-well polystyrene µClear® plate (Greiner, #655090). Resazurin (R7017) was added to each sample at a final concentration of 20 µM. Plates were then incubated with 5 % CO₂ at 37 °C. The absorbance of resorufin, generated following reduction of resazurin, was read using a spectrofluorometer (FLUOstar Galaxy, BMG Labtech) at 595 nm every 30 minutes for 4 hours, and compared to blank controls.

2.10 Real-Time Quantitative Polymerase Chain Reaction (qPCR)

2.10.1 qPCR Primers

Table 2 – qPCR primers, primer sequences for reference genes and genes of interest.

	Gene	Forward/Reverse	Sequence
Reference Gene	ACTB	F	GCAGAAAACAAGATGAGATTGGC
		R	TGTGAACTTTGGGGGATGCT
	GAPDH	F	CAACAGCCTCAAGATCATCAGC
		R	TGGCATGGACTGTGGTCATGAG
	EEF1A1	F	GGACACGTAGATTCGGGCAA
		R	CACGTTACGCTCAGCTTTC
	TUBB	F	ACTTTGTATTTGGTCAGTCT
		R	TTGCTGATAAGGAGAGTGCCC
Gene of Interest	PMAIP1	F	ACTGTTCGTGTTTCAGCTCGC
		R	GAGTAGCACACTCGACTTCCA
	BIRC4	F	GTGTCTGGTAAGAACTACTGAGA
		R	CCTTGAACTGAACCCCATTCG

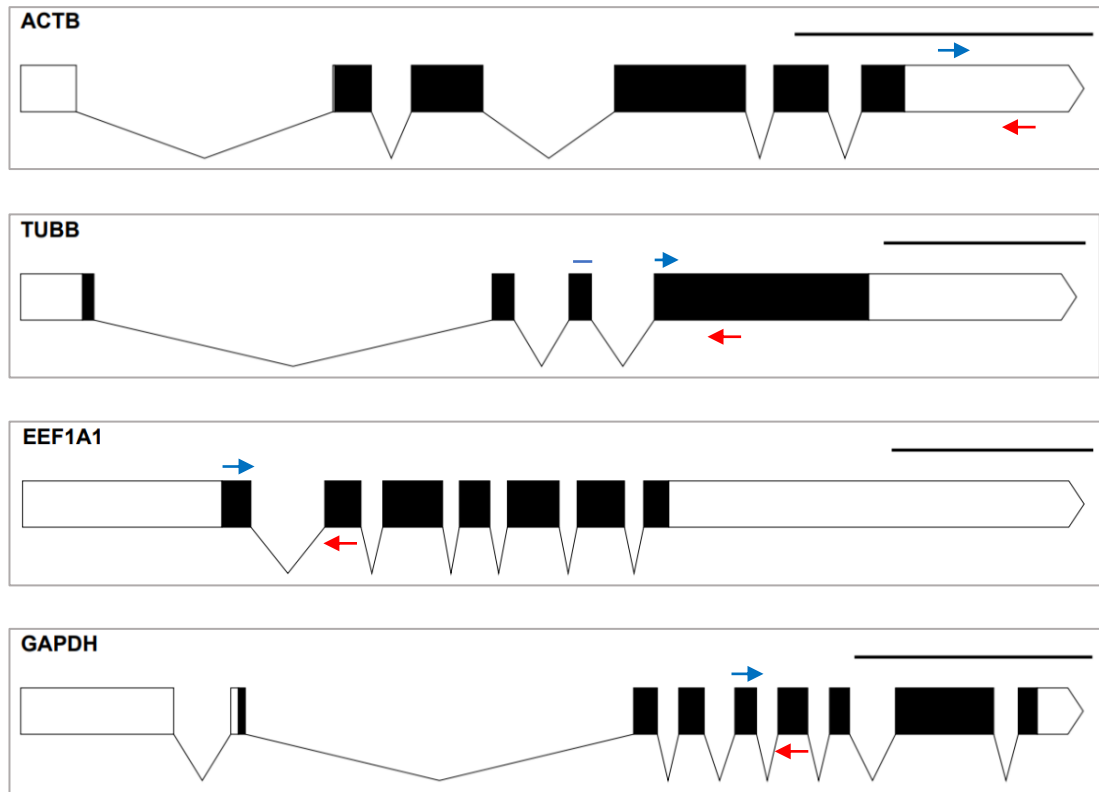


Figure 10 – Reference gene structures and PCR amplicons. Genes are positioned 5'-3' with black regions as exons, joining lines as introns, and white regions as untranslated regions (UTR). Blue and Red arrows represent forward and reverse primers, respectively. Scale bars are 1000 bp. Primer amplicons for all other genes can be found in the appendix (see 7.2.4.1).

2.10.2 Comparative qPCR

qPCR was performed using iTaq™ Universal SYBR® Green Supermix (BIORAD) as per the manufacturer's instructions on a qPCR thermocycler (7500 fast real-time qPCR, Applied Biosciences). Complementary DNA (cDNA) final concentration was tailored to each gene's expression, typically $83.33 \text{ pg} \cdot \mu\text{l}^{-1}$, however increased to $333.33 \text{ pg} \cdot \mu\text{l}^{-1}$ for low abundance messenger RNA (mRNA) with 250 nM of primers. Data was analysed, and reference genes verified using the Pfaffl method (Pfaffl, 2001) and REST (Pfaffl, 2002) software (QIAGEN).

2.11 Statistical Analysis

2.11.1 Regression analysis

Statistical method that examines the relationship between two or more variables (i.e. UV dosage and cell type). Fits the data to a non-linear or

linear model depending on the type of data. linear regression analysis provided information for any statistical differences between the slopes and/or intercepts of data for HeLa GFP and SvnGFP cell lines.

2.11.2 Two-way ANOVA

Determines how a response is affected by two factors. Correct for multiple comparisons using statistical hypothesis testing: Turkey (within each column, compare rows) and Sidak (compare each cell mean within each mean in that row).

2.12 Antibody Specifications

Table 3 – Antibody and molecular stain specifications and sources

Specificity	Host Species	Technique	Dilution	Media	Source
Antibodies					
α-Tubulin (TUBB)	Mouse	WB	1:2000	5% Milk TBST	Sigma T5168
BCL2	Mouse	WB	1:500	5% Milk TBST	Abcam ab692
CYC	Rabbit	IF	1:1000	10% Goat Serum	Abcam ab110417
DRP1	Rabbit	WB	1:1000	5% BSA TBST	CST D6C7
FIS1	Rabbit	WB	1:250	5% Milk TBST	Sigma HPA017430
GFP	Rabbit	WB	1:500	5% Milk TBST	Homemade (Wheatley Lab)
TATA Binding Protein (TBP)	Mouse	WB	1:2000	5% Milk TBST	CST #8515S
VDAC	Rabbit	WB	1:1000	5% Milk TBST	CST #4661S (D73D12)
Rabbit HRP conjugated	Goat	WB	1:1000	5% Milk TBST	Dako (PO217)
Mouse HRP conjugated	Rabbit	WB	1:1000	5% Milk TBST	Dako (PO260)
Texas Red	Goat	IF	1:1000	PBA Buffer	Vector Labs TI-100
Cy5	Goat	IF	1:1000	PBA Buffer	Vector Labs CY-1500
Molecular Stains					
DAPI	N/A	IF	1:500	PBA Buffer	
MTR	N/A	IF	1:2000	DMEM (D6429)	Invitrogen™ (M22425)
MTDR	N/A	IF	1:3000	DMEM (D6429)	Invitrogen™ (M22426)

2.14 Online Resources

Table 3 – Summary of online databases and resources used

Resource	Use	Website
PubMed	Protein/gene literature search engine	https://www.ncbi.nlm.nih.gov/pubmed/
UniProt	Protein database	https://www.uniprot.org/
Addgene	Plasmid repository	https://www.addgene.org/
GeneCards	Protein/gene database	https://www.genecards.org/
WormWeb	Exon/intron gene maps	http://wormweb.org/exonintron

CHAPTER 3: Results

3.1 UVC dosage response of HeLa cells ectopically expressing Svn

In order to determine a suitable UV treatment dose for proceeding experimentation, a dose response experiment was performed testing a variety of dosages, ranging from 0.001 to 5.0 J·cm², with analysis at 24 hours post-treatment using resazurin reagent (see 2.3). Data points were corrected by subtracting the mean control value, determined based on calculated standard deviation data, and appropriate outliers were removed (outliers ≥ 1.5 x interquartile range). The results were fitted to a sigmoidal curve of percentage cell viability (%) against log [UVC], J·cm² (Figure 11). The data generated indicate no significant differences between the response of GFP- and SvnGFP-overexpressing HeLa cells upon exposure to varying amounts of UVC radiation. This was tested by performing non-linear regression analysis which determined that a single curve adequately fit both data sets. The results of the non-linear fit yielded R² values of 0.8236 for GFP and 0.8623 for SvnGFP.

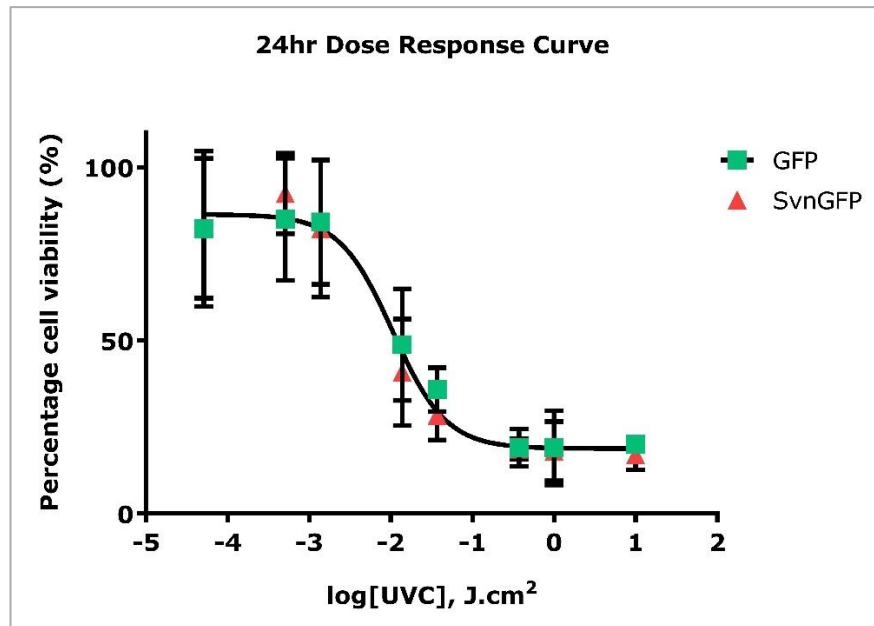


Figure 11 – 24-hour dose-response of HeLa cell lines to UVC treatment. Triplicates of HeLa GFP (green) and SvnGFP (red) cell lines were subject to varying dosages of UVC radiation (0.001 to 5.0 J·cm²). Resazurin assay samples were analysed after 24 hours using a spectrophotometer (595 nm). Results presented as percentage cell viability (%). Non-linear regression analysis determined that a single curve adequately fit both data set. Results of the non-linear fit yielded R² values of 0.8236 and 0.8623 for GFP and SvnGFP data sets, respectively. (N=9; n=3).

3.2 Mitochondrial metabolism of HeLa cell lines

Although the dose response data indicated that there was no significant difference in the response of GFP- and SvnGFP-overexpressing HeLa cells, previous research has indicated that survivin alters mitochondrial metabolism (Hagenbuchner *et al.*, 2013; Rivadeneira *et al.*, 2015). This is important because the resazurin reagent used to determine cell viability in the dose response experiment relies on properly functioning metabolism to allow for its efficient reduction to resorufin. Furthermore, when comparing between cell lines, it is assumed that both have similar metabolic activities. Therefore, another experiment was performed to identify differences in the metabolisms of GFP- and SvnGFP-overexpressing HeLa cells (Figure 12). The results indicate that those cells over-expressing SvnGFP have a notably reduced metabolic capability compared to the GFP expressing cells.

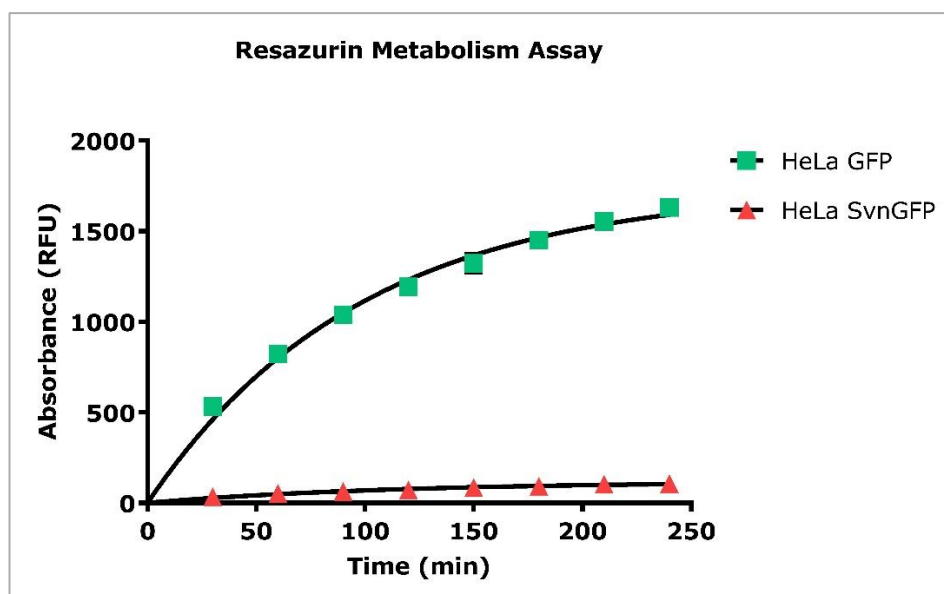


Figure 12 – Over-expression of SvnGFP in HeLa cells results in diminished mitochondrial metabolism. Mitochondria-enriched fractions of HeLa GFP (green) and SvnGFP (red) cell lines were harvested via subcellular fractionation. Samples were aliquoted (20 μ g) in triplicate and incubated with 20 μ M resazurin reagent for 4 hours, with samples being analysed every 30 minutes. Results presented as absorbance in relative fluorescence units (RFU). (N=3; n=1).

3.3 Response to UVC irradiation: apoptosis morphology markers

With the knowledge that Svn does indeed alter mitochondrial metabolism, it was then important to investigate whether Svn could alter mitochondrial derived apoptosis stimulation. However, first it was necessary to identify and document the changes incurred in cellular morphology during cell death. Therefore, an imaging experiment was performed which looked at specific markers of apoptosis before and after treatment with UVC radiation (Figure 13). These markers included the morphology of the mitochondrial network, the localisation of CYC to the mitochondria or cytoplasm, and nuclear morphology.

Following treatment with 180 mJ·cm² of UVC radiation, HeLa cells expressing GFP were incubated for 0, 4, and 8 hours before being stained, with MTDR (mitochondrial stain), and fixed. Cells were prepared for imaging by probing with antibodies against CYC and incubating with a nuclear stain (DAPI).

The observed changes were as follows: increased mitochondrial fragmentation (Figure 13A, B and C), expulsion of CYC from the mitochondria (Figure 13D, E and F), and increased nuclear condensation (Figure 13G, H and I). Fragmentation of the mitochondria is shown by the change in morphology from long, tubular networks to small punctate structures which are dispersed across the cell. Prior to irradiation, the localisation of CYC appears to be co-distributed in a similar manner to that of the mitochondria. However, following treatment the signal for CYC is distributed throughout the cell. Condensation of the nuclei is shown by an increase in the intensity of the DAPI signal as well as a reduction in size. Another observation is that, following treatment, cells appear more rounded and less spread out.

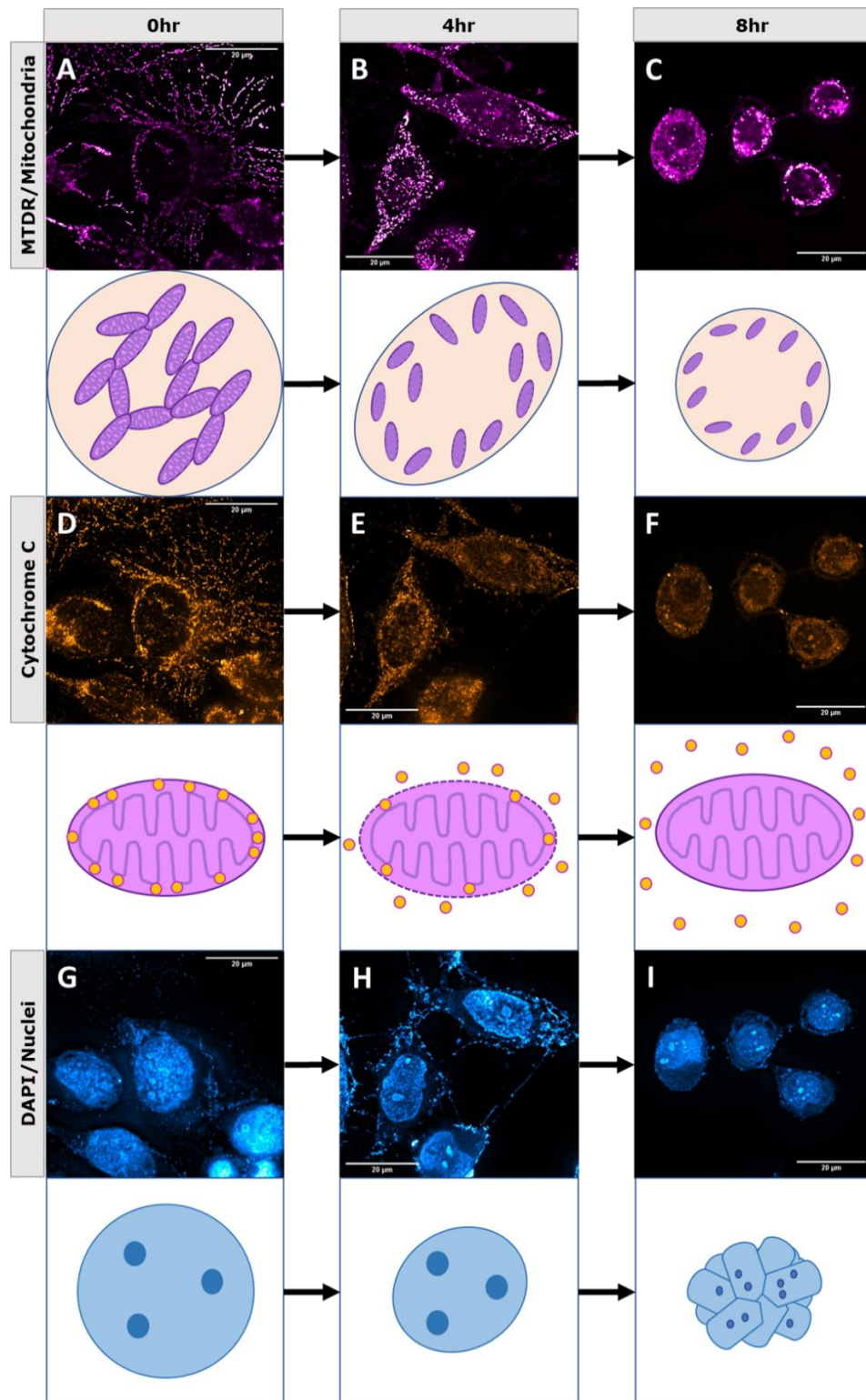
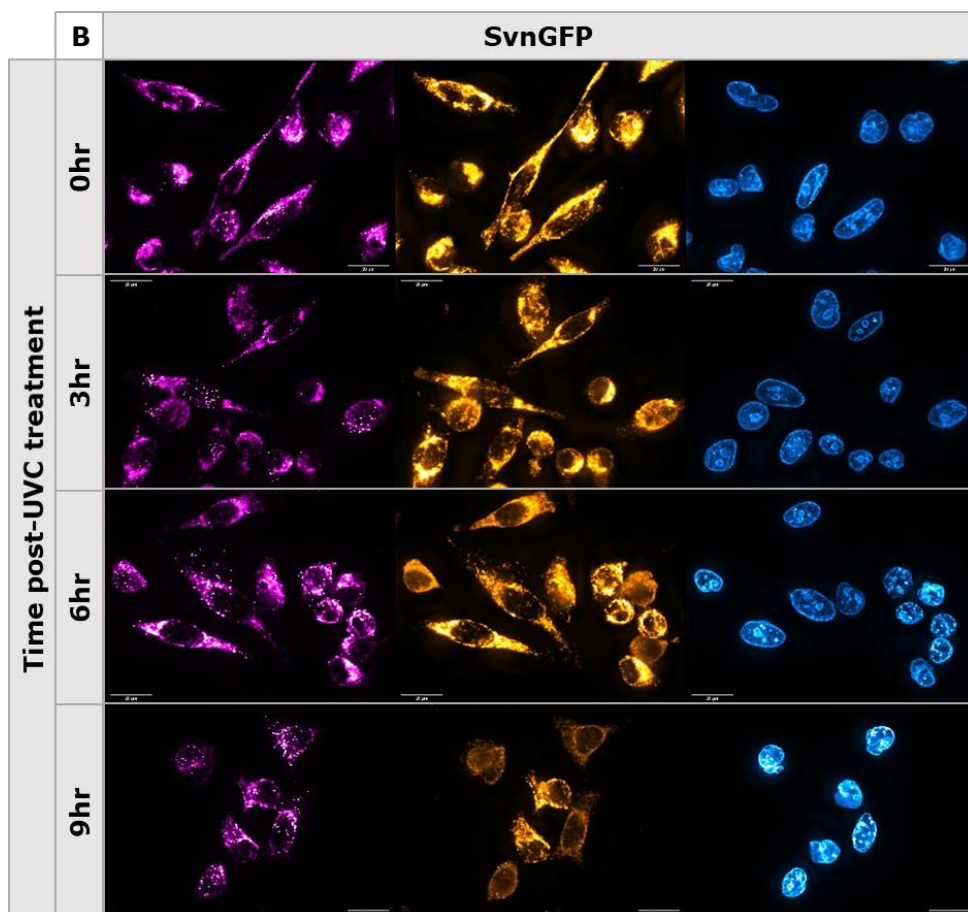
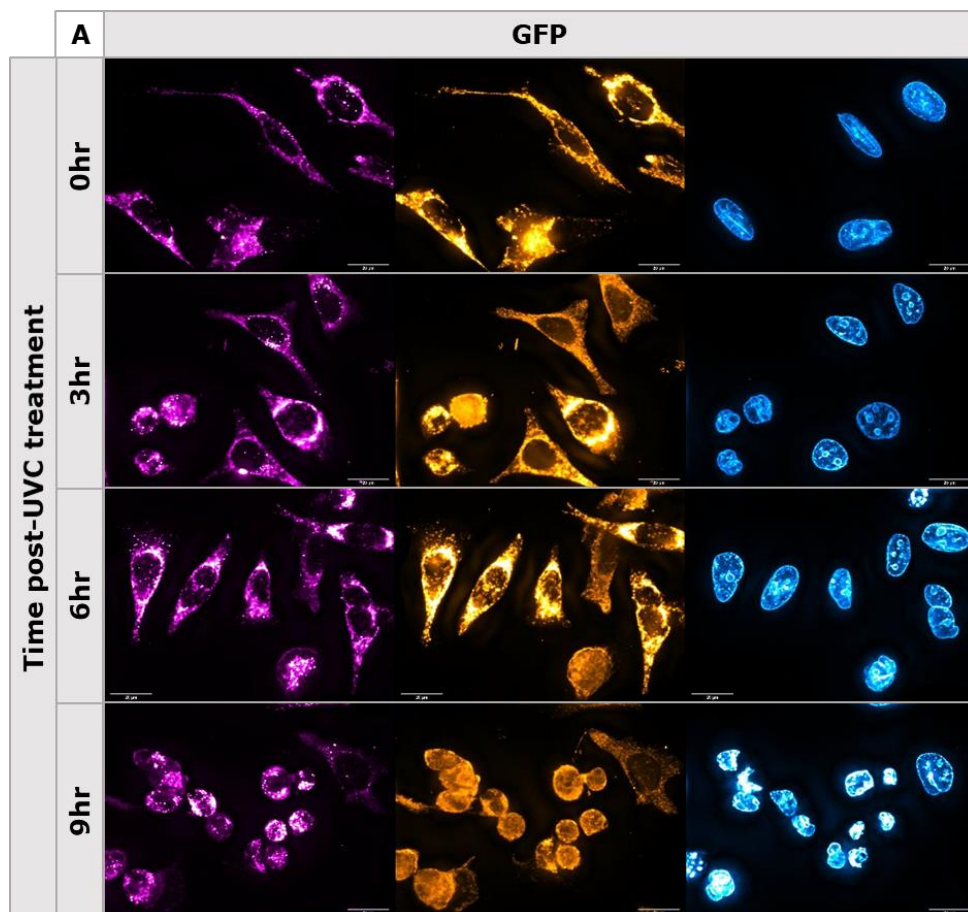


Figure 13 – Morphological markers for apoptosis observed prior to and post-UVC irradiation. Extended mitochondrial network (A) undergoes fragmentation (B and C) following induction of apoptosis. CYC localised to the mitochondrial intermembrane space (D) is released from the mitochondria (E) following MOMP and disperses throughout the cell (F). Activation of caspases initiates transformation of healthy nuclei (G) to those with fragmented and condensed structures (H and I). Mitochondria (purple), CYC (orange), nuclei (blue). Dark blue circles represent nucleoli. Dashed lines represent membrane permeabilisation. Scale bar represents 15 μm .

3.4 Co-localisation of VDAC and CYC in HeLa cells lines

With the knowledge of the morphological changes that occur following exposure to UVC radiation it was decided that one way in which a delay in apoptosis could be identified was through fixing cells at different timepoints following treatment. HeLa GFP and SvnGFP-overexpressing cells were treated with 180 mJ·cm² of UVC (254 nm) radiation and fixed at 0, 3, 6, and 9 hours post-treatment before being imaged (see 2.5.2). Following deconvolution (see 2.5.3) (Figure 14A and B), images were analysed using a macro (created by Alex Fezovich, Wheatley Lab) (see 7.1) that identifies co-localisation between VDAC and CYC. To do this, the macro plots the signal intensity of a pixel in the first channel (VDAC/Cy5) against the signal intensity of the same pixel in the second channel (CYC/TRITC). This is performed for all pixels in the sample image. The macro then attempts to fit a line of best fit to the plot and calculates its R² value. The higher the R², the better the linear fit describes the data points, and therefore the greater the co-localisation between the two channels.

As cells were fixed at a range of timepoints (Figure 14A and B) it was possible to compare the results of the macro (Figure 14C and D) to identify whether the changes in co-localisation between VDAC and CYC, over time, were different between GFP and SvnGFP expressing cells. The results indicate a significant difference between the 9-hour timepoints (Figure 15A), with reduced co-localisation between VDAC and CYC in SvnGFP expressing HeLa cells compared to GFP controls. However, the results of a linear regression analysis revealed no significant difference between the slopes for each data set (Figure 15B).



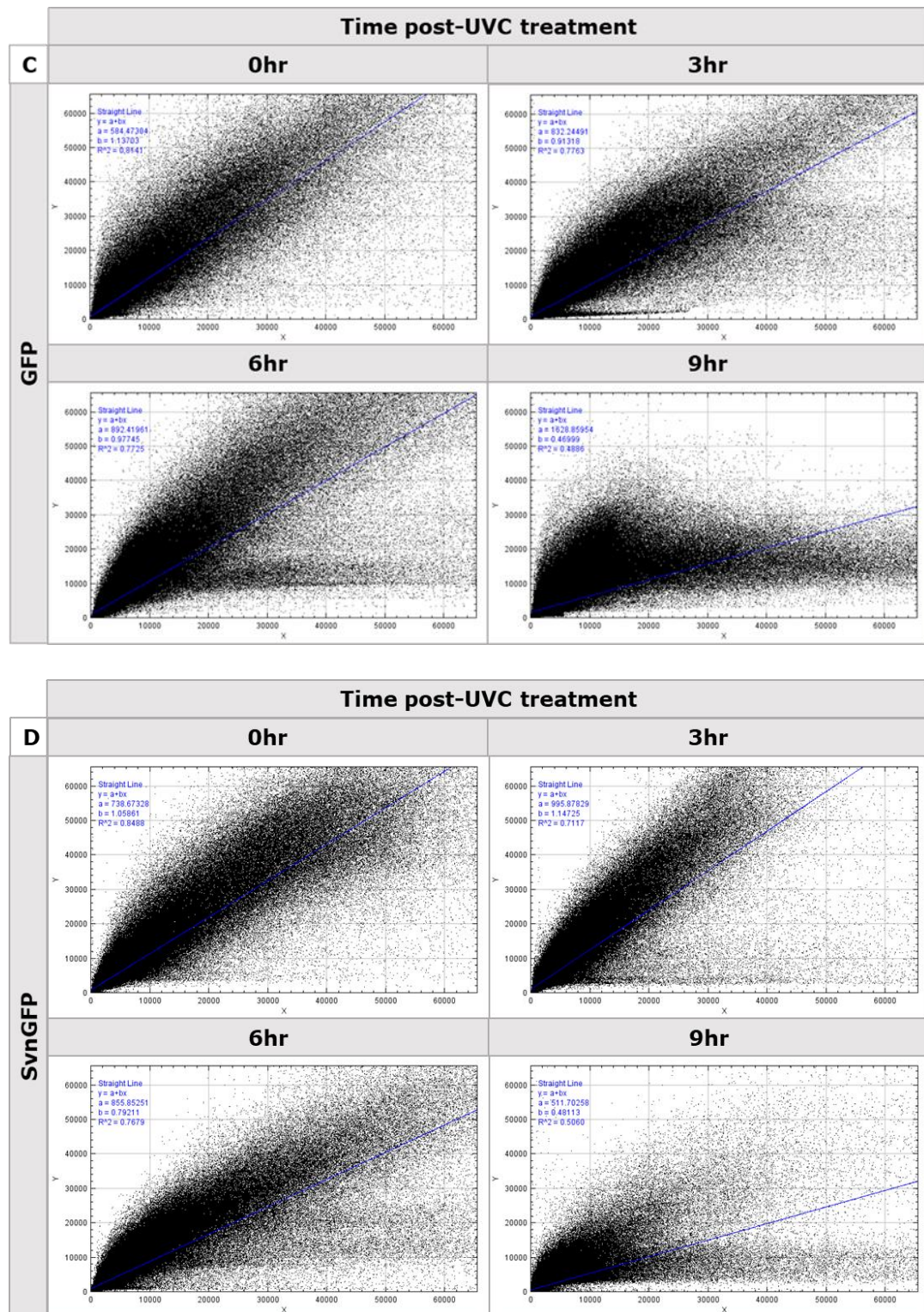


Figure 14 – Microscope images and scatter plots of co-localisation between VDAC and CYC in HeLa GFP and SvnGFP cell lines. *HeLa cells expressing GFP and SvnGFP were treated with 180 mJ·cm² UVC (254 nm) radiation before being fixed every 3 hours over a 9-hour period. Microscope images of GFP (A) and SvnGFP (B) expressing HeLa cells were processed using Huygens deconvolution software before being analysed using a co-localisation macro. Scatter plots for GFP (C) and SvnGFP (D) image samples were generated and R² value for each plot determined. Mitochondria (purple), CYC (orange), nuclei (blue). (N=3; n=1).*

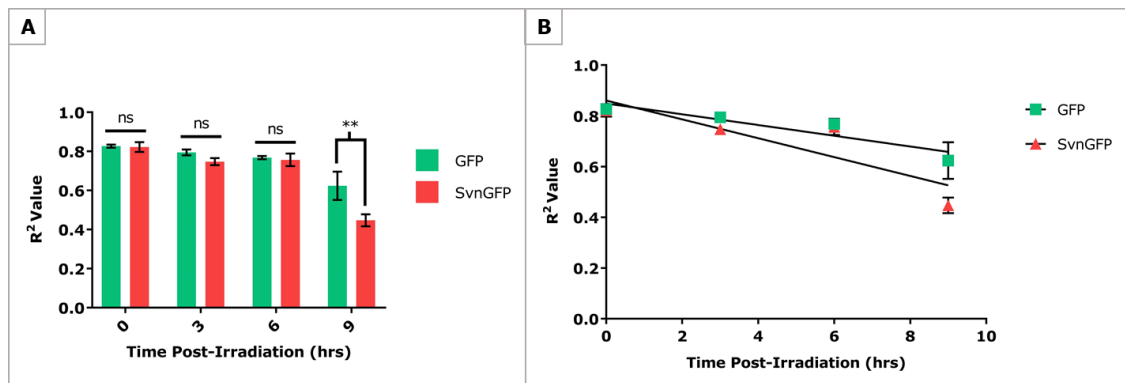


Figure 15 – Statistical analysis of co-localisation data obtained for HeLa cell lines following treatment with UVC radiation. Statistical differences between R^2 values for each timepoint were calculated using (A) two-way ANOVA or (B) linear Regression analysis (ns, not significant; $p < 0.01 = **$). ($N=3$; $n=1$).

3.5 Caspase activity in Svn expressing HeLa cells

As the previous experiment indicated no difference in the co-localisation of VDAC and CYC between GFP and SvnGFP expressing cells, we decided to perform two further experiments to determine if Svn was truly inhibiting apoptosis in our cell lines. These experiments focussed on two events in the apoptosis pathway. These include activation of caspases, following formation of the apoptosome, and exposure of phosphatidylserine at the outer membrane.

The caspase assay detects activation of CASP3 and CASP7 using NucView®; a high-affinity DNA-binding dye which is linked to a caspase substrate. In addition, 7-AAD is used to indicate the structural integrity of the PM; when the PM has lost integrity (i.e. late apoptotic/dead and necrotic cells) 7-AAD permeates the cell membrane and is detected. Samples were treated with 180 mJ·cm² of 245 nm UVC radiation before harvesting via trypsinisation at 0, 3, 6 and 9-hours post-treatment. Samples were analysed on a Muse Cell Analyser. Cells were categorised as either live, early apoptotic, late apoptotic/dead, or necrotic based on the presence or absence of either or both caspase-3/7 and 7-AAD markers (see Figures 16-18).

Following acquisition of the CASP3/7 data, the gating for each sample 'Apoptosis Profile' was altered based on the live cell population in the GFP control (0 hour) samples. This allowed for clear identification of those cell populations in the (early) apoptotic, late apoptotic/dead and dead (necrotic) categories (Figure 16).

Data acquired for the caspase activity assay (Figure 17A and B) was subject to statistical analysis using a two-way ANOVA which compared the differences between the same timepoint for each data set (e.g. 3hr GFP Vs 3hr SvnGFP). This analysis showed that there were significantly more SvnGFP expressing HeLa cells that were still viable at 9 hours post-irradiation than the GFP controls (Figure 17A). At 6 and 9 hours post-UVC treatment, the 'Percentage Gated (%)' of HeLa SvnGFP cells in the 'Late Apoptotic' fraction is significantly less than that of the GFP control cells (Figure 17D). Statistical analysis of the combined early and late apoptotic

fractions also showed a significant difference between the two HeLa cells lines at 3, 6, and 9 hours post-irradiation (Figure 17C).

Linear regression analysis was also performed for the live and combined early-late data. The results of this analysis showed that far more SvnGFP over-expressing HeLa cells were viable 9-hours post-treatment (Figure 18B) and that the total number of apoptotic SvnGFP cells was approximately half that of the GFP only control cells at 9-hours post-treatment (Figure 18D).

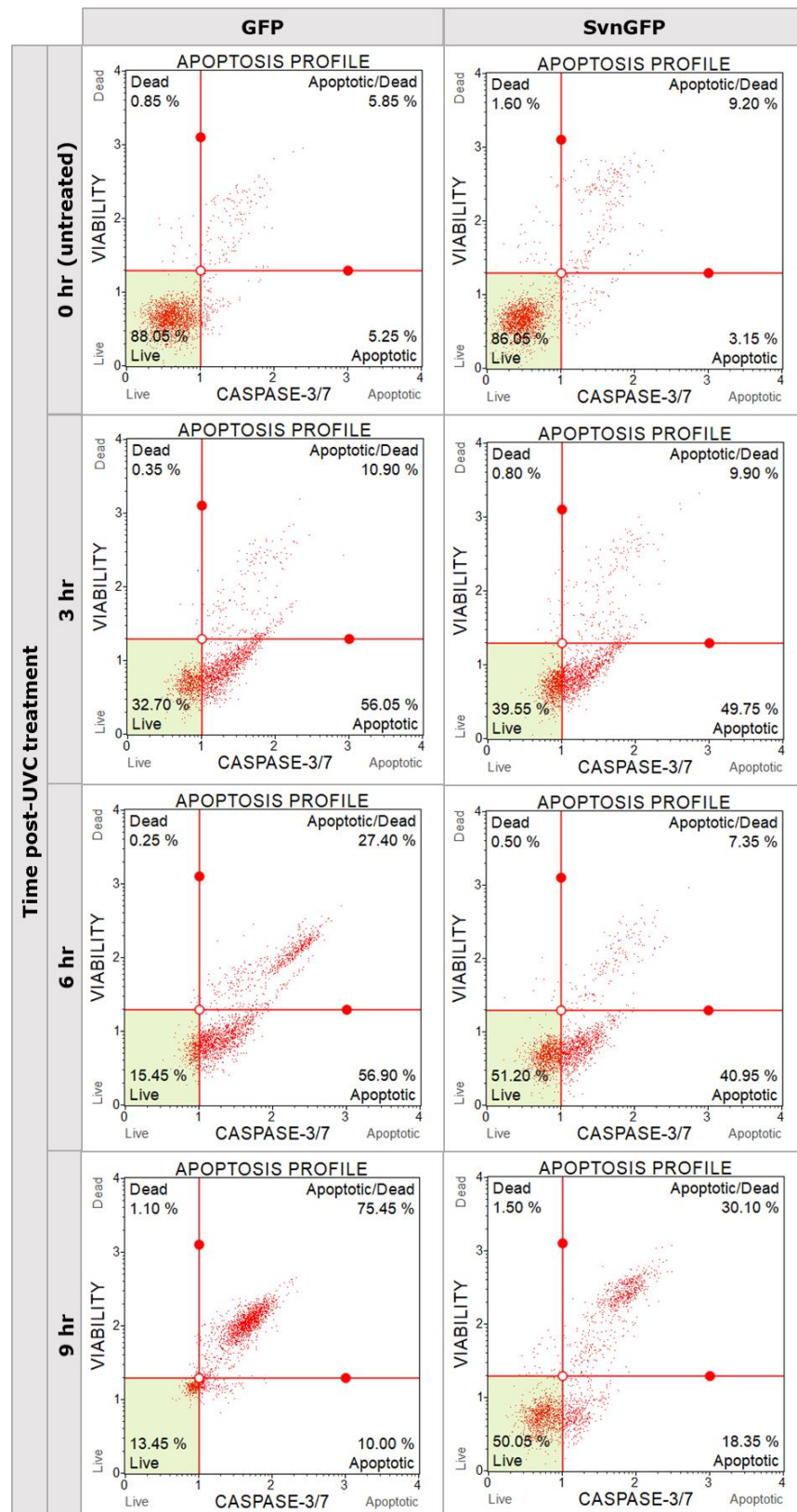


Figure 16 – FACS profiles of Caspase-3/7 assay data for HeLa GFP and SvnGFP cell lines post-UVC treatment. *HeLa GFP and SvnGFP cells lines treated with 180 mJ·cm² UVC (254 nm) radiation and harvested every 3 hours over a 9-hour period. Gates set based on '0 hr (untreated)' GFP controls in order to separate four distinct categories: live, early apoptotic, late apoptotic/dead and dead.*

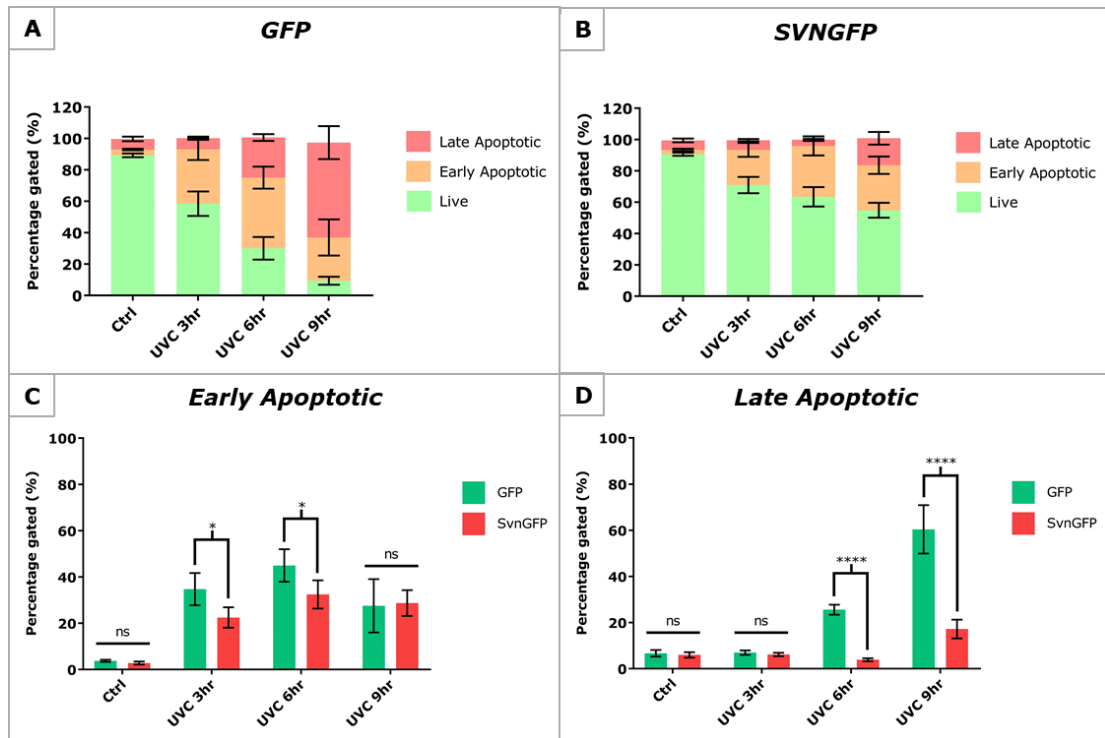


Figure 17 – Combined caspase-3/7 data and analysis of early and late apoptotic events. *HeLa* cells expressing GFP and SvnGFP were treated with 180 mJ·cm² of UVC (254 nm) radiation before being harvested every 3 hours over a 9-hour period. The combined data for 'Live', 'Early' and 'Late' fractions for GFP (A) and SvnGFP (B) expressing *HeLa* cells. Statistical differences for early apoptotic (C) and late apoptotic fractions were calculated using a two-way ANOVA (ns, not significant; $p < 0.05$ = * and $p < 0.0001$ = ****). (N=9; n=3).

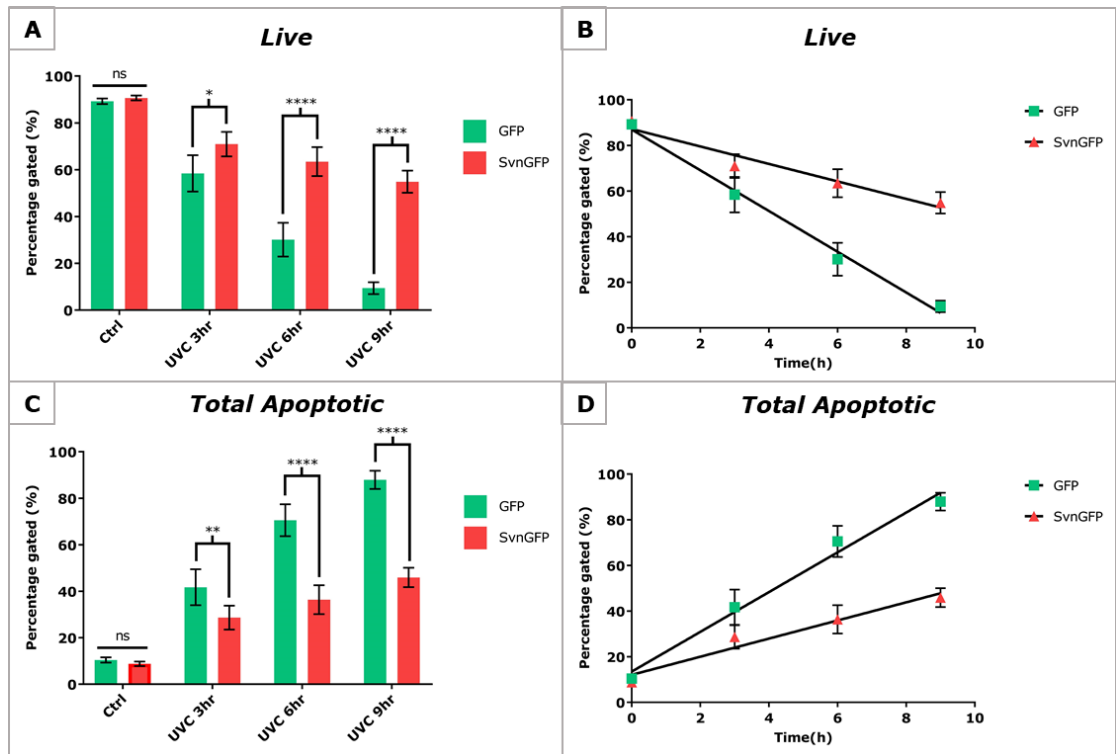


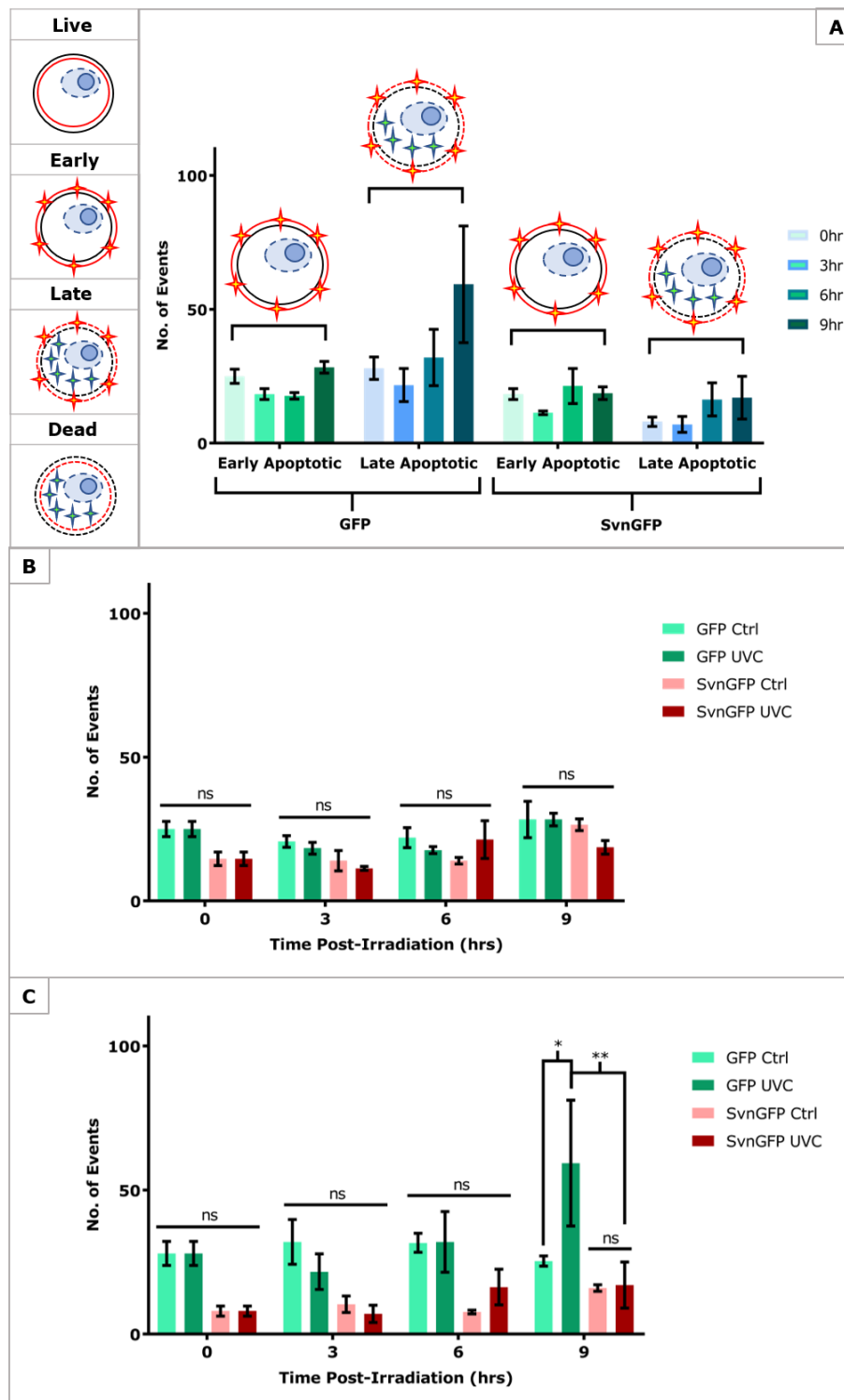
Figure 18 – Caspase-3/7 activity of HeLa GFP and SvnGFP cell lines following treatment with UVC radiation. *HeLa cells expressing GFP and SvnGFP were treated with 180 mJ·cm² of UVC (254 nm) radiation before being harvested every 3 hours over a 9-hour period. Data for (A) 'Live' and (C) 'Total Apoptotic' fractions was subject to statistical analysis using a two-way ANOVA (ns, not significant; $p < 0.05 = *$, $p < 0.01 = **$ and $p < 0.0001 = ****$). linear regression analysis was performed for (B) 'Live' and (D) 'Total Apoptotic' fractions. (N=9; n=3).*

3.6 Phosphatidylserine exposure in Svn expressing HeLa cells

During apoptosis, phosphatidylserine (PS), a lipid found in the body of the PM, is transferred to the outer membrane to act as an “eat-me” signal to local phagocytes. Therefore, exposure of PS to the outer membrane, and subsequent binding of Annexin V, has been used here to identify successful induction of apoptosis (early apoptotic and late apoptotic/dead cells). In addition, uptake of 7-AAD has been used to identify those cells with compromised PM integrity (late apoptotic/dead and dead cells).

Samples were treated with 180 mJ.cm² of 245 nm UVC radiation before harvesting via trypsinisation at 0, 3, 6 and 9-hours post-treatment. Samples were analysed on a Muse Cell Analyser. Cells were categorised as either live, early apoptotic, late apoptotic, or dead based on the presence or absence of either or both Annexin V and 7-AAD markers (Figure 19A).

The results of the Annexin V assay were analysed using a two-way ANOVA which compared the differences in the mean of each data set (i.e. GFP Ctrl Vs GFP UVC, GFP UVC Vs SvnGFP UVC etc.). The data (Figure 19A and B) shows that the number of early apoptotic events is indifferent between HeLa GFP and SvnGFP cells following UVC treatment. However, at 9 hours post-UVC treatment there are significantly more HeLa GFP cells in late apoptosis compared to control cells (Figure 19A and C). Alongside this, the number of HeLa SvnGFP cells in late apoptosis, at 9 hours post-UVC treatment, is significantly less than their HeLa GFP counterparts. There is also no significant difference between UVC-treated HeLa SvnGFP cells and controls cells in early or late apoptosis (Figure 19B and C).



3.7 Expression of mitochondrial & apoptotic proteins in HeLa cells

To begin to uncover the molecular mechanism behind the death delaying activity of survivin, expression of proteins involved in mitochondrial fission (DRP1 and FIS1) and apoptosis (BCL2, AIF1 and VDAC) was analysed, via western blotting. Whole cell extracts for HeLa GFP, SvnGFP and Svn¹⁻¹⁰GFP expressing cells were run on 12% SDS-PAGE gels (see Figure 20A). Loading controls included TUBB and TBP. Membranes were also probed with an anti-GFP antibody to identify correct expression of GFP, SvnGFP and Svn¹⁻¹⁰GFP constructs. Protein bands were analysed in ImageJ by obtaining the pixel intensity of each band and normalising the area under the peak to that of the HeLa GFP control cells. The statistical significance was tested using a Two-Way ANOVA.

Results indicate that DRP1 (Figure 20B) in the HeLa GFP extracts did not change significantly following treatment with UVC radiation. However, it significantly increases in HeLa SvnGFP and Svn¹⁻¹⁰GFP cell extracts when compared to the controls. Of further interest is that the amount of DRP1 present in Svn¹⁻¹⁰GFP control cell extracts is significantly less than that in the HeLa GFP control. There was no significant change in the amount of FIS1 (Figure 20C) between any of the HeLa cell lines following UVC-irradiation.

In control cells expressing GFP, BCL2 (Figure 20D) increases significantly following exposure to UVC. This change is also observed for both SvnGFP and Svn¹⁻¹⁰GFP expressing cell lines, although the apparent increase in BCL2 seen in SvnGFP cells is not significant. There is significantly more AIF1 (Figure 20E) present in SvnGFP cells subject to UVC treatment than in the GFP treated samples. However, this trend is not observed for those cells expressing Svn¹⁻¹⁰GFP. The amount of VDAC (Figure 20F) present in the GFP cells is relatively stable even after treatment with UVC radiation. However, there is significantly less VDAC present in the control samples of SvnGFP and Svn¹⁻¹⁰GFP expressing HeLa cells, when compared to the GFP control. Furthermore, there is a slight increase in VDAC in both SvnGFP and Svn¹⁻¹⁰GFP cell lines following irradiation, but this appears to not be significant.

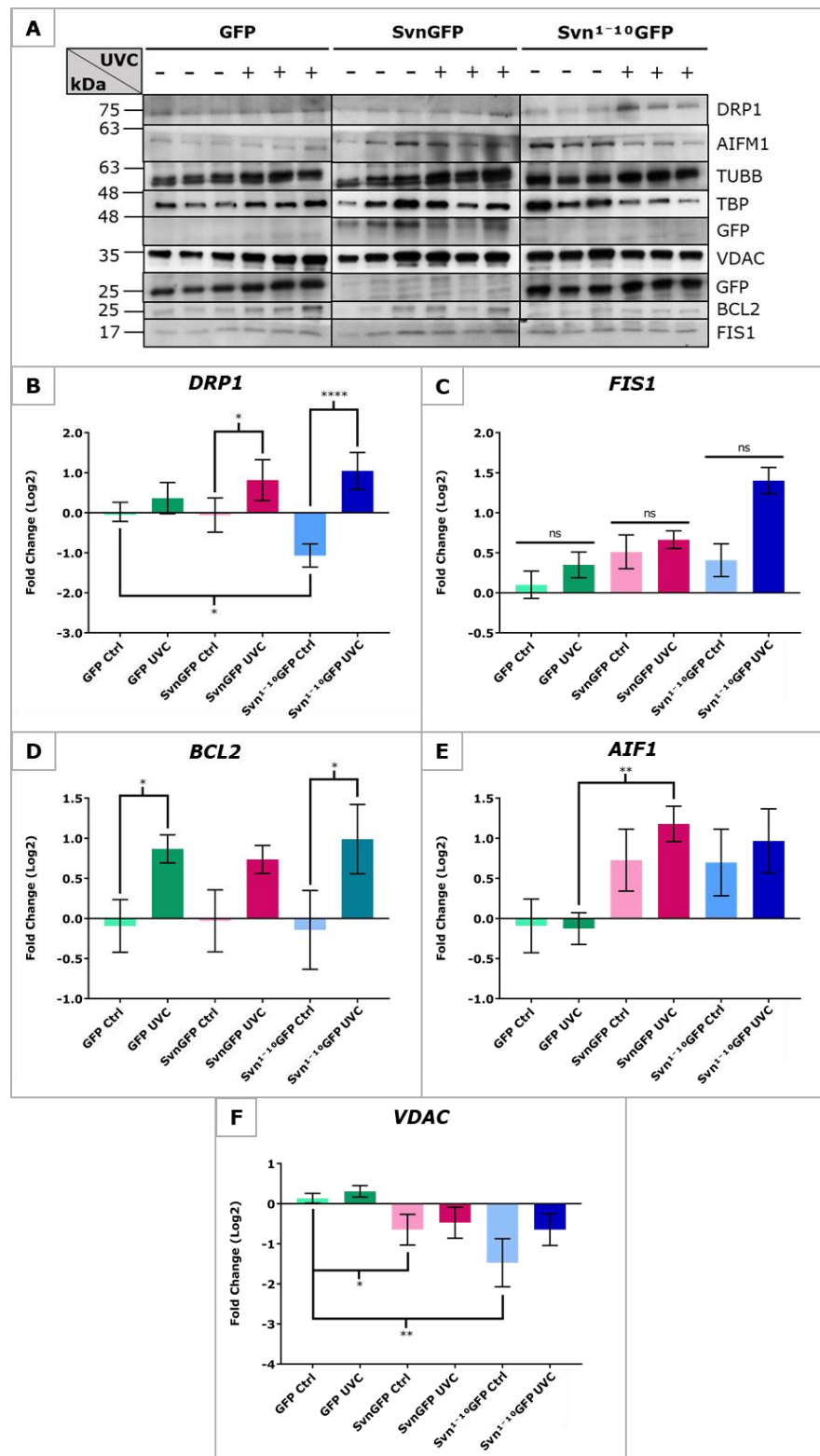


Figure 20 – Western blot analysis of HeLa GFP whole cell extracts (A) Western blot performed for whole cell extracts of HeLa GFP, SvnGFP and Svn¹⁻¹⁰GFP cell lines treated with 180 mJ·cm² UVC (254 nm) radiation. Results were normalised to GFP Ctrl. (B) to (F) show the log fold change in the amount of each protein probed for during western blotting. (B) DRP1. (C) FIS1. (D) BCL2. (E) AIF1. (F) VDAC. Loading controls included TUBB and TBP. Statistical differences were calculated using a two-way ANOVA (ns, not significant; $p < 0.05 = *$, $p < 0.01 = **$ and $p < 0.0001 = ****$). (N=9; n=3).

3.8 Changes in the mitochondrial network of irradiated HeLa cells

DRP1 and FIS1 are both members of the mitochondrial fission machinery and therefore any change in the levels of either of these proteins could affect the structure of the mitochondrial network. It is known that fission is an early event in the intrinsic pathway of apoptosis. From this, it was hypothesised that the changes in DRP1/FIS1 protein levels, revealed through western blotting (Figure 21), could lead to an observed change in mitochondrial morphology and networking. Therefore, a live imaging experiment was performed with GFP and SvnGFP expressing HeLa cells to test this hypothesis. Cells were treated with 180 mJ·cm² of UVC radiation and incubated for 5 hours before imaging on a DeltaVision Elite microscope (see 2.5.1) (Figure 21). Images generated following deconvolution showed no apparent difference in mitochondrial morphology between the two HeLa cell lines.

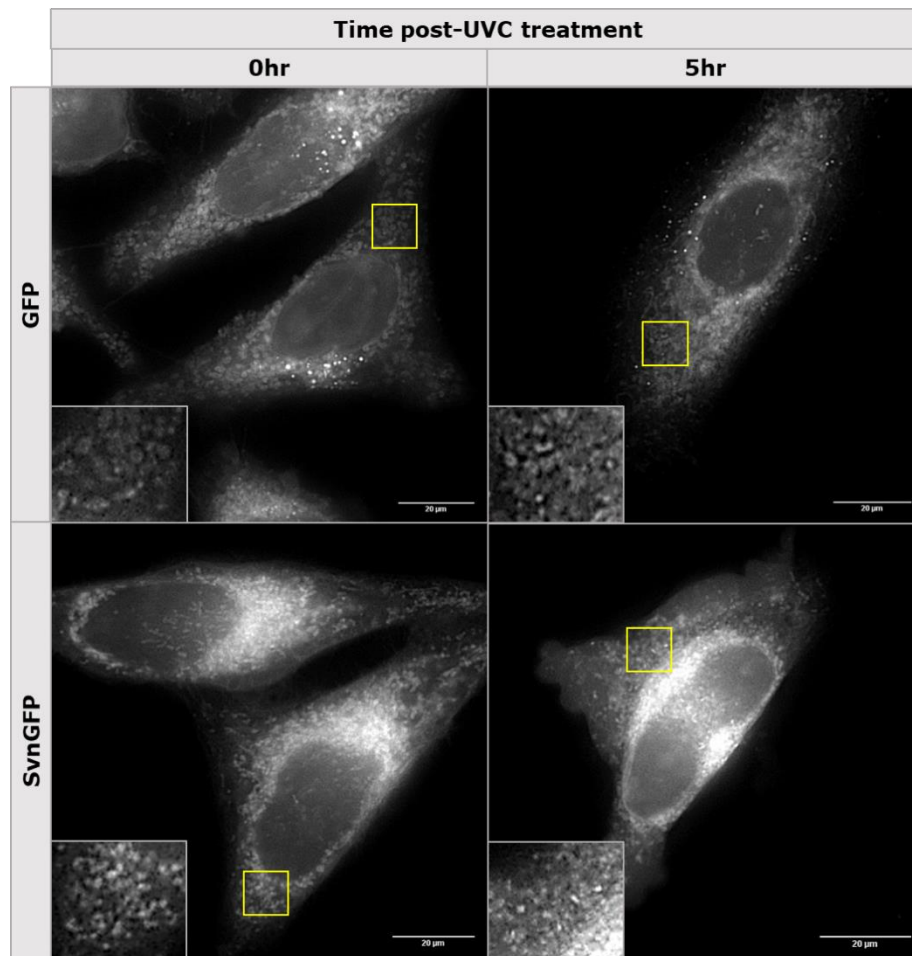


Figure 21 – Mitochondrial morphology before and after treatment of HeLa cells with UVC radiation. *HeLa cells expressing GFP and SvnGFP were treated with 180 mJ·cm² UVC (254 nm) radiation and left to incubate for 5 hours post-treatment. Cells were stained with MTR prior to imaging of the mitochondria. Control cells are denoted as '0hr' post-UVC treatment. (N=3; n=1).*

3.9 qPCR analysis of HeLa cells ectopically expressing Svn

Based on the data gathered from western blot analysis, we decided that further information about the anti-apoptotic effect of Svn could be revealed through qPCR analysis. Due to time constraints, previously optimised primers were used as they were readily available. As there appeared to be no difference in BCL2 between the cell lines, NOXA, an inhibitor of MCL1, was analysed to identify if Svn was affecting the role of this pro-apoptotic protein. XIAP was chosen for analysis as it is widely thought that XIAP complexes with Svn and promotes its anti-apoptotic effect. All data obtained from the qPCR procedure was analysed using analytical techniques integrated into the REST software (see 2.10.2).

We observed a statistically significant increase in the amount of NOXA mRNA in HeLa cells over-expressing SvnGFP when compared to the GFP only control. However, there appeared to be no significant difference in the levels of XIAP mRNA between the two HeLa cell lines.

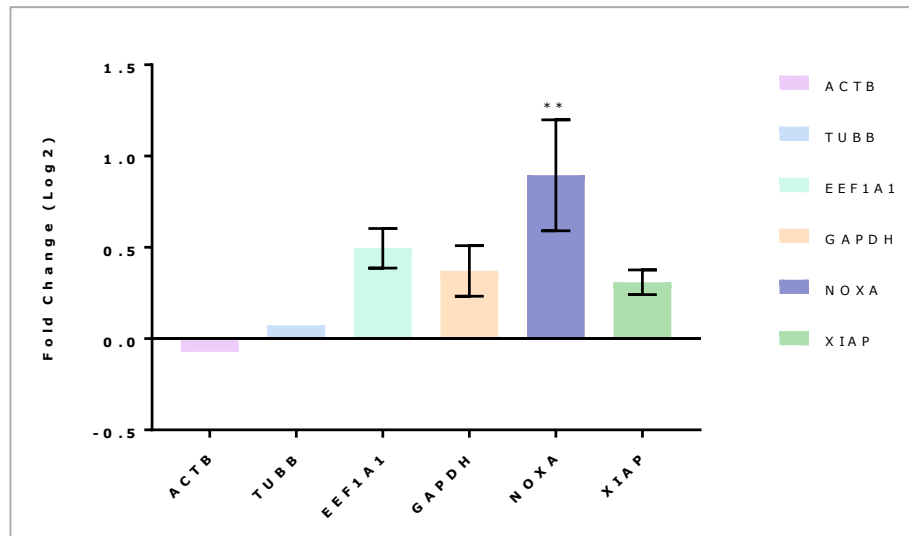


Figure 22 – qPCR analysis of HeLa cell lines. Comparative qPCR analysis was performed with mRNA samples purified from HeLa cells overexpressing either GFP or SvnGFP. Results for NOXA and XIAP were normalised to reference genes ACTB (actin) and TUBB (tubulin). Statistical differences were calculated using randomisation and bootstrapping methods integrated into the REST software ($p < 0.01 = **$). ($N=9$; $n=3$).

CHAPTER 4: Discussion

Previous research has highlighted a role for Svn in tumorigenesis as its overexpression promotes cell proliferation and resistance to apoptosis (Chen *et al.*, 2016; Li, Hu and Li, 2018). Therefore, an area of increased interest is the development of anti-cancer agents that can specifically target Svn and inhibit its tumour-promoting activities (Garg *et al.*, 2016; Rathore *et al.*, 2017). Further to this, it has been observed that removal of the MTS of Svn affects its ability to inhibit cell death processes (Dohi, Beltrami, *et al.*, 2004; Wheatley, 2015; Dunajová *et al.*, 2016), but the exact mechanism behind this is yet to be determined. However, Svn and the mitochondria are both involved in intrinsic apoptosis, and therefore we decided to further investigate their roles in this pathway.

It has been previously documented that UVC radiation, unlike UVA and UVB, induces only the intrinsic (mitochondrial) pathway of apoptosis (Takasawa *et al.*, 2005). Therefore, by studying the response of HeLa cells ectopically expressing Svn to UVC treatment, we hoped to increase our understanding of the role for mitochondrial Svn in apoptosis inhibition, and ultimately provide further insight into its function in cancer.

4.1 Mitochondrial metabolism of Svn expressing HeLa cells affects reduction of resazurin

Although the results of the dose response experiment indicated no significant difference in the response of GFP and SvnGFP expressing HeLa cells, this data may not be entirely accurate. Previous research (Wheatley, 2015) has shown that when exposed to X-irradiation, HeLa cells expressing wild-type SvnGFP were able to promote survival, but control cells expressing just GFP were unable to protect against such apoptotic insults. Hence, the data obtained from the UVC dose response experiment is not consistent with that of previous findings. It is possible that the reason there is no difference observed between the GFP and SvnGFP expressing cell lines to UVC irradiation is that the basis of this assay, resazurin, is affected by altered mitochondrial metabolism, of which Svn has been demonstrated to affect (Townley & Wheatley, unpublished). Resazurin is typically used to monitor cell viability, however a large assumption of this assay is that

cellular metabolism across samples is similar. Resazurin is converted to resorufin in the presence of reducing reactions within the cell. Therefore, if there are differences in the number of viable cells between two samples, then the detected difference in the amount of resorufin will reflect this; greater volume of cells equals greater reduction of resazurin (Perrot *et al.*, 2003). However, differences in mitochondrial metabolism can also mimic this effect. (Aleshin *et al.*, 2015; Uzarski *et al.*, 2017).

In order to clarify if Svn is altering mitochondrial metabolism in our system, we performed a metabolism assay (Hagenbuchner *et al.*, 2013; Rivadeneira *et al.*, 2015). The results of the metabolism assay confirmed that the mitochondria of those cells expressing SvnGFP had reduced metabolic activity. This led to the conclusion that the accuracy of the dose response results may be compromised, and that the true response of cells expressing Svn would be significantly different to that of the GFP controls. Therefore, future dose response experiments may consider using an alternative assay to detect viability that does not depend on mitochondrial metabolism.

4.2 Ectopically expressed Svn delays caspase activation

Activation of the caspase cascade is the result of upstream induction of apoptosis (see 1.6.1). To determine whether ectopic expression of Svn leads to inhibition or delay of intrinsic apoptosis (see 1.6.1.2), activation of caspases is a key step to be studied. Release of CYC, following MOMP, leads to formation of the apoptosome and subsequent activation of CASP9. If these preparative steps have occurred successfully, CASP9 then activates CASP3 and CASP7, which are key for the execution phase of apoptosis. Therefore, we next studied the activity of these two executioner caspases by flow cytometry.

The results of the caspase assay revealed that overexpression of SvnGFP by HeLa cells promotes survival by delaying, rather than inhibiting, activation of the executioner caspases. This was confirmed by the presence of some SvnGFP expressing cells in both the early and late stages of apoptosis. Interestingly, although there were fewer SvnGFP expressing cells in apoptosis, compared to the GFP controls, there was little difference

between the early apoptotic fractions of the two HeLa cell lines. This may suggest that Svn may also be promoting survival of cells even after apoptosis has been triggered, which could explain why there is no significant difference between the percentage of GFP and SvnGFP cells in early apoptosis but also why there is then a significant discrepancy between the two cell lines during late stage apoptosis.

Furthermore, by calculating the percentage of total apoptotic cells that were late apoptotic/dead (Figure 23), it was found that there were significantly fewer SvnGFP expressing HeLa cells that died as a result of induction of apoptosis when compared to the GFP controls.

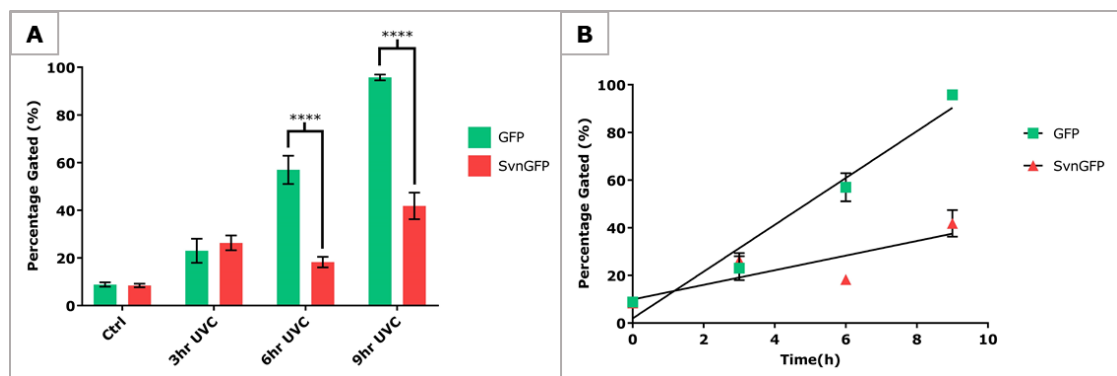


Figure 23 – Percentage of total apoptotic cells that were late apoptotic/dead. HeLa cells expressing GFP and SvnGFP were treated with 180 mJ·cm² of UVC (254 nm) radiation before being harvested every 3 hours over a 9-hour period. Data for late apoptotic/dead fractions (Figure 17) were normalised against the total number of apoptotic cells (Figure 18). Statistical differences were calculated using (A) two-way ANOVA or (B) linear regression analysis ($p < 0.0001$ = ****). (N=9; n=3).

This analysis further supports the hypothesis that Svn is promoting survival of cells even after apoptosis induction. In fact, recent research has revealed a possible new pathway that results in the recovery of cells from apoptosis. This pathway is known as “Anastasis”, meaning ‘rising to life’ in Greek (Tang and Tang, 2018). Interesting, of the proteins believed to be involved in executing anastasis, sequestosome 1 (SQSTM1), autophagy-related protein Atg12 (ATG12), HSP90 and HSP27 (Sun *et al.*, 2017; Tang *et al.*, 2017) have all been documented to either interact directly with Svn or to be indirectly regulated by Svn activity. For example, Svn has been found to bind to SQSTM1 (Chan *et al.*, 2017; Huang *et al.*, 2017) leading to alterations in autophagic flux. Furthermore, Svn has been shown to not

only inhibit excessive autophagy (Humphry and Wheatley, 2018), but also to repress autophagosome elongation by negatively regulating formation of ATG12 conjugates with autophagy-related protein Atg5 (Chan *et al.*, 2017; Huang *et al.*, 2017). As aforementioned (see 1.8.3), it is hypothesised that the interaction of Svn with HSP90 is required for its localisation to the mitochondria, as inhibition of HSP90 or disruption of the Svn-HSP90 complex results in degradation of Svn by the proteasome and apoptosis via the mitochondrial (intrinsic) pathway (Fortugno *et al.*, 2003). Finally, research has shown that co-localisation of Svn with HSP25/27 results in inhibition of active CASP3 (Villapol *et al.*, 2008).

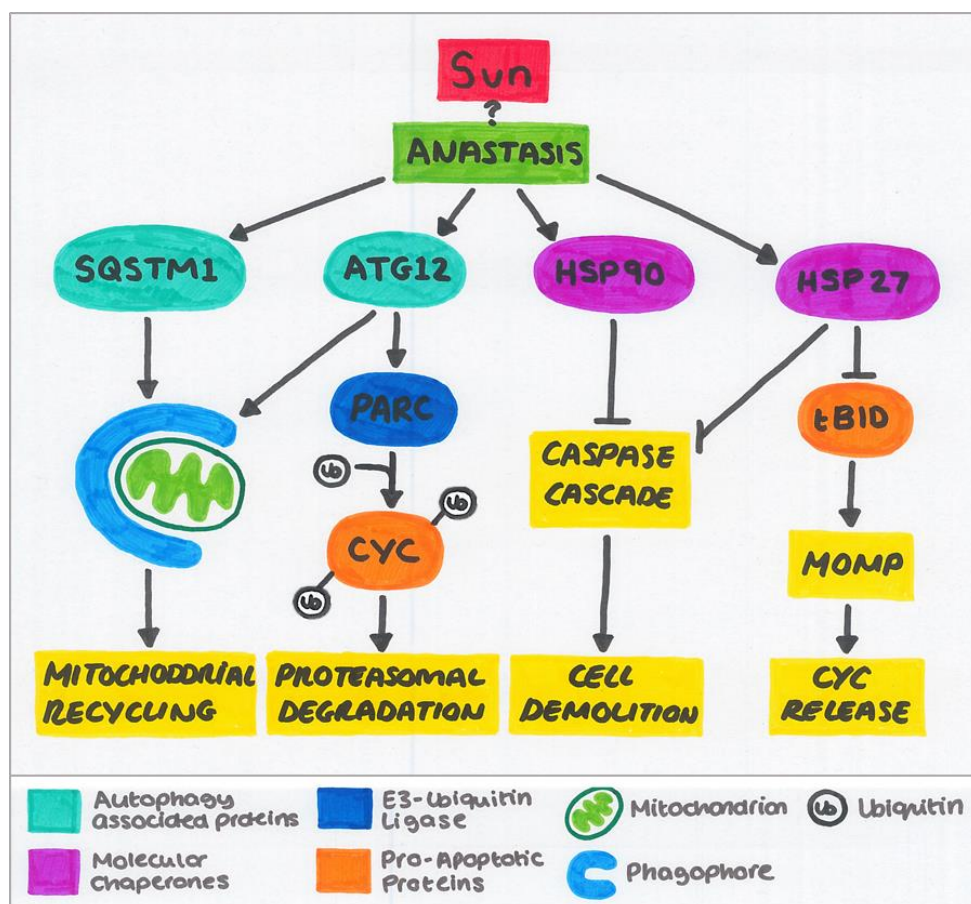


Figure 24 – Theoretical model for Svn-mediated anastasis. Ectopic expression of Svn appears to be aiding in cell revival following entry into apoptosis. Anastasis is the currently understood mechanism by which dying cells return to their original state prior to induction of cell death. Research has indicated that Svn can interact with several proteins that are thought to regulate anastasis including SQSTM1, ATG12, HSP90 and HSP27, therefore suggesting that Svn may be utilising this pathway to avoid cell death completion. Arrowed lines indicate activation steps. Flat-headed lines indicate inhibitory steps. Drawn by author (adapted from Tang and Tang, 2018).

In anastatic cells, SQSTM1 and ATG12 are believed to promote recycling of damaged mitochondria following expulsion of CYC (Geisler *et al.*, 2010; Okatsu *et al.*, 2010; Radoshevich *et al.*, 2010). Furthermore, ATG12 may also promote degradation of cytoplasmic CYC by PARC (Gama *et al.*, 2014; Lopez and Tait, 2014) which is also interestingly involved in sequestering TP53 (see 1.6.1.3) (Nikolaev *et al.*, 2003). On the other, HSP27 is thought to inhibit translocation of tBID to the mitochondria and release of CYC (Paul *et al.*, 2002), and may also cooperate with HSP90 to prevent activation of the caspase cascade following formation of the apoptosome (Garrido *et al.*, 1999; Bruey *et al.*, 2000; Pandey, 2000).

Therefore, it is possible that the anti-apoptotic activity of Svn not only promotes survival by inhibiting initiation of apoptosis, but also by interacting with proteins involved in anastasis. To support this further, future work should involve analysing cells at later timepoints to see if cells expressing Svn make a full recovery or whether they eventually die.

4.3 Co-localisation of VDAC & CYC is the same across HeLa cell lines

To further confirm results which suggested a pro-survival role for Svn, we decided to perform an imaging experiment to visually clarify the results of the caspase assay through identification of apoptotic markers. In particular, the release of CYC from, and therefore the loss of co-localisation with, the mitochondria (identified by the presence of VDAC) was used here to identify apoptosis induction. Microscope images were taken of HeLa GFP and SvnGFP cell lines, following UVC treatment, and the co-distribution of VDAC and CYC was analysed.

Although the results indicate that there was little or no difference in the change in co-localisation of VDAC and CYC between GFP and SvnGFP expressing HeLa cells, this is not consistent with previous research (Wheatley, 2015) or the results of the caspase assay which both suggest a pro-survival role for Svn. This could be due to the lack of sample images that could be analysed, and therefore it is possible that the data extracted from this analysis may not be completely representative of the true effect that Svn overexpression is having on HeLa cell survival. Hence, it is likely that the results of the statistical analysis are not accurate in either

circumstance (Two-Way ANOVA or linear Regression) as only one independent repeat was performed for this experiment. Therefore, future experiments will require at least three biological repeats and a minimum of 50 cells should be imaged per repeat to ensure that the sample size is adequate.

4.4 Phosphatidylserine exposure is altered in HeLa cells ectopically expressing Svn

The arrangement of the PM is the result of differential localisation of specific phospholipid subtypes. For example, the outer leaflet of the PM is enriched with sphingomyelin and phosphatidylcholine, whereas phosphatidylethanolamine and PS are normally confined to the inner leaflet (Clark, 2011; Hankins *et al.*, 2015). To maintain such asymmetric distributions, flippase and floppase enzymes (ATP-dependent transporter proteins) promote internalisation and externalisation of phospholipids, respectively. Scramblase enzymes on the other hand promote passive, bidirectional movement of phospholipids along their concentration gradients (Clark, 2011). Preserving phospholipid localisation is important for regulation of several cellular processes, including cell signalling.

Previous research has reported that, following initiation of apoptosis, activated caspases alter the distribution of PS across the PM in order to promote phagocytosis. It is hypothesised that externalisation of PS to the outer leaflet is the result of caspase-dependent activation and inactivation of scramblase and flippase, respectively (Segawa and Nagata, 2015). Furthermore, Annexin V, which is normally localised to the cytosol, has been shown to bind to externalised PS. Transport of Annexin V to the outer leaflet has also been shown to rely on scramblase activity (Stewart *et al.*, 2018).

Fewer SvnGFP expressing HeLa cells were in late apoptosis at 9 hours when compared to the GFP controls. This suggests that Svn may be promoting survival by preventing entry into the later stages of apoptosis. This further supports the previous hypothesis that Svn may be positively regulating anastasis. Furthermore, it is likely that cells undergoing anastasis will have to remove PS from the exterior of the cell in order to prevent engulfment

(Hammill, Uhr and Scheuermann, 1999; Tang *et al.*, 2012). This could be achieved by internalisation of PS back to the inner leaflet (Kenis *et al.*, 2010), but this has not as yet been verified. Therefore, future work may benefit from analysing cells at later time points, post-irradiation, to see if the levels of PS decrease following its externalisation.

However, it is worth noting that the results of this assay were not as clear as we would have liked as the data shows a lack of cells in any stage of apoptosis. This may be because later timepoints were required to analyse PS exposure as it is thought to be a late event in the apoptosis pathway, occurring after caspase activation (Pozarowski *et al.*, 2003; Segawa and Nagata, 2015), although this has not yet been clarified and there is evidence that suggests PS exposure precedes MOMP and CYC release (Denecker *et al.*, 2000).

4.5 Svn alters the expression of some mitochondrial and apoptotic proteins in HeLa cells

In order to further assess how Svn could alter the intrinsic pathway of apoptosis, we decided to evaluate how up-regulation of Svn altered the expression of a range of mitochondrial and apoptotic proteins. Research indicates that changes in the expression of mitochondrial fission and fusion proteins can alter the response of cells to apoptotic stimuli. Therefore, we also performed a live imaging experiment to identify if Svn over-expression caused changes in any of the apoptotic markers identified previously.

Fission Proteins

Cells ectopically expressing SvnGFP and Svn¹⁻¹⁰GFP have significantly less DRP1 than GFP control cells. However, following treatment with UVC radiation, the levels of DRP1 present in SvnGFP and Svn¹⁻¹⁰GFP expressing HeLa cells was significantly greater than that observed following treatment of the control cells. On the other hand, there was no significant change in FIS1 levels following treatment of any of the HeLa cell lines.

It was initially hypothesised that the presence of Svn in the mitochondria might affect their dynamics, and that this could possibly explain the ability of Svn¹⁻¹⁰GFP to promote survival following initiation of the intrinsic

pathway of apoptosis. Furthermore, it has been previously documented that fragmentation of the mitochondria is an early step in the intrinsic pathway (Yuan *et al.*, 2007) and that the fission machinery (i.e. DRP1 and FIS1) actively participates in this process. Therefore, one mechanism by which Svn could delay apoptosis could be by inhibiting mitochondrial fragmentation. This hypothesis is consistent with the results of this analysis as, although the levels of DRP1 appear to increase following treatment with UVC, FIS1 does not change. FIS1 is a receptor for the DRP1 yeast orthologue, DNM1 (Mozdy, McCaffery and Shaw, 2000), and therefore it has been suggested that FIS1 may perform a similar role in mammalian cells (Yoon *et al.*, 2003). Furthermore, both FIS1 and DRP1 are involved in mitochondrial fission. Therefore, no significant change in FIS1 could mean that any increase in DRP1, as a result of Svn¹⁻¹⁰GFP expression, is ineffective at promoting mitochondrial fission.

Furthermore, up-regulation of FIS1 has also been reported to promote accumulation of the autophagy-related protein LC3 and trigger mitophagy (Twig *et al.*, 2008; Shen *et al.*, 2014). This could also explain why there is no significant change in FIS1 following exposure of Svn expressing cells to UVC treatment, as Svn is known to inhibit excessive autophagy (Humphry and Wheatley, 2018).

Other research has also suggested that reduced expression of DRP1 promotes processing of OPA1, which results in tightening of the cristae junctions and inhibition of CYC release following exposure to apoptotic stimuli (Estaquier and Arnoult, 2007). Since the analysis has revealed a significant decrease in DRP1 between GFP and Svn¹⁻¹⁰GFP expressing cells, prior to treatment, this could suggest that, under “normal” conditions, Svn is down-regulating DRP1 to promote the previously suggested scenario. However, this is not consistent with the increase in DRP1 that was observed following treatment with UVC radiation. That said, when taken with the reduction in FIS1, it could be suggested that if DRP1 is not being recruited to the mitochondria for fragmentation, any increase in its levels may be promoting its function in an alternate pathway. For example, previous research has stated that although Svn increases the expression of DRP1, which still results in fragmentation of the mitochondria, this does not result

in apoptosis, and instead leads to increased utilisation of the glycolytic pathway (Hagenbuchner *et al.*, 2013). Alternatively, recent research (Lima *et al.*, 2018) has suggested that over-expression of DRP1 does not result in mitochondrial fission as the activity of DRP1 is in fact dependent upon activation by a variety of post-translational modifications; different modifications yield different activities (see 1.8.6.2).

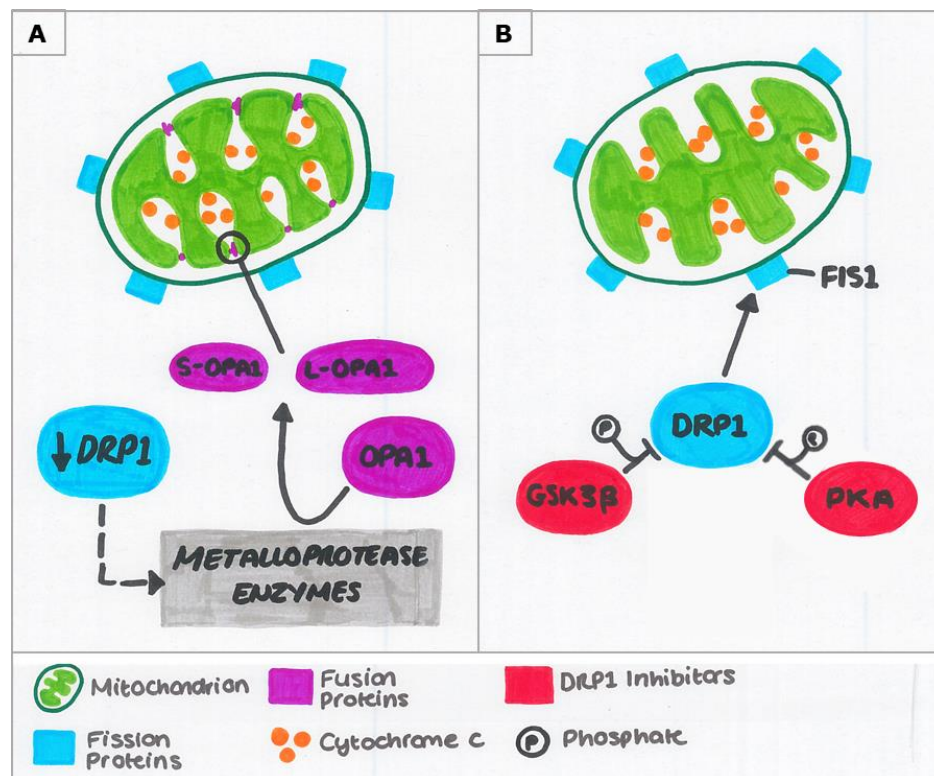


Figure 25 – Theoretical model for DRP1 activity in cells ectopically expressing Svn1-10GFP. (A) Untreated cells presented with less DRP1. Research has indicated that down-regulation of DRP1 can promote cleavage of OPA1 by metalloprotease enzymes. The cleaved products (S-OPA1 and L-OPA1) are thought to be involved in regulating cristae structure. Increased cleavage of OPA1 results in tightening of the cristae junctions which could inhibit CYC release following initiation of apoptosis. (B) Cells treated with UVC had elevated levels of DRP1, but live fluorescence imaging showed no excessive mitochondrial fragmentation as a result. DRP1 is known to be post-translationally modified by PKA and GSK3 β , resulting in its inactivation. Therefore, the lack of fission may be the result of DRP1 inactivity and loss of interaction with FIS1. Arrowed lines indicate activation steps. Flat-headed lines indicate inhibitory steps. Dashed line indicates indirect activation. Drawn by author.

To further clarify the previously mentioned hypotheses, future research may benefit from investigating the effect of Svn expression on the mitochondrial fission factor and E3 ubiquitin-protein ligase MARCH5, as these proteins are involved in DRP1 recruitment to the mitochondria and subcellular trafficking of DRP1, respectively (Karbowski, Neutzner and

Youle, 2007; Otera *et al.*, 2010; Liu and Chan, 2015). Furthermore, identification of any post-translational modifications to DRP1, in the presence of ectopically expressed Svn, could aid our understanding of the effect of Svn on DRP1 activity (Chang and Blackstone, 2010).

Apoptotic Proteins

Statistical analysis revealed no significant difference between the amount of AIF1 present in GFP, SvnGFP or Svn¹⁻¹⁰GFP expressing cells. However, the results did show that there was significantly more AIF1 present in SvnGFP expressing cells after UVC treatment when compared to the GFP treated control cells. This result is not consistent with the previously suggested hypothesis for an anti-apoptotic role for Svn, as AIF1 is a pro-apoptotic protein. However, this increase in AIF1 could also be the result of cells attempting to further promote apoptosis when faced with Svn-mediated delays. For example, research has indicated that expression of AIF1 can promote transcription of CASP3 (Liu *et al.*, 2018). However, in line with the previously suggested involvement for Svn in anastasis, any increase in CASP3 may be inhibited and therefore cell death averted, regardless of an increase in AIF1. In concordance with this, HSP70, another protein believed to be involved in anastasis, is thought to suppress AIF1 to prevent DNA fragmentation following MOMP, thus allowing anastatic cells to revert back to "normal" (Ravagnan *et al.*, 2001; Gurbuxani *et al.*, 2003). Although no evidence has yet been found for an interaction between Svn and HSP70, the previously suggested role for Svn in promoting anastasis (see 4.2) could still result in activation of this protein.

Like Svn, BCL2 is an anti-apoptotic protein which is over-expressed in several cancers, and is believed to perform its function by inhibiting oligomerisation of BAX and subsequent pore formation in the mitochondrial outer membrane (Kalkavan and Green, 2018). Western blot analysis revealed a significant increase in BCL2 between the untreated and treated samples for GFP expressing HeLa cells. However, this increase, although significant, does not appear to protect GFP control cells from cell death, as indicated by the results of the caspase assay. Furthermore, a similar increase in BCL2 was also observed for both SvnGFP and Svn¹⁻¹⁰GFP

expressing cells, suggesting that the role of Svn in delaying cell death does not involve BCL2.

Finally, we wanted to identify if there were any changes in VDAC expression for those cell lines over-expressing Svn as this could provide information about changes in mitochondrial mass between the cell lines, giving insight into any alterations to mitophagy or mitochondrial biogenesis. In fact, the results indicate that there was less VDAC in SvnGFP and Svn¹⁻¹⁰GFP expressing HeLa cells prior to treatment. On the other hand, there was no significant change in VDAC levels following exposure to UVC radiation. Also, the presence of mitochondrial Svn¹⁻¹⁰ appears to perturb VDAC further, suggesting that the effect of Svn on VDAC is linked to its localisation to the mitochondria. One possible explanation for the observed reduction in VDAC is that this protein is normally involved in promoting removal of dysfunctional mitochondria, via mitophagy, by recruiting Parkin to the outer mitochondrial membrane (Sun *et al.*, 2012). Svn has been shown to inhibit excessive autophagy in order to promote cell survival (Humphry and Wheatley, 2018). This could explain why there is less VDAC in those cells ectopically expressing Svn as this could help to prevent removal of dysfunctional mitochondria by mitophagy. Furthermore, VDAC1 and VDAC2 have both been shown to interact with pro-apoptotic BAX (Galluzzi and Kroemer, 2007; McCommis and Baines, 2012). However, whilst VDAC1 appears to promote release of CYC via its association with BAX (Magrì, Reina and De Pinto, 2018), VDAC2 appears to inhibit these events (Cheng *et al.*, 2003; Shore, 2009). Therefore, less VDAC1 in cells over-expressing Svn would make sense due to the anti-apoptotic role of Svn, but the same cannot be said for VDAC2. Moreover, since the antibody used is unable to distinguish between the VDAC isoforms it is unclear as to the individual levels of either VDAC1 or VDAC2 in Svn expressing cells. Therefore, future research could attempt to determine the different levels of VDAC isoforms present in Svn expressing cells to determine the true effect of Svn on VDAC activity.

4.6 The mitochondrial network of HeLa cell lines responds similarly following irradiation

Due to the observed changes in DRP1 in SvnGFP and Svn¹⁻¹⁰GFP expressing cells, fluorescence microscopy was used to see if these changes resulted in alterations to the mitochondrial network. From the images acquired of GFP and SvnGFP expressing HeLa cells there appears to be no differences in the response of the mitochondrial network to UVC treatment. This could support the hypothesis that increased DRP1, as a result of Svn over-expression, does not result in excessive mitochondrial fission (Lima *et al.*, 2018). However, further analysis is required to confirm this hypothesis.

4.7 Svn over-expression causes increased transcription of NOXA

Based on the results of the western blot analysis (see 4.5) it was proposed that qPCR analysis of a BCL2 regulator could reveal why the levels of BCL2 increased in all three HeLa cell lines following UVC treatment. However, due to time constraints it would not have been possible to design and optimise new primers for such a regulator. Therefore, primers for NOXA, which can inhibit anti-apoptotic MCL1 (Kale, Osterlund and Andrews, 2018), and XIAP, which were already optimised, were chosen as both proteins are involved in apoptosis and XIAP is also thought to stabilise Svn and promote its anti-apoptotic function.

The results of the comparative analysis revealed that there was a significantly higher expression of NOXA in HeLa cells expressing SvnGFP. Conversely, there was no difference in the amount of XIAP mRNA present in either cell line.

Increased transcription of NOXA would normally support a scenario involving increased inhibition of MCL1 and subsequent apoptosis. However, this scenario does not fit with the previously suggested anti-apoptotic role for Svn. Therefore, it could be suggested that although the levels of NOXA mRNA are higher in cells ectopically expressing Svn, this does not directly translate to increased activity. For example, it has been shown in haematological cancer cells that, due to upregulated glucose metabolism, which is a common result of Svn over-expression (Hagenbuchner *et al.*,

2013), NOXA can be phosphorylated by CDK5, leading to sequestration and subsequent suppression of its pro-apoptotic activity (Lowman *et al.*, 2010).

Furthermore, although XIAP is thought to complex with Svn and promote its anti-apoptotic activity (Dohi, Okada, *et al.*, 2004), it also promotes degradation of Svn when complexed with XIAP-associated factor 1 (XAF1) (Arora *et al.*, 2007). It is suggested that by sequestering XIAP, XAF1 inhibits its anti-caspase function and activates its E3-ligase activity, therefore promoting direct ubiquitination and subsequent degradation of Svn. Hence, overexpression of SvnGFP may not promote XIAP expression because of its documented anti-Svn function.

CHAPTER 5: Conclusion

The aim of this investigation was to increase our understanding of the role for Svn and the mitochondria in apoptosis. To do this, HeLa cells expressing SvnGFP were analysed to reveal their response to treatment with UVC radiation. Experimentation showed that Svn promotes survival of cells following exposure to apoptotic stimuli. Not only does Svn delay entry into apoptosis but results also suggest that it is aiding in the recovery of apoptotic cells by promoting the activity of proteins involved in anastasis. Furthermore, analysis of protein levels indicate that Svn may be down-regulating DRP1, to promote tightening of cristae junctions and subsequent inhibition of CYC release, but also inhibiting mitochondrial fragmentation. The latter is suggested to occur either by preventing interaction of DRP1 with the outer membrane receptor protein FIS1, or by altering the post-translational modification of DRP1, possibly diverting its activity towards alternate pathways. Therefore, it is concluded that, in cancer, mitochondrial Svn is inhibiting apoptosis by altering mitochondrial dynamics and interacting with several proteins involved in both the intrinsic pathway and anastasis.

Chapter 6: References

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Zitouni, S. *et al.* (2014) 'Polo-like kinases: Structural variations lead to multiple functions', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm3819.

Chapter 7: Appendix & Supplementary Information

7.1 Co-localisation Analysis Macro

```
requires("1.52n");
  dir = getDirectory("Choose a Directory ");

#@ String (label = "File suffix", value = ".tif") suffix
#@ Float (label = "Which is the first channel to analyse?", value = "1") First
#@ Float (label = "Min value", value = "0") FirstMin
#@ Float (label = "Max value", value = "1") FirstMax
#@ Float (label = "Which is the second channel to analyse?", value = "2") Second
#@ Float (label = "Min value", value = "0") SecondMin
#@ Float (label = "Max value", value = "1") SecondMax
  setBatchMode(true);
  count = 0;
  countFiles(dir);
  n = 0;
  processFiles(dir);
  //print(count+" files processed");
  function countFiles(dir) {
    list = getFileList(dir);
    for (i=0; i<list.length; i++) {
      if (endsWith(list[i], "/"))
        countFiles(""+dir+list[i]);
      else
        count++;
    }
  }
  function processFiles(dir) {
    list = getFileList(dir);
    for (i=0; i<list.length; i++) {
      if (endsWith(list[i], "/"))
        processFiles(""+dir+list[i]);
      else {
        showProgress(n++, count);
        path = dir+list[i];
        processFile(path);
      }
    }
  }
  function processFile(path) {
    if (endsWith(path, suffix)) {
      open(path);
      getSelectionBounds(xbase, ybase, width, height);
      imageID=getTitle();
      selectWindow(imageID);
      run("Z Project...", "projection=[Max Intensity]");
      selectWindow(imageID);
      close();
      selectWindow("MAX_" + imageID);
      run("Stack to Images");
      selectImage(First);
      run("Color Balance...");
      setMinAndMax(FirstMin, FirstMax);
      run("16-bit");
    }
  }
}
```

```

run("Grays");
run("Apply LUT");
save(path + "-First.png");
labelsx = newArray(width);
for (j=0; j<width; j++)
labelsx[j] = toString(xbase+j);
labelsy = newArray(height);
for (k=0; k<height; k++)
labelsy[k] = toString(ybase+k);
number = ((width + 1) * (height + 1));
pixelsx = newArray(number);
for (m=0; m<height; m++)
for (l=0; l<width; l++)
pixelsx[((m * (width + 1)) + l)] = getPixel(labelsx[l], labelsy[m]);
selectImage(Second);
run("Color Balance...");
setMinAndMax(SecondMin, SecondMax);
run("16-bit");
run("Grays");
run("Apply LUT");
save(path + "-Second.png");
pixelsy = newArray(number);
for (m=0; m<height; m++)
for (l=0; l<width; l++)
pixelsy[((m * (width + 1)) + l)] = getPixel(labelsx[l], labelsy[m]);
Fit.doFit("Straight Line", pixelsx, pixelsy);
print(getTitle());
Fit.rSquared;
selectWindow("Log");
save(path + "-Colocalisation R^2 Log.txt");
Fit.plot;
Plot.setStyle(1, "black,black,1.0,Dot");
Plot.setLogScaleX(false);
Plot.setLogScaleY(false);
Plot.setLimits(0,65535,0,65535);
selectWindow("y = a+bx");
save(path + "-Colocalisation Plot.png");
close();
        while (nImages>0) {
            selectImage(nImages);
            close();
        }
    }
}

```

7.2 qPCR

All qPCR optimisation steps were performed by Amelia Townley (DTP student, Wheatley Lab) and/or Dr James Grey who both kindly provided samples and primers required to perform comparative qPCR.

7.2.1 RNA extraction

7×10^6 cells were harvested by scraping (on ice) into 0.2 ml of media, and then 1 ml TRI-reagent added and incubated for 5 minutes at RT. 200 μ l 1-Bromo-3-chloropropane (BCP; 11.76 % (v/v) in TRI reagent and A₂ media) was then added, vigorously shaken and incubated for 3 minutes at RT. Samples were centrifuged at 10,000 x g for 15 minutes at 4 °C. The upper colourless layer was removed and incubated overnight at -20 °C in acidified isopropanol solution (256 mM sodium acetate pH 4.0 and 36 % isopropanol (v/v)). Samples were then centrifuged at 17,000 x g for 15 minutes at 4 °C, the supernatant discarded, and pellet washed in 70 % ethanol, before further centrifugation to pellet. Samples were then DNase treated with RNase-free DNase I (Qiagen Cat#79254) according to the manufacturer's instructions. DNase treated RNA was isolated by phenol-chloroform-isoamyl-alcohol (50 % phenol-chloroform-isoamyl alcohol (v/v) 125:24:1 in SDW). Samples were vigorously shaken and incubated for 3 minutes at RT, before centrifugation at 11,000 x g for 9 minutes at 12 °C. The upper aqueous phase was transferred to a fresh tube and precipitated overnight at -20 °C in acidified ethanol solution (323 mM sodium acetate pH 5.2 and 65 % ethanol (v/v)). Samples were centrifuged at 17,200 x g for 15 minutes at 4 °C, supernatant discarded, and pellet washed in 70 % ethanol. The pellet was then dried, dissolved in SDW and RNA concentration determined using by spectrophotometry (Nanodrop 2000, Thermo Scientific) before storage at -80 °C.

7.2.2 RNA Integrity

To determine RNA integrity, samples were prepared in RNA loading buffer (400 mM formamide, 20 mM MoPS, 8 mM NaAc, 1 mM EDTA, 4.44% formaldehyde (w/v), 40 ng· μ l RNA, 1X purple loading buffer (NEB)), and denatured at 65 °C for 15 minutes. A denaturing 1.5 % agarose gel was

prepared (1.5 % agarose (w/v), 6.7 % formaldehyde (w/v), 0.02 % SYBR safe (v/v), 20 mM MoPS, 8 mM NaAc, 1 mM EDTA) was made and pre-ran at 55 V for 5 minutes in 1 x RNA running buffer (100 mM MoPS, 40 mM NaAc, 5 mM EDTA), before addition of samples. Gels were pre-ran at 55 V for 2 hours, before visualisation under UV light (UVP). Samples that did not show two clear bands upon the RNA gel were re-prepared.

7.2.3 cDNA synthesis

1 µg of each RNA sample was reverse transcribed to produce cDNA using the iScript™ cDNA synthesis kit (BIORAD) according to the manufacturer's instructions. Reactions were run in duplicate, one containing the complete mixture as described (RT+), and the second lacking the reverse transcriptase enzyme (RT-) and a negative control. Both RT+ and RT- samples were then stored at -20 °C.

7.2.4 qPCR Primer Information

7.2.4.1 qPCR Amplicons

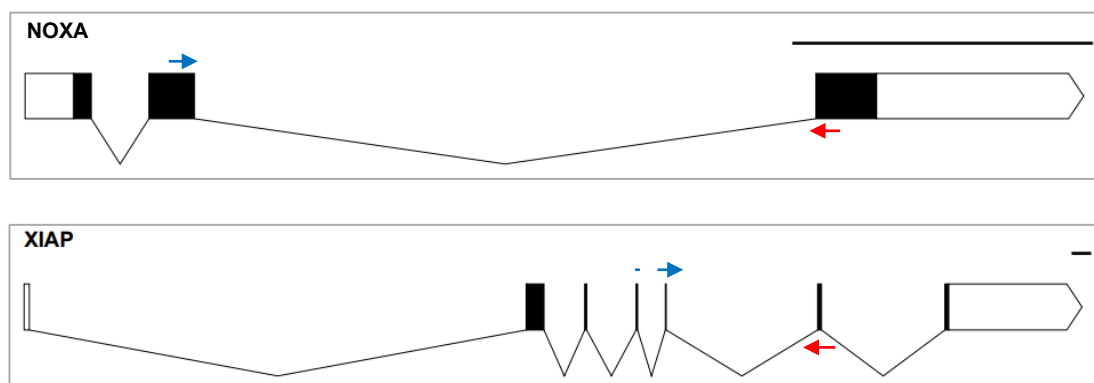


Figure 26 – PCR amplicons for genes of interest. Genes are positioned 5'-3' with black regions as exons, joining lines as introns, and white regions as UTRs. Blue and Red arrows represent forward and reverse primers, respectively. Scale bars are 1000 bp.

7.2.4.2 Primer Annealing Temperature Optimisation by PCR

To ensure primer amplification is optimal at 60 °C, a PCR reaction was performed using 312.5 pg·µl of cDNA, 250nM primer mix, 3.2 µM dNTPs, 1 x RedTaq buffer and 0.05 units of RedTaq® DNA polymerase. PCR reactions were run at 64, 62, 60 and 58 °C upon a Thermocycler (T100,

BIORAD) utilising a temperature gradient to optimise the annealing temperature. Reaction mixtures, final concentrations and PCR cycles are summarised below in Table 5 and Table 6, respectively.

Table 5 – Annealing temperature optimisation: reaction stoichiometry

Constituent	Volume (ml)	Final Concentration
10X REDTaq Buffer	2	1X
Forward Primer	0.5	250nM
Reverse Primer	0.5	250nM
dNTPs	0.5	250µM
Template	1	312.5pg/µl
REDTaq® Polymerase	1	0.05U/µl
DEPC treated ddH2O	14.5	-
Final Volume	20	

Table 6 – Annealing temperature optimisation: PCR programme

Step	1	2	3	4	5	6	7
Temperature (°C)	95	95	64/62/60/58	72	Return to 2	72	12
Time (s)	180	30	30	60	X39	300	∞

7.2.5 DNA Agarose Gel Electrophoresis

DNA samples were prepared in 6x Gel loading dye (NEB) and ran on a 1.2 % agarose gel (w/v prepared in 1 x TAE buffer (40 mM Tris, 20 mM Acetate and 1 mM EDTA) with SYBR safe gel stain (Invitrogen)) in 1 x TAE buffer at 100 V for 30-45 minutes. DNA was visualised using a UV trans-illuminator.

7.2.6 qPCR Primer Efficiency

qPCR primer efficiency was calculated by quantitative analysis of the threshold breaking cycle (CT) achieved during a PCR reaction for six, threefold serial dilutions of 2500 pg·µl of template containing the same primers, alongside blank and RT- reactions ran in duplicate in a Thermocycler (7500 Fast Real-Time PCR, Applied Biosciences). The final

concentration of each reaction and PCR programme are shown below in Table 4 and Table 5, respectively. The gradient of the standard curve was then used to calculate the reaction efficiency according to the MIQE guidelines (2011) using the following equation:

$$qPCR\ efficiency = (10 - 1^{gradient}) - 1$$

Reaction mix, final concentrations (Table 7) and PCR cycle (Table 8) is summarised below. Table 9 summarises the calculated efficiencies for all qPCR primers used.

Table 7 – Primer efficiency optimisation: reaction stoichiometry

Constituent	Volume (µl)	Final Concentration
2X iTaq™ Universal SYBR® Green Supermix	10	1x
Forward Primer	0.5	250nM (500nM gDNA standards and 375 GFP/SvnGFP)
Reverse Primer	0.5	250nM (500nM gDNA standards and 375 GFP/SvnGFP)
Template	1	-
DEPC treated ddH2O	7.5	-
Final Volume	20	

Table 8 – Primer efficiency optimisation: thermocycler programme

Step	1	2	3	4	5	6
Temperature (°C)	50	95	95	60	Return to 3	Default melt curve
Time (s)	120	60	15	60	X 39	

Table 9 – qPCR primer efficiencies

Reference Gene mRNA	Gradient	Efficiency	R²	Concentration (nM)
Reference Gene mRNA				
EEF1A1	-3.584	0.9012	0.9998	250
ACTB	-3.673	0.8718	0.9994	250
GAPDH	-3.581	0.9021	0.998	250
TUBB	-3.624	0.8877	0.9988	250
GOI mRNA				
PMAIP1	-3.515	0.9253	0.9868	250
XIAP	-3.504	0.9292	0.9878	250

7.3 Statistical Analyses

Tabular results for statistical analysis with two-way ANOVA or regression performed using GraphPad Prism 7 software.

7.3.1 UVC dosage response of HeLa cells ectopically expressing Svn

Two-way ANOVA (multiple comparisons)

 Nonlin fit	A	B	C
	GFP	SvnGFP	Global (shared)
	Y	Y	Y
1	Comparison of Fits		
2	Null hypothesis		One curve for all data sets
3	Alternative hypothesis		Different curve for each data set
4	P value		0.7348
5	Conclusion (alpha = 0.05)		Do not reject null hypothesis
6	Preferred model		One curve for all data sets
7	F (DFn, DFd)		0.5013 (4, 68)
8			

7.3.2 Mitochondrial Metabolism of HeLa cell lines

Two-way ANOVA (multiple comparisons)

Nonlin fit		A	B	C
		HeLa GFP	HeLa SvnGFP	Global (shared)
		Y	Y	Y
1	Comparison of Fits			
2	Null hypothesis			One curve for all data sets
3	Alternative hypothesis			Different curve for each data set
4	P value			<0.0001
5	Conclusion (alpha = 0.05)			Reject null hypothesis
6	Preferred model			Different curve for each data set
7	F (DFn, DFd)			5707 (2, 44)
8				

7.3.3 Co-localisation of VDAC and CYC in HeLa cells lines

Two-way ANOVA (multiple comparisons)

2way ANOVA Multiple comparisons						
1	Compare each cell mean with the other cell mean in that row					
2						
3	Number of families	1				
4	Number of comparisons per family	4				
5	Alpha	0.05				
6						
7	Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
8						
9	GFP - SvnGFP					
10	0	0.004867	-0.1245 to 0.1343	No	ns	>0.9999
11	3	0.04713	-0.08227 to 0.1765	No	ns	0.7891
12	6	0.01153	-0.1179 to 0.1409	No	ns	0.9986
13	9	0.1768	0.04743 to 0.3062	Yes	**	0.0059
14						

Linear regression

Are the slopes equal?

F = 0.8936. DFn = 1, DFd = 4
P=0.3980

If the overall slopes were identical, there is a 39.80% chance of randomly choosing data points with slopes this different. You can conclude that the differences between the slopes are not significant.

Since the slopes are not significantly different, it is possible to calculate one slope for all the data. The pooled slope equals -0.02917.

Are the elevations or intercepts equal?

F = 1.143. DFn = 1, DFd = 5
P=0.3338

If the overall elevations were identical, there is a 33.38% chance of randomly choosing data points with elevations this different. You can conclude that the differences between the elevations are not significant.

Since the Y intercepts are not significantly different, it is possible to calculate one Y intercept for all the data. The pooled intercept equals 0.8545.

7.3.4 Caspase activity in Svn expressing HeLa cells

Live fraction – Two-way ANOVA (multiple comparisons)

2way ANOVA Multiple comparisons						
1	Compare each cell mean with the other cell mean in that row					
2						
3	Number of families	1				
4	Number of comparisons per family	4				
5	Alpha	0.05				
6						
7	Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
8						
9	GFP - SvnGFP					
10	Ctrl	-1.422	-11.8 to 8.952	No	ns	0.9938
11	UVC 3hr	-12.5	-22.87 to -2.126	Yes	*	0.0130
12	UVC 6hr	-33.38	-43.75 to -23	Yes	****	<0.0001
13	UVC 9hr	-45.47	-55.84 to -35.09	Yes	****	<0.0001
14						

Live fraction – Linear regression

Are the slopes equal?

$F = 34.94$, $DFn = 1$, $DFd = 4$

$P = 0.0041$

If the overall slopes were identical, there is a 0.41% chance of randomly choosing data points with slopes this different. You can conclude that the differences between the slopes are very significant.

Because the slopes differ so much, it is not possible to test whether the intercepts differ significantly.

Early apoptotic fraction – Two-way ANOVA (multiple comparisons)

2way ANOVA Multiple comparisons						
1	Compare each cell mean with the other cell mean in that row					
2						
3	Number of families	1				
4	Number of comparisons per family	4				
5	Alpha	0.05				
6						
7	Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
8						
9	GFP - SvnGFP					
10	Ctrl	0.9556	-10.95 to 12.86	No	ns	0.9992
11	UVC 3hr	12.27	0.362 to 24.17	Yes	*	0.0413
12	UVC 6hr	12.5	0.5953 to 24.4	Yes	*	0.0364
13	UVC 9hr	-1.2	-13.1 to 10.7	No	ns	0.9981
14						

Late apoptotic/dead fraction – Two-way ANOVA (multiple comparisons)

2way ANOVA Multiple comparisons						
1	Compare each cell mean with the other cell mean in that row					
2						
3	Number of families	1				
4	Number of comparisons per family	4				
5	Alpha	0.05				
6						
7	Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
8						
9	GFP - SvnGFP					
10	Ctrl	0.7111	-9.352 to 10.77	No	ns	0.9995
11	UVC 3hr	0.7889	-9.274 to 10.85	No	ns	0.9993
12	UVC 6hr	21.69	11.63 to 31.75	Yes	****	<0.0001
13	UVC 9hr	43.21	33.15 to 53.27	Yes	****	<0.0001
14						

Combined apoptotic fractions – Two-way ANOVA (multiple comparisons)

2way ANOVA Multiple comparisons						
1	Compare each cell mean with the other cell mean in that row					
2						
3	Number of families	1				
4	Number of comparisons per family	4				
5	Alpha	0.05				
6						
7	Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
8						
9	GFP - SvnGFP					
10	Ctrl	1.667	-7.574 to 10.91	No	ns	0.9827
11	UVC 3hr	13.06	3.815 to 22.3	Yes	**	0.0030
12	UVC 6hr	34.19	24.95 to 43.43	Yes	****	<0.0001
13	UVC 9hr	42.01	32.77 to 51.25	Yes	****	<0.0001
14						

Combined apoptotic fractions – Linear regression

Are the slopes equal?

F = 22.8. DFn = 1, DFd = 4

P=0.0088

If the overall slopes were identical, there is a 0.88% chance of randomly choosing data points with slopes this different. You can conclude that the differences between the slopes are very significant.

Because the slopes differ so much, it is not possible to test whether the intercepts differ significantly.

Percentage of apoptotic cells that were late apoptotic/dead – Two-way ANOVA (multiple comparisons)

2way ANOVA Multiple comparisons						
1	Compare each cell mean with the other cell mean in that row					
2						
3	Number of families	1				
4	Number of comparisons per family	4				
5	Alpha	0.05				
6						
7	Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
8						
9	GFP - SvnGFP					
10	Ctrl	0.4111	-13.42 to 14.25	No	ns	>0.9999
11	3hr UVC	-3.302	-17.14 to 10.53	No	ns	0.9525
12	6hr UVC	38.71	24.88 to 52.55	Yes	****	<0.0001
13	9hr UVC	53.94	40.11 to 67.78	Yes	****	<0.0001
14						

Percentage of apoptotic cells that were late apoptotic/dead – Linear regression

Are the slopes equal?
F = 12.22. DF _n = 1, DF _d = 4
P=0.0250
If the overall slopes were identical, there is a 2.50% chance of randomly choosing data points with slopes this different. You can conclude that the differences between the slopes are significant.
Because the slopes differ so much, it is not possible to test whether the intercepts differ significantly.

7.3.5 Phosphatidylserine exposure in Svn expressing HeLa cells

Early apoptotic events – Two-way ANOVA (multiple comparisons)

2way ANOVA Multiple comparisons						
1	Within each row, compare columns (simple effects within rows)					
2						
3	Number of families	4				
4	Number of comparisons per family	6				
5	Alpha	0.05				
6						
7	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
8						
9	0					
10	GFP Ctrl vs. GFP UVC	0	-12.05 to 12.05	No	ns	>0.9999
11	GFP Ctrl vs. SvnGFP Ctrl	10.33	-1.715 to 22.38	No	ns	0.1138
12	GFP Ctrl vs. SvnGFP UVC	10.33	-1.715 to 22.38	No	ns	0.1138
13	GFP UVC vs. SvnGFP Ctrl	10.33	-1.715 to 22.38	No	ns	0.1138
14	GFP UVC vs. SvnGFP UVC	10.33	-1.715 to 22.38	No	ns	0.1138
15	SvnGFP Ctrl vs. SvnGFP UVC	0	-12.05 to 12.05	No	ns	>0.9999
16						
17	3					
18	GFP Ctrl vs. GFP UVC	2.333	-9.715 to 14.38	No	ns	0.9525
19	GFP Ctrl vs. SvnGFP Ctrl	6.667	-5.381 to 18.71	No	ns	0.4498
20	GFP Ctrl vs. SvnGFP UVC	9.333	-2.715 to 21.38	No	ns	0.1752
21	GFP UVC vs. SvnGFP Ctrl	4.333	-7.715 to 16.38	No	ns	0.7648
22	GFP UVC vs. SvnGFP UVC	7	-5.048 to 19.05	No	ns	0.4072
23	SvnGFP Ctrl vs. SvnGFP UVC	2.667	-9.381 to 14.71	No	ns	0.9314
24						
25	6					
26	GFP Ctrl vs. GFP UVC	4.333	-7.715 to 16.38	No	ns	0.7648
27	GFP Ctrl vs. SvnGFP Ctrl	8	-4.048 to 20.05	No	ns	0.2924
28	GFP Ctrl vs. SvnGFP UVC	0.6667	-11.38 to 12.71	No	ns	0.9968
29	GFP UVC vs. SvnGFP Ctrl	3.667	-8.381 to 15.71	No	ns	0.8424
30	GFP UVC vs. SvnGFP UVC	-3.667	-15.71 to 8.381	No	ns	0.8424
31	SvnGFP Ctrl vs. SvnGFP UVC	-7.333	-19.38 to 4.715	No	ns	0.3668
32						
33	9					
34	GFP Ctrl vs. GFP UVC	0	-12.05 to 12.05	No	ns	>0.9999
35	GFP Ctrl vs. SvnGFP Ctrl	1.833	-10.21 to 13.88	No	ns	0.9760
36	GFP Ctrl vs. SvnGFP UVC	9.667	-2.381 to 21.71	No	ns	0.1524
37	GFP UVC vs. SvnGFP Ctrl	1.833	-10.21 to 13.88	No	ns	0.9760
38	GFP UVC vs. SvnGFP UVC	9.667	-2.381 to 21.71	No	ns	0.1524
39	SvnGFP Ctrl vs. SvnGFP UVC	7.833	-4.215 to 19.88	No	ns	0.3100
40						

Late apoptotic events – Two-way ANOVA (multiple comparisons)

2way ANOVA Multiple comparisons						
1	Within each row, compare columns (simple effects within rows)					
2						
3	Number of families	4				
4	Number of comparisons per family	6				
5	Alpha	0.05				
6						
7	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
8						
9	0					
10	GFP Ctrl vs. GFP UVC	0	-28.11 to 28.11	No	ns	>0.9999
11	GFP Ctrl vs. SvmGFP Ctrl	20	-8.115 to 48.11	No	ns	0.2371
12	GFP Ctrl vs. SvmGFP UVC	20	-8.115 to 48.11	No	ns	0.2371
13	GFP UVC vs. SvmGFP Ctrl	20	-8.115 to 48.11	No	ns	0.2371
14	GFP UVC vs. SvmGFP UVC	20	-8.115 to 48.11	No	ns	0.2371
15	SvmGFP Ctrl vs. SvmGFP UVC	0	-28.11 to 28.11	No	ns	>0.9999
16						
17	3					
18	GFP Ctrl vs. GFP UVC	10.33	-17.78 to 38.45	No	ns	0.7528
19	GFP Ctrl vs. SvmGFP Ctrl	21.67	-8.448 to 49.78	No	ns	0.1787
20	GFP Ctrl vs. SvmGFP UVC	25	-3.115 to 53.11	No	ns	0.0956
21	GFP UVC vs. SvmGFP Ctrl	11.33	-16.78 to 39.45	No	ns	0.6968
22	GFP UVC vs. SvmGFP UVC	14.67	-13.45 to 42.78	No	ns	0.5005
23	SvmGFP Ctrl vs. SvmGFP UVC	3.333	-24.78 to 31.45	No	ns	0.9883
24						
25	6					
26	GFP Ctrl vs. GFP UVC	-0.3333	-28.45 to 27.78	No	ns	>0.9999
27	GFP Ctrl vs. SvmGFP Ctrl	24	-4.115 to 52.11	No	ns	0.1163
28	GFP Ctrl vs. SvmGFP UVC	15.33	-12.78 to 43.45	No	ns	0.4623
29	GFP UVC vs. SvmGFP Ctrl	24.33	-3.782 to 52.45	No	ns	0.1090
30	GFP UVC vs. SvmGFP UVC	15.67	-12.45 to 43.78	No	ns	0.4437
31	SvmGFP Ctrl vs. SvmGFP UVC	-8.667	-36.78 to 19.45	No	ns	0.8373
32						
33	9					
34	GFP Ctrl vs. GFP UVC	-34	-62.11 to -5.885	Yes	*	0.0128
35	GFP Ctrl vs. SvmGFP Ctrl	9.333	-18.78 to 37.45	No	ns	0.8052
36	GFP Ctrl vs. SvmGFP UVC	8.333	-19.78 to 36.45	No	ns	0.8525
37	GFP UVC vs. SvmGFP Ctrl	43.33	15.22 to 71.45	Yes	**	0.0012
38	GFP UVC vs. SvmGFP UVC	42.33	14.22 to 70.45	Yes	**	0.0015
39	SvmGFP Ctrl vs. SvmGFP UVC	-1	-29.11 to 27.11	No	ns	0.9997
40						

7.3.6 Expression of mitochondrial & apoptotic proteins in HeLa cells

TBP – Two-way ANOVA (multiple comparisons)

2way ANOVA Multiple comparisons						
1	Within each row, compare columns (simple effects within rows)					
2						
3	Number of families	6				
4	Number of comparisons per family	15				
5	Alpha	0.05				
6						
7	Uncorrected Fisher's LSD	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Individual P Value
8						
9	TBP					
10	GFP Ctrl vs. GFP UVC	-0.1714	-1.016 to 0.6731	No	ns	0.6896
11	GFP Ctrl vs. SvnGFP Ctrl	-0.003255	-0.8478 to 0.8413	No	ns	0.9939
12	GFP Ctrl vs. SvnGFP UVC	-0.1792	-1.024 to 0.6654	No	ns	0.6763
13	GFP Ctrl vs. S158 Ctrl	0.03489	-0.9037 to 0.9735	No	ns	0.9417
14	GFP Ctrl vs. S158 UVC	0.2786	-0.6601 to 1.217	No	ns	0.5593
15	GFP UVC vs. SvnGFP Ctrl	0.1681	-0.6512 to 0.9875	No	ns	0.6864
16	GFP UVC vs. SvnGFP UVC	-0.007776	-0.8271 to 0.8115	No	ns	0.9851
17	GFP UVC vs. S158 Ctrl	0.2063	-0.7097 to 1.122	No	ns	0.6577
18	GFP UVC vs. S158 UVC	0.45	-0.466 to 1.366	No	ns	0.3342
19	SvnGFP Ctrl vs. SvnGFP UVC	-0.1759	-0.9952 to 0.6434	No	ns	0.6727
20	SvnGFP Ctrl vs. S158 Ctrl	0.03815	-0.8779 to 0.9542	No	ns	0.9347
21	SvnGFP Ctrl vs. S158 UVC	0.2818	-0.6342 to 1.198	No	ns	0.5450
22	SvnGFP UVC vs. S158 Ctrl	0.2141	-0.702 to 1.13	No	ns	0.6457
23	SvnGFP UVC vs. S158 UVC	0.4577	-0.4583 to 1.374	No	ns	0.3259
24	S158 Ctrl vs. S158 UVC	0.2437	-0.7598 to 1.247	No	ns	0.6328
25						

DRP1 – Two-way ANOVA (multiple comparisons)

26	DRP1					
27	GFP Ctrl vs. GFP UVC	-0.3422	-1.187 to 0.5023	No	ns	0.4255
28	GFP Ctrl vs. SvnGFP Ctrl	0.08129	-0.7632 to 0.9258	No	ns	0.8498
29	GFP Ctrl vs. SvnGFP UVC	-0.7914	-1.636 to 0.05315	No	ns	0.0661
30	GFP Ctrl vs. S158 Ctrl	1.09	0.1517 to 2.029	Yes	*	0.0230
31	GFP Ctrl vs. S158 UVC	-1.023	-1.962 to -0.08449	Yes	*	0.0328
32	GFP UVC vs. SvnGFP Ctrl	0.4235	-0.3958 to 1.243	No	ns	0.3096
33	GFP UVC vs. SvnGFP UVC	-0.4492	-1.268 to 0.3702	No	ns	0.2812
34	GFP UVC vs. S158 Ctrl	1.433	0.5166 to 2.349	Yes	**	0.0023
35	GFP UVC vs. S158 UVC	-0.6809	-1.597 to 0.2351	No	ns	0.1444
36	SvnGFP Ctrl vs. SvnGFP UVC	-0.8727	-1.692 to -0.05335	Yes	*	0.0369
37	SvnGFP Ctrl vs. S158 Ctrl	1.009	0.09308 to 1.925	Yes	*	0.0310
38	SvnGFP Ctrl vs. S158 UVC	-1.104	-2.02 to -0.1884	Yes	*	0.0183
39	SvnGFP UVC vs. S158 Ctrl	1.882	0.9657 to 2.798	Yes	****	<0.0001
40	SvnGFP UVC vs. S158 UVC	-0.2318	-1.148 to 0.6843	No	ns	0.6186
41	S158 Ctrl vs. S158 UVC	-2.114	-3.117 to -1.11	Yes	****	<0.0001
42						

BCL2 – Two-way ANOVA (multiple comparisons)

43	BCL-2					
44	GFP Ctrl vs. GFP UVC	-0.9602	-1.805 to -0.1157	Yes	*	0.0260
45	GFP Ctrl vs. SvnGFP Ctrl	-0.06277	-0.9073 to 0.7818	No	ns	0.8837
46	GFP Ctrl vs. SvnGFP UVC	-0.8289	-1.673 to 0.01565	No	ns	0.0544
47	GFP Ctrl vs. S158 Ctrl	0.04991	-0.8887 to 0.9885	No	ns	0.9167
48	GFP Ctrl vs. S158 UVC	-1.082	-2.02 to -0.1431	Yes	*	0.0241
49	GFP UVC vs. SvnGFP Ctrl	0.8974	0.07811 to 1.717	Yes	*	0.0319
50	GFP UVC vs. SvnGFP UVC	0.1313	-0.688 to 0.9506	No	ns	0.7525
51	GFP UVC vs. S158 Ctrl	1.01	0.09408 to 1.926	Yes	*	0.0308
52	GFP UVC vs. S158 UVC	-0.1216	-1.038 to 0.7944	No	ns	0.7940
53	SvnGFP Ctrl vs. SvnGFP UVC	-0.7661	-1.585 to 0.05321	No	ns	0.0667
54	SvnGFP Ctrl vs. S158 Ctrl	0.1127	-0.8033 to 1.029	No	ns	0.8087
55	SvnGFP Ctrl vs. S158 UVC	-1.019	-1.935 to -0.103	Yes	*	0.0294
56	SvnGFP UVC vs. S158 Ctrl	0.8788	-0.03724 to 1.795	No	ns	0.0600
57	SvnGFP UVC vs. S158 UVC	-0.2529	-1.169 to 0.6631	No	ns	0.5870
58	S158 Ctrl vs. S158 UVC	-1.132	-2.135 to -0.1282	Yes	*	0.0272
59						

FIS1 – Two-way ANOVA (multiple comparisons)

60	FIS1					
61	GFP Ctrl vs. GFP UVC	-0.2484	-1.093 to 0.5962	No	ns	0.5629
62	GFP Ctrl vs. SvnGFP Ctrl	-0.411	-1.255 to 0.4336	No	ns	0.3387
63	GFP Ctrl vs. SvnGFP UVC	-0.5631	-1.408 to 0.2814	No	ns	0.1902
64	GFP Ctrl vs. S158 Ctrl	-0.307	-1.246 to 0.6317	No	ns	0.5200
65	GFP Ctrl vs. S158 UVC	-1.3	-2.238 to -0.3612	Yes	**	0.0068
66	GFP UVC vs. SvnGFP Ctrl	-0.1626	-0.9819 to 0.6567	No	ns	0.6961
67	GFP UVC vs. SvnGFP UVC	-0.3148	-1.134 to 0.5045	No	ns	0.4499
68	GFP UVC vs. S158 Ctrl	-0.0586	-0.9746 to 0.8574	No	ns	0.8998
69	GFP UVC vs. S158 UVC	-1.051	-1.967 to -0.1355	Yes	*	0.0246
70	SvnGFP Ctrl vs. SvnGFP UVC	-0.1522	-0.9715 to 0.6671	No	ns	0.7148
71	SvnGFP Ctrl vs. S158 Ctrl	0.104	-0.812 to 1.02	No	ns	0.8232
72	SvnGFP Ctrl vs. S158 UVC	-0.8889	-1.805 to 0.02715	No	ns	0.0571
73	SvnGFP UVC vs. S158 Ctrl	0.2562	-0.6598 to 1.172	No	ns	0.5822
74	SvnGFP UVC vs. S158 UVC	-0.7367	-1.653 to 0.1793	No	ns	0.1144
75	S158 Ctrl vs. S158 UVC	-0.9929	-1.996 to 0.01057	No	ns	0.0524
76						

AIF1 – Two-way ANOVA (multiple comparisons)

77	AIFM1					
78	GFP Ctrl vs. GFP UVC	0.03484	-0.8097 to 0.8794	No	ns	0.9353
79	GFP Ctrl vs. SvnGFP Ctrl	-0.8193	-1.664 to 0.02527	No	ns	0.0572
80	GFP Ctrl vs. SvnGFP UVC	-1.271	-2.116 to -0.4269	Yes	**	0.0033
81	GFP Ctrl vs. S158 Ctrl	-0.7909	-1.73 to 0.1477	No	ns	0.0982
82	GFP Ctrl vs. S158 UVC	-1.059	-1.997 to -0.12	Yes	*	0.0272
83	GFP UVC vs. SvnGFP Ctrl	-0.8541	-1.673 to -0.03478	Yes	*	0.0411
84	GFP UVC vs. SvnGFP UVC	-1.306	-2.126 to -0.487	Yes	**	0.0019
85	GFP UVC vs. S158 Ctrl	-0.8258	-1.742 to 0.09026	No	ns	0.0770
86	GFP UVC vs. S158 UVC	-1.093	-2.009 to -0.1774	Yes	*	0.0195
87	SvnGFP Ctrl vs. SvnGFP UVC	-0.4522	-1.272 to 0.3671	No	ns	0.2780
88	SvnGFP Ctrl vs. S158 Ctrl	0.02834	-0.8877 to 0.9444	No	ns	0.9514
89	SvnGFP Ctrl vs. S158 UVC	-0.2393	-1.155 to 0.6767	No	ns	0.6072
90	SvnGFP UVC vs. S158 Ctrl	0.4805	-0.4355 to 1.397	No	ns	0.3024
91	SvnGFP UVC vs. S158 UVC	0.2129	-0.7031 to 1.129	No	ns	0.6475
92	S158 Ctrl vs. S158 UVC	-0.2677	-1.271 to 0.7358	No	ns	0.5997
93						

VDAC – Two-way ANOVA (multiple comparisons)

94	VDAC					
95	GFP Ctrl vs. GFP UVC	-0.2275	-1.231 to 0.776	No	ns	0.6556
96	GFP Ctrl vs. SvnGFP Ctrl	1.061	0.05753 to 2.064	Yes	*	0.0383
97	GFP Ctrl vs. SvnGFP UVC	0.9965	-0.006914 to 2	No	ns	0.0516
98	GFP Ctrl vs. S158 Ctrl	1.461	0.4572 to 2.464	Yes	**	0.0045
99	GFP Ctrl vs. S158 UVC	0.6343	-0.3692 to 1.638	No	ns	0.2143
100	GFP UVC vs. SvnGFP Ctrl	1.288	0.285 to 2.292	Yes	*	0.0121
101	GFP UVC vs. SvnGFP UVC	1.224	0.2206 to 2.227	Yes	*	0.0170
102	GFP UVC vs. S158 Ctrl	1.688	0.6847 to 2.692	Yes	**	0.0011
103	GFP UVC vs. S158 UVC	0.8618	-0.1417 to 1.865	No	ns	0.0920
104	SvnGFP Ctrl vs. SvnGFP UVC	-0.06445	-1.068 to 0.939	No	ns	0.8994
105	SvnGFP Ctrl vs. S158 Ctrl	0.3997	-0.6037 to 1.403	No	ns	0.4334
106	SvnGFP Ctrl vs. S158 UVC	-0.4267	-1.43 to 0.5767	No	ns	0.4030
107	SvnGFP UVC vs. S158 Ctrl	0.4642	-0.5393 to 1.468	No	ns	0.3631
108	SvnGFP UVC vs. S158 UVC	-0.3623	-1.366 to 0.6412	No	ns	0.4776
109	S158 Ctrl vs. S158 UVC	-0.8264	-1.83 to 0.177	No	ns	0.1060
110						