INVESTIGATING THE ROLE OF SURVIVIN IN MITOCHONDRIAL HEALTH.

AMELIA RUTH TOWNLEY, BSc

Biochemistry, School of Life Sciences, Medical School,

Queen's Medical Centre,

Nottingham,

NG7 2UH

Thesis Submitted to the University of Nottingham for the degree of Masters by Research

SEPTEMBER 2016

Abstract

Survivin is a multi-functional protein with roles as both an apoptotic inhibitor and cell cycle regulator. Survivin is also one of the most tumour specific molecules, offering cancerous cells resistance to both chemotherapy and radiotherapy. It is localised to either the nucleus or cytoplasm, however a small pool with an unknown function is detected in cancerous cell lines within the mitochondria. Through the use of binding experiments we have unveiled two new binding partners of survivin, the tyrosine kinase c-Src and the glycerol-phospholipid conversion enzyme phosphatidylserine decarboxylase. Firstly, we have found that the survivin$_{1\text{-}10}$ NH_2 terminus is a *bona fide* mitochondrial targeting sequence and allows for its binding to c-Src. Secondly, we have previously found that survivin binds to phosphatidylserine decarboxylase, and now we find that upon threonine 34 phosphorylation survivin inhibits phosphatidylethanolamine production within the mitochondria, causing drastic changes to mitochondrial architecture and cell growth. These novel molecular insights suggest that this multi-faceted protein may be even more diverse in its action than previously known. Furthermore, its current roles in cancer could further be clarified through the consideration of its influence upon membrane architecture, cell signalling and metabolism. We also provide insight to its contribution to metabolic disorders through the regulation of inner mitochondrial membrane integrity.

Published papers:

Dunajová, L. Cash, E. Markus, R. Rochette, S. Townley, A. R. Wheatley, S. P. The NH_2 terminus of survivin is a mitochondrial targeting sequence and c-Src regulator, *J Cell Sci* 2016.

Key Words:

Survivin, Phosphatidylethanolamine, Phosphatidylserine decarboxylase, c-Src, Mitochondria, Warburg effect.

Acknowledgements:

I would like to thank the members of the C5 office for their guidance and continued support over the duration of this thesis. I would particularly like to thank the members of the Wheatley Lab for their hospitality, kindness, and insight into my project.

Table of Contents

CHAPTER 1: Introduction

1.1 Overview

Our understanding of the cancer related protein survivin, which functions within the cell cycle and apoptosis (Ambrosini *et al* 1997), has expanded rapidly since the discovery of its emerging roles in both autophagy and mitochondrial health (Wang *et al* 2011, Dohi *et al* 2004). Over recent years its role in cancer propagation has also become more apparent due to an appreciation of the vast network of pro-survival functions it orchestrates. Not only is the expression of the survivin gene significantly higher in malignant cells, but it has also been shown to confer tumours with chemotherapy resistance to a variety of pro-apoptotic stimuli (Tirrò *et al* 2006). Its high level of expression in cancer cells in comparison to low or absent expression in normal cell lines therefore makes it an attractive target for cancer therapy. However, the consequences of survivin inhibition in non-malignant cell lines have not been fully defined; understanding its full mechanism of action is essential in the development of survivin anti-tumour therapies.

Research within the Wheatley Lab has unveiled two new potential binding partners of the protein survivin, the tyrosine kinase c-Src and the glyero-phospholipid conversion enzyme phosphatidylserine decarboxylase. Below follows a summary of the cell cycle, cell death, survivin, the process of phospholipid metabolism and the proto-oncogene c-Src.

1.2.1 Cell cycle summary

The most basic function of the cell is the ability to divide its genetic material accurately and pass its genome identically to two daughter cells. It is essential for both cellular and organism survival that this process occurs as efficiently and precisely as possible. The cell cycle in multi-cellular organisms is essential in both development and in the maintenance of cell number within tissues and organs. The cell cycle is tightly controlled to preserve DNA integrity and ensure correct genetic material is inherited (Barnum *et al* 2014).

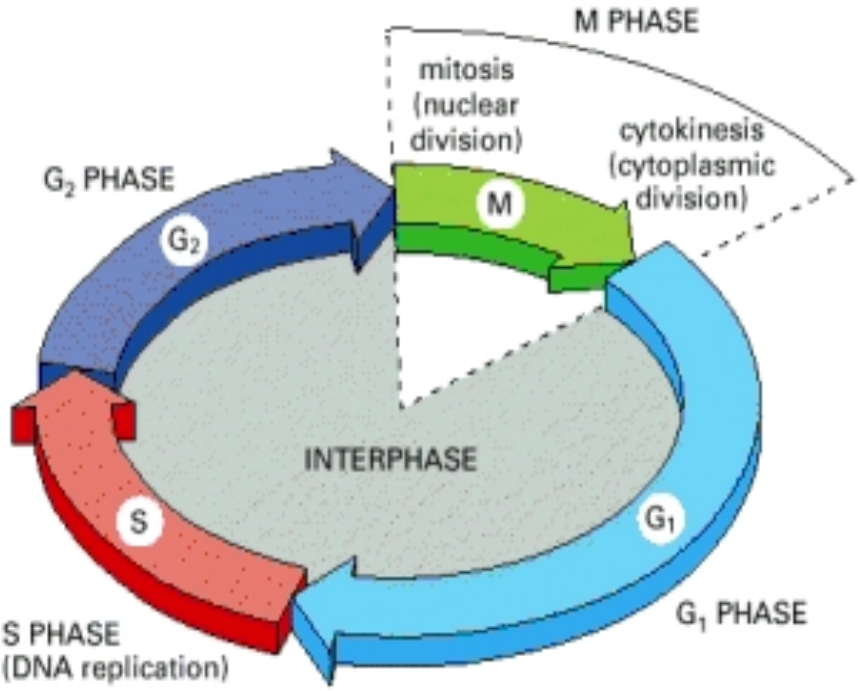


Figure.1. The Cell Cycle. Taken from Molecular Biology of the Cell Fourth Edition

Cell replication is initiated proceeding interphase, followed by a gap phase, G1, before DNA replication is instigated during S phase (Figure 1). This initial gap phase, G1, allows a cell to monitor its extracellular environment ensuring conditions are correct for to progress into DNA replication. If not suitable, the cell can enter G0 phase where it can wait for an unspecified

amount of time until conditions allow for DNA replication to begin. The progression into S phase is influenced by cell cycle proteins, for example cyclin-dependent kinases (Cdks), that promote cell cycle advancement in response to favourable replication conditions such as adequate energy reserves, nutrients and sufficient cell size (Neganova *et al* 2008). A second gap phase, G2, then follows DNA replication in which cells prepare for the process of splitting; allowing time for protein and organelle amplification thus ensuring the appropriate quantity is passed to each daughter cell. Cell division then occurs during M phase, described as 6 sequential events, prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis.

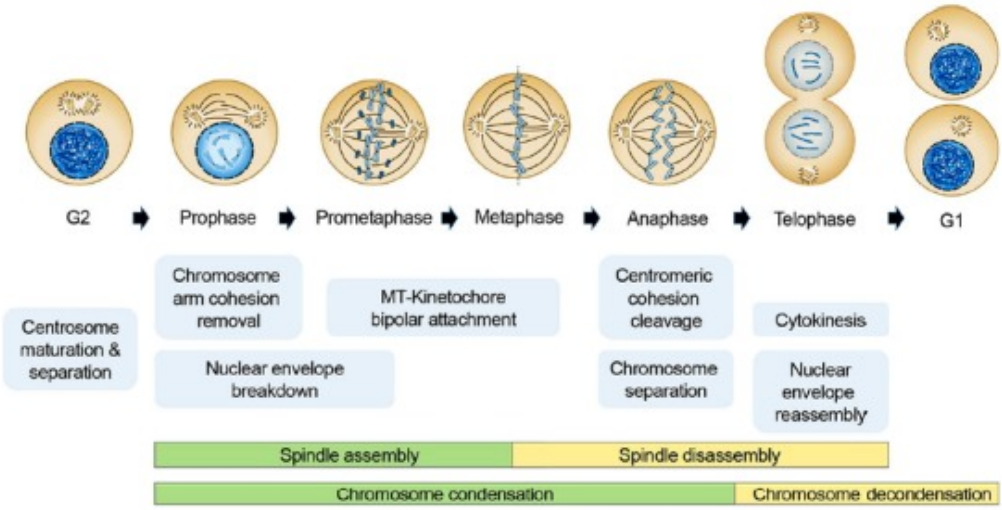


Figure 2. The six stages of mitosis. From gap phase 2, G2, cells enter prophase where the nuclear envelope disintegrates and chromosomes condense. Prometaphase follows in which chromosomes attach to microtubules (MT) via their kinetochores, which are aligned down the cell centre at metaphase. Chromosomes are separated in anaphase, and telophase commences as cell separation begins, initiating cytokinesis. Adapted from Kim *et al* 2016.

Mitosis initiates with prophase (Figure 2), where the nuclear envelope disintegrates and sister chromatids condense to form chromosomes attached by a centromere. Prometaphase is defined when duplicated centrosomes separate to opposing

poles and nucleate microtubules. Kinetochore microtubules formed at the centromere then attach to structures on chromosomes called kinetochores, allowing for their bipolar attachment (Cheeseman *et al* 2008) The tension created across these microtubules causes chromosomes to align at the cell centre or 'metaphase plate'. The onset of anaphase is activated by the 'Anaphase promoting complex/cyclosome' (APC/C), a multi-subunit E3 ubiquitin ligase that ubiquinates the protein securin targeting it for degradation. This releases the enzyme separase (Wirth *et al* 2006), which then acts to cleave the subunit 'cc1' of cohesion, a protein complex that allows for the cohesion of sister chromatids. Sister chromatid separation is initiated as spindle microtubules begin to shorten and telophase commences as an actin/myosin ring forms at the cell equator, causing cytoplasmic cleavage or cytokinesis (Straight *et al* 2003). The nuclear envelope reforms and chromosomes de-condense to form two identical sister cells.

1.2.2 Cell cycle checkpoints and regulation

To ensure the accuracy of each stage of the cell cycle is maintained before its progression is permitted, it is regulated by a series of checkpoints throughout its advancement. Failures in monitoring the cell cycle can result in catastrophic genetic alterations, resulting in the production of aberrant, dysfunctional and possibly cancerous cells. An important stage of mitosis that requires monitoring is during the separation of sister chromatids at anaphase, necessitating co-ordinated chromosome segregation before cytokinesis is initiated. The cell cycle checkpoint which regulates this stage is the mitotic spindle checkpoint or spindle assembly checkpoint (SAC) (Musacchino 2015).

1.2.3 Chromosomal passenger complex (CPC)

The process of cell division requires the well-timed organisation of chromosomes, the cytoskeleton and membranes to ensure its correct completion. A complex of essential proteins called the chromosomal passenger proteins (CPP) are responsible for monitoring numerous stages during mitosis and cytokinesis to co-ordinate these processes (Bolton *et al* 2002, Gerben Vader *et al* 2006). Proteins comprising the CPC include the serine/threonine protein kinase Aurora B (Glover et al 1995), and the targeting/regulatory components survivin, borealin and inner centromeric protein (INCENP) (Bolton *et al* 2002). The structure of the CPC comprises of two regions, the localisation module and the kinase module, based around the scaffold structure of INCENP (Carmena *et al* 2012).

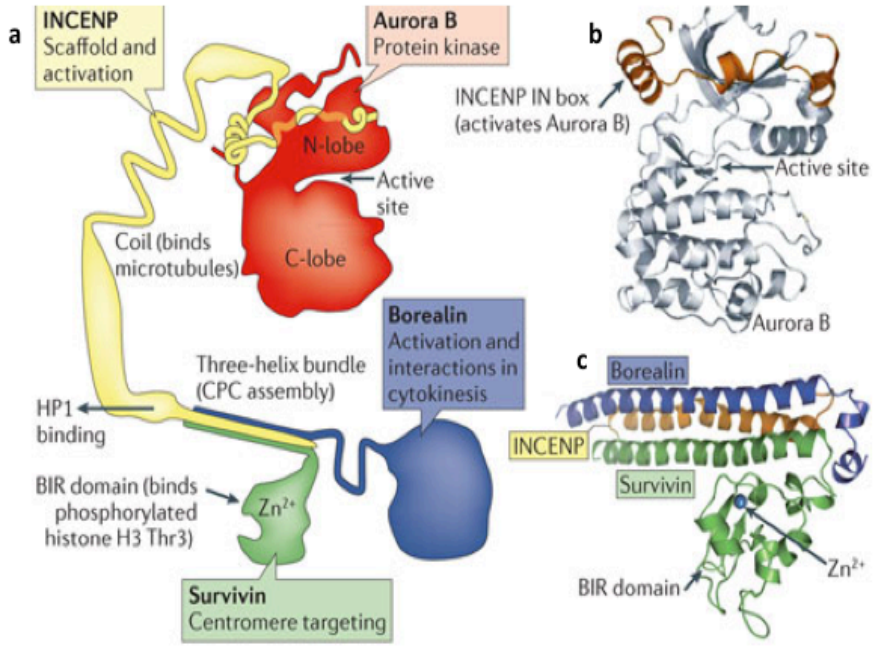


Figure 3 A) Diagram of the Chromosomal Passenger Complex, formed by Aurora B, inner centromeric protein (INCENP), survivin and borealin. The function of each module is displayed. B) The crystal structure of the scaffold module INCENP with Aurora B kinase. C) The crystal structure of the three helix bundle of survivin, borealin and INCENP. Adapted from Carmena *et al* 2012.

As shown in Figure 3, the kinase module contains the enzymatic Aurora B, which docks with the C-terminal highly conserved IN box of INCENP. The localisation module comprises of the INCENP N-terminus, survivin and borealin, which interact in a three-helix bundle (Jeyaprakash *et al* 2011). This structure allows for the 'Baculovirus IAP repeat' (BIR) domain of survivin and the C-terminus of borealin to remain exposed within close proximity. Targeting of this complex is therefore achieved through the recognition of substrates through these two domains (Jeyaprakash *et al* 2007, Gerben Vader *et al* 2006). Survivin can also interact with Aurora B directly suggesting it could perform a regulatory function over the enzyme (Wheatley *et al* 2001).

At the onset of mitosis, the CPC kinase domain is activated, enabling for its dynamic re-localisation in co-ordination with each stage of the cell cycle, to phosphorylate key cell cycle substrates. The precise movement of the CPC throughout mitosis/cytokinesis provides an effective means of activating distinct proteins at specific stages of the cell cycle (Carmena *et al* 2012). The CPC initially accumulates at centromeres, where it corrects aberrant kinetochore-microtubule attachments resulting in accurate chromosome alignment at the metaphase plate and cell cycle progression into anaphase (Ruchaud *et al* 2007). At the metaphase-anaphase transition it then relocates to the cytoplasmic mid-body where it promotes the formation of the cleavage furrow, shortening of spindle microtubules thus separating sister chromosomes, the reformation of the nuclear envelope and then abscission of the two resultant daughter cells (Kitagawa *et al* 2015).

1.3. Programmed cell death

Programmed cell death (PCD) defines a set of molecular pathways that stimulates cell death in response to a series of death promoting signals (Lockshin *et al* 1974). Cell numbers within an organism is tightly controlled through a collaboration of the processes of PCD and cell division. Primarily, cell death is a means to sustain tissue integrity and homeostasis through the removal of old, infected or mutated cells (Fuchs *et al* 2015). Inappropriate cell death (excessive or lack of) can play a role in neurodegenerative diseases, autoimmune disorders and the formation of aberrant cells, thus malignant transformation in cancer (Elmore, 2007). PCD also plays a very important role in embryonic development; removing organelles that are no longer required and sculpting various organs and limbs such as the digits (Jacobson *et al* 1997).

The main types of PCD include apoptosis, necrosis and autophagic cell death. The type of cell death stimuli the cell receives can contribute to the type of PCD a cell executes. It is generally accepted that apoptosis forms the most important and prominent means of cell death; through systematic and defined cellular elimination (Elmore, 2007.)

1.3.1 Apoptosis

Apoptosis is the most well characterised form of PCD, so much so that the terms are used inter-changeably. It is active during development, removing cells to allow for organ and digit formation (Jacobson *et al* 1997), and throughout the normal process of cell turnover to maintain appropriate healthy cellular levels (Fuchs *et al* 2015). It also acts as a defence mechanism to prevent damaged or aberrant cells becoming too prevalent within the population. Key traits of apoptosis

include cell shrinkage, membrane blebbing, chromatin condensing and nuclear fragmentation through endonuclease cleavage resulting in the formation of apoptotic bodies (Kerr *et al* 1972, Elmore 2007). It is generally considered that one of the first stages of apoptosis is the exposure of phosphatidylserine upon apoptotic bodies resulting in their engulfment by phagocytic cells such as macrophages. This acts as an effective mechanism of disposing of the cell waste, which triggers no inflammatory response (Fadok *et al* 1992).

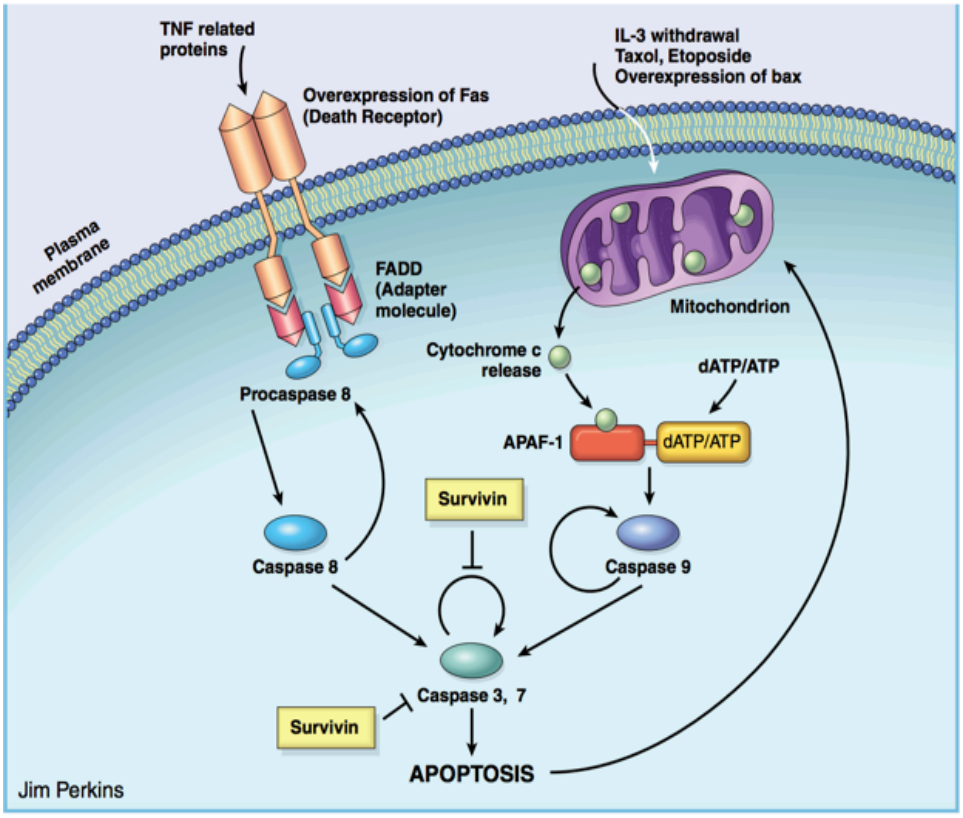


Figure 4. The extrinsic and intrinsic pathways of apoptosis. The extrinsic pathway responds to extra-cellular pro-death signals via cell death receptors at the plasma membrane, which then activate executioner caspases. The intrinsic pathway is centred at the mitochondria, and responds to intra-cellular death signals, releasing cytochrome c to achieve the cell death response. Adapted from Escuín *et al* 1999.

Apoptosis is co-ordinated by a class of serine proteases called caspases, which upon activation cleave target proteins at aspartic acid residues to co-ordinate the effects of apoptosis

(Cryns *et al* 1998). To ensure apoptosis does not trigger inappropriately, they are produced and held in an inactive state until activation by a pro-death signal, achieved through cleavage by pro-caspases or activation by dimerization. A cascade of activation then occurs, rapidly amplifying the pro-death signal; once a cell death signal is activated it is therefore hard to stop the process (Thornberry *et al* 1998). Apoptotic mechanisms are exceedingly complex but can be classified into two pathways, the intrinsic and extrinsic pathway (Figure 4). Originally thought to be distinct in their action, it is now a generally accepted view that cross talk occurs between them (Roy *et al* 2000).

The extrinsic pathway is activated through external pro-death signals acting upon cell surface death receptors belonging to the Tumour necrosis family (TNF) (Locksley *et al* 2001). Members of this family, including the receptors TNF 1/2, Fas, and DR 4/5, all share an 80 amino acid cytoplasmic death domain essential for the transmission of extra-cellular pro-death signals to intra-cellular pathways (Ashkenazi *et al* 1998). Association of a death ligand to its respective receptor triggers the formation of the death-inducing signalling complex (DISC) by death domain dimerization, resulting in the binding, cleavage and activation of pro-caspase 8 (Wajant *et al* 2002). Once apoptosis is triggered, executioner caspases 3 and 7 are cleaved and activated. The most noted death ligand/receptors are the FasL/FasR, TNF-*α*/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 systems (Elmore 2007).

The intrinsic pathway is based upon a network of non-receptor mediated signals centred through the mitochondria, a multifaceted organelle responsible for both cell growth and cell death. The mitochondrion not only triggers cell death, but also

takes a vital role in the decision of cell death commitment through the numerous pro-death pathways that spatially converge there (Wei *et al* 2001). The intrinsic pathway is activated by a variety of intracellular cell stimuli, such as DNA damage, growth factor withdrawal (Elmore, 2007) or oxidative stress promoting the accumulation of reactive oxygen species (ROS). High concentrations of ROS damage cellular macromolecules and proteins, which not only triggers apoptosis but also leads to mitochondrial impairment (Ott *et al* 2007). These stimuli cause drastic changes in the inner mitochondrial membrane; opening mitochondrial permeability transition (MPT) pores leading to a loss of membrane potential and the release of pro-apoptotic proteins (cytochrome c, Smac/DIABLO and apoptosis-inducing factor) in a mechanism referred to as 'mitochondrial outer membrane permeabilization' (MOMP) (Saelens *et al* 2004). Cytochrome c then binds to Apaf-1 (Apoptotic protease activating factor 1) and pro-caspase 9 in the presence of ATP, causing their oligomerization into a heptamer known as the apopotosome (Pan *et al* 1998). The grouping of pro-caspase 9 in this way leads to caspase 9 activation hence effector caspases 3 and 7. This therefore stimulates apoptosis initiation but also severely compromises the mitochondrial electron transport chain through the generation of damaging superoxide ions, thus impairing the dying cells energy production capabilities (Cai *et al* 1998).

Control of the intrinsic pathway and MOMP is orchestrated by a collection of proteins known as the Bcl-2 protein family. The levels of their pro- and anti-apoptotic proteins localised at the mitochondria determine whether a cell survives or initiates cell death. Up to twenty-five Bcl-2 proteins have been identified,

sharing from one to four of the BCL-2 homology (BH) domains essential for their activity (BH1, BH2, BH3, BH4) (Cory *et al* 2002). The activation of the two pro-apoptotic proteins BAX and BAK are essential for MOMP, oligomerizing to open pores in the mitochondrial outer membrane causing cytochrome c release (Gross *et al* 1998, Wei *et al* 2000). Numerous pro-apoptotic and anti-apoptotic Bcl-2 family proteins control the activation of BAX and BAK, the most notable of which are Bid and Bim. The pro-apoptotic protein Bid provides an essential cross talk between the extrinsic pathway and MOMP, caused by activation of the Fas pathway. Fas activates caspase 8, which cleaves Bid into its truncated form tBid, resulting in the inhibition of BAX and BAK inhibitors and subsequent cytochrome c release and apoptosis activation (Cory *et al* 2002, Wei *et al* 2001).

A variety of other proteins regulate the apoptotic process by inhibiting both the intrinsic and extrinsic pathways, the so called 'Inhibitor of apoptosis protein' family (IAPs) (Deveraux *et al* 1999), characterized by the presence of at least one BIR domain (Baculovirus IAP repeat). Of the 8 identified inhibitors, four contain three BIR domains; c-IAP1, c-IAP2, XIAP, NAIP, whilst ML-IAP, survivin, Bruce and hILP2 contain only one. The BIR domain consists of a 70aa segment folded into four α-helixes and three β-sheets. Inhibitors of apoptosis are thought to bind directly to caspases 3, 7 and or 9, preventing their activation (Verdecia *et al* 2000).

1.3.2 Necrosis

In contrast to the controlled process of apoptosis, a more toxic form of cell death can occur, so called Necrosis or Necroptosis. Originally thought to be a passive and accidental, recent

evidence now suggests that it is a regulated means of cell death. A variety of upstream pro-and anti-death signals are shared in both apoptosis and necroptosis, including cellular inhibitors of apoptosis and pro-death receptor/ligands complexes (Linkermann *et al* 2014). Necroptosis activation results in the formation of the necrosome, an intracellular amyloid-like structure that acts as a cell death signalling transducer. The initiation of necroptosis causes the features of necrosis: cellular swelling, plasma membrane rupture and cell leakage. Because of this, necroptosis is linked to a variety of neurodegenerative diseases (Dunai *et al* 2011).

1.3.3 Autophagy

Autophagy is primarily a catabolic mechanism of recycling to dispose of damaged or redundant cell matter in response to anti-survival signals or the presence of harmful substances released through mitochondrial damage or stress (Xie *et al* 2007). This process of recycling is not only used to produce energy and supplement building materials for new cellular constructs, but also to promote cell survival through the removal of potentially damaging cellular matter (Debnath *et al* 2005). Autophagy is therefore essential for cell homeostasis through the maintenance of fully functioning cellular material. Defects in autophagy can result in diseases such as cancer, neurodegenerative and inflammatory diseases (Levine *et al* 2008).

Even though autophagy is a pro-survival mechanism, if prolonged it can result in cell death, termed 'autophagic cell death' or PCD II. Initial evidence for this phenomenon arose from observations of high incidence of cell death associated with large numbers of autophagosomes and active autophagy,

however no definitive evidence has been found to link autophagy directly to a specific mechanism of cell death (Yonekawa *et al* 2014). It is unclear if autophagy promotes cell death directly, collaborates with another process such as apoptosis, or if cell death occurs due to increased degradation of cellular components (Denton *et al* 2012, Debnath *et al* 2005).

Three types of autophagy occur in mammalian cells: macro-autophagy, micro-autophagy and chaperone-mediated autophagy (CMA), all of which support lysosomal degradation. Macro-autophagy is defined by the shuttling of cytoplasmic components to a lysosome through an intermediate double membrane structure called an autophagosome. These fuse with a lysosome, forming an autolysosome, delivering its contents and facilitating their degradation (Xie *et al* 2007). This is in contrast to micro-autophagy, which involves a direct engulfment of small cytosolic components by lysosomes through extrusions of the lysosomal membrane. In CMA, chaperones are used to identify proteins for degradation, which are then directly transported to the lysosome (Parzych *et al* 2014). Due to the understanding of how macroautophagy plays roles in disease, the mechanism of macroautophagy degradation will be further elaborated.

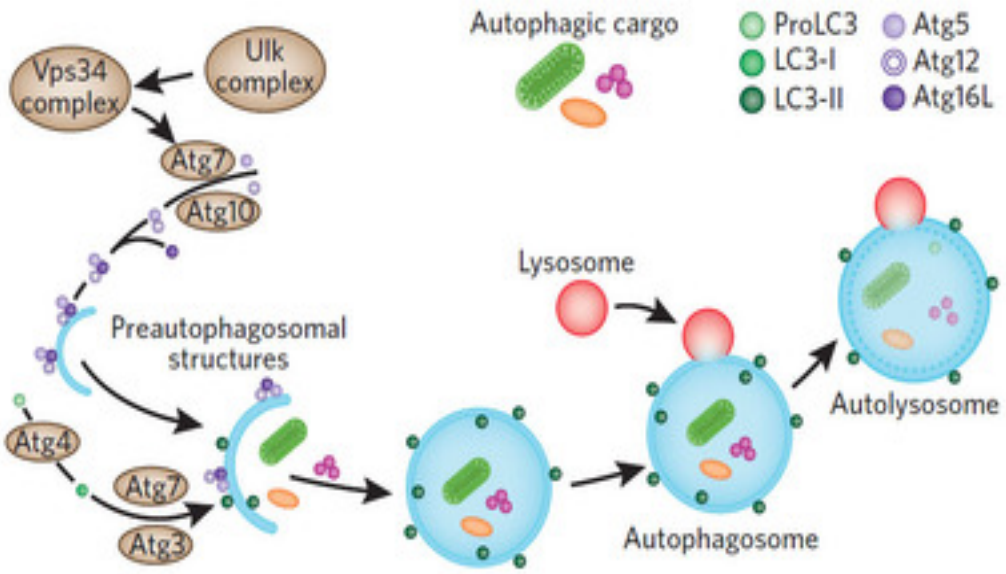


Figure 5. Overview of autophagy in mammalian cells. Formation of pre-autophagosomal structures occurs through a combination of unc-51-like kinase 1 and 2 with Vsp34. Expansion of the membrane occurs through the Atg5-Atg12-Atg16L and LC3 systems. The autophagosome then fuses with a lysosome to degrade engulfed molecules. Adapted from Fleming *et al* 2011.

The origin of phagophore formation in mammalian cells is suggested to occur through one of two mechanisms: either from organelles such as the endoplasmic reticulum (ER) or Golgi apparatus (Hayashi-Nishino *et al* 2009), or the through their de novo formation. However, a lack of organelle membrane proteins within phagophores highlights their de novo formation as more probable (Rubinsztein *et al* 2012). The phagophore formation proteins were identified in yeast as the Atg protein family, with mammalian homologues identified soon after (Matsuura *et al* 1997, Mizushima *et al* 1998). Autophagy commences through cell signalling stimulation of two mammalian homologues of Atg1, unc-51-like kinase 1 and 2 (ULK1, ULK2), which are essential for autophagosome formation (Yang *et al* 2010). ULK1 and 2 complex with Vps34 (mammalian class phosphatidylinositol 3-kinase, PI3K) to

initiate the formation of phagophore assembly sites/pre-autophagosomal structures (PAS). Vps34 along with its partner Beclin-1, selectively uses PI to produce PI3K, which is supplemented to the double membrane structure of the pre-autophagosomes (Funderburk *et al* 2010). The phagophore membrane then expands through the activity of two ubiquitin-like conjugation systems, the Atg5-Atg12-Atg16L and the LC3 system (Figure 5). Atg7, acting as an E1 ubiquitin-activating enzyme activates Atg12, which is transferred to Atg10, an E2 ubiquitin carrier protein that promotes an interaction with Atg5. Coupled Atg5-Atg12 interacts with Atg16L forming complexes that associate with the expanding phagophore membrane (Yang *et al* 2010, Glick *et al* 2010). The second membrane expansion system involves the microtubule-associated protein light chain 3 (LC3). Firstly, LC3 undergoes cleavage by Atg4 to expose a C-terminal glycine residue (LC3-I). This is then activated by the E1-like Atg7 and transferred to the E2 like carrier protein Atg3, before a conjugation with the glycero-phospholipid phosphatidylethanolamine (PE) to form LC3-II (Tanida *et al* 2004). PE is a negative membrane-curving agent upon membranes, promoting curvature and the eventual closure of the forming phagophore membrane. LC3-II is then added to the phagophore membrane through a complex formation with Atg16L, which acts as a scaffold for lipid addition to the membrane. Finally the autophagosome fuses with a lysosome, causing the degradation of engulfed molecules (Glick *et al* 2010).

1.4. Survivin

Since its discovery in 1997 (Ambrosini *et al* 1997), our understanding of the functions of survivin has shifted drastically from a simple apoptotic inhibitor (IAP) to a protein that orchestrates numerous cell homeostasis pathways. Survivin is functionally distinct from other anti-apoptotic proteins, in that it not only controls the process of cell death but also cell proliferation through its role as both an apoptotic inhibitor and member of the CPC (Ambrosini *et al* 1997, Altieri *et al* 2008). It is ordinarily expressed at the G2 & M phase of the cell cycle, as a result of which it is highly detected in cells with rapid growth rates, such as foetal tissues, the kidney, lung, liver and also in cancer cells (Altieri *et al* 2003, Escuin *et al* 1999).

Survivin is one of the most tumour specific molecules (Velculescu *et al* 1999), associated with both chemotherapy and radiotherapy resistance and an unfavourable outcome in cancer (Tirrò *et al* 2006). This makes it a highly attractive cancer target for developing treatments such as vaccines and therapeutic drugs. Mounting evidence has demonstrated the crucial roles survivin plays in non-cancerous differentiated cell lines, including T-cells (Okada *et al* 2004, Andersso*n et al* 2015) and endothelial cells (Blanc-brude *et al* 2003). Understanding the complete arsenal of processes in which survivin functions is therefore critical to the development of anti-survivin treatments with minimal impact upon non-malignant cells. Cancer treatment most regularly fails due to a lack of cell death in response to chemotherapy or radiotherapy, in part because of caspase inactivation (Igney *et al* 2002). In this sense targeting apoptotic inhibitors such as

survivin may provide clinical applications that will allow not only the improvement of pre-existing treatments, but also the development of novel ones. The survivin gene transcription inhibitor YM155 has currently reached Phase II clinical trials, highlighting the potential of survivin treatment in cancer (Rauch *et al* 2014).

1.4.1.1 Structure of survivin

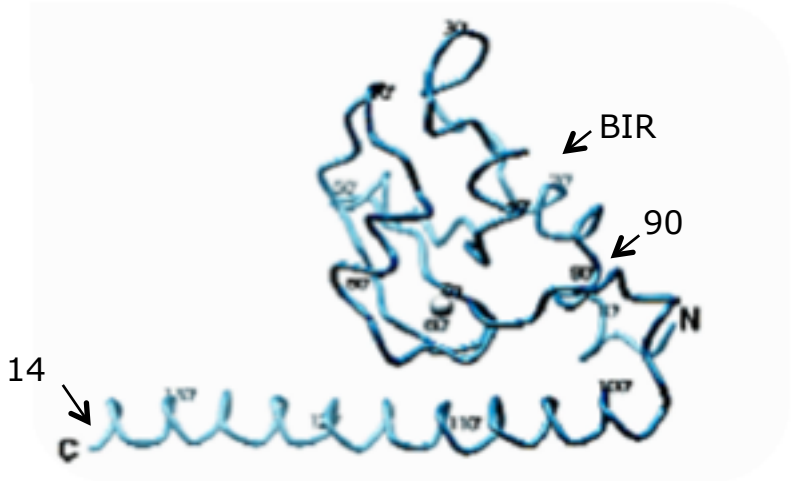


Figure 6. Crystal Structure of survivin showing its BIR domain, a globular region of the protein found linked to a C-terminal α-helix coiled-coil tail. Shown as described by Verdecia *et al* 2000.

Survivin is the smallest member of the inhibitor of apoptosis (IAP) family, a protein of only 16.5kDa in size comprising of 142 amino acids that primarily exists as a homo-dimer *in vivo* (Verdecia *et al* 2000). Its key interacting structure is the Baculovirus IAP repeat (BIR) domain (Figure 6), found linked to an extended COOH-terminal α-helix coiled-coil domain (Altieri *et al* 2006). The 70 essential amino acids of the BIR domain provide the protein with a region that facilitates both its function in the CPC and its anti-apoptotic properties (Carmena *et al* 2012, Ambrosini *et al* 1997). Unique in its structure as an apoptotic inhibitor, it contains a single IAP

repeat and lacks a carboxy-terminal RING zinc-finger domain, unlike other apoptotic inhibitors such as XIAP and cIAP1/2 (Verdecia *et al* 2000).

The human survivin gene, encoded on chromosome 17q25 (Ambrosini *et al* 1997), allows for the formation of four isoforms of survivin, survivin-2α, survivin-2B, survivin-3B and survivin-ΔEx3. Each isoform has its own individual cellular localisation and structure (Mahotka *et al* 1999). The expression of each splice variants its gene encodes is controlled by numerous post-translational mechanisms, including acetylation, ubiquitination and phosphorylation (see 1.4.3) (Vong *et al* 2005, O'Connor *et al* 2000).

1.4.1.2 Survivin localisation

The expression of survivin in both foetal and differentiated cells is normally restricted to G2 phase and mitosis, where its expression increases due to its essential contribution to mitosis. It initially localises at centromeres within the nucleus at the onset of mitosis and upon entry to anaphase it dynamically re-locates to the spindle mid-zone (Wheatley *et al* 2001).

The role of survivin within cancer cells appears to be functionally beneficial to their survival, due to its up-regulation not only within the cell cycle but also throughout interphase (Altieri *et al* 2008). In cancer, survivin is found predominantly either within the nucleus or intermittently within the cytoplasm, with shuttling observed between these pools controlled by the exportin CRM1 (Colnaghi *et al* 2006). The cytoplasmic pool of survivin provides a cytoprotective role for cancer cells; numerous studies have demonstrated that survivin restricted to the nucleus through a mutation in the

nuclear export signal (NES) could not achieve its anti-apoptotic function (Stauber *et al* 2006, Colnaghi *et al* 2006). This therefore highlights the importance of nuclear exportation of survivin from the nucleus to the cytoplasm as a key requirement for its anti-apoptotic function.

A small pool of survivin is also detected in the mitochondria of cancer cells, not present in non-malignant cells (Dohi *et al* 2004). This pool is only detectable biochemically; the cytoplasmic pool normally eclipses its observation under microscopy (Duvajona *et al* 2016). Although the true function of this mitochondrial pool is not yet characterised, evidence suggests it acts as another compartmentalized store of anti-apoptotic survivin, which is released in response to a pro-cell survivial signal (Dohi *et al* 2007). The existence of multiple spatial pools of survivin is consistent with its multifaceted functions in varying complex pathways.

1.4.2 Roles of Survivin

1.4.2.1 Survivin and the CPC

Survivin is a vital component of the CPC necessary for directing the complex at the onset of prometaphase to the centromere, where it can then act to correct faulty kinetochore-microtubule attachments (Gerben Vader *et al* 2006). Its role within the complex is crucial to ensure its correct localisation, and errors within this process can cause catastrophic cellular damage. Targeting of the CPC is enabled through survivin and borealin co-operatively recognising specific mitotic phosphorylation marks on histones within centromeric chromatin (Jeyaprakash *et al* 2007). The cohesin-associated kinase Haspin phosphorylates histone 3 which can be directly bound by its BIR domain, particularly residues H80

and K62 (Niedzialkowska *et al* 2011). The binding of survivin to the complex has been linked with the regulation of the enzymatic component Aurora B, thus also the activity of the CPC (Bolton *et al* 2002).

1.4.2.2 Survivin and apoptosis

The over-expression of survivin has been associated with inhibition of both the extrinsic and intrinsic pathway of apoptosis (Ambrosini *et al* 1997), and it has since been identified that its cytoplasmic pool provides this function (Stauber *et al* 2006, Connell *et al* 2008). The mechanism in which survivin achieves apoptosis inhibition is not fully characterised; research provides evidence for both a direct and an indirect action with apoptotic executioner proteins, caspases. Binding studies have demonstrated an interaction of survivin with effector caspases 3 and 7 (Shin *et al* 2001, Liu *et al* 2004), however survivin lacks structures present in other IAPs that allow for caspase interaction (Riedl *et al* 2001). Survivin also binds to other apoptotic proteins such as XIAP, enhancing their stability and protecting it from proteasomal degradation, which could function to increase their anti-apoptotic activity (Marusawa *et al* 2003).

1.4.2.3 Survivin and Autophagy

The down-regulation of survivin is linked with the induction of apoptosis through mechanisms associated with autophagy. This process has been replicated using the survivin suppressor YM155 in prostate cancer cell lines where the induction of autophagy was observed (Wang *et al* 2011). siRNA treatment of survivin and treatment using Arsenic Trioxide in U118-MG cells also caused a down regulation of survivin and induced autophagic cell death (Chui *et al* 2011). The evidence

henceforth provided suggests that survivin could act as a negative regulator of autophagy.

In addition to this, evidence suggests that survivin interacts with the Bcl2 protein Beclin-1. Beclin-1 phosphorylates and activates Vsp34 to allow formation of the autophagosome (Funderburk *et al* 2010), see Figure 5. It also interacts and is sequestered by pro-survival Bcl2 proteins, which prevent Beclin-1 release and thus inhibit autophagy. Nui *et al* 2010 demonstrated that Beclin-1 knockdown caused a down regulation of survivin which sensitised glioma cells to TRAIL-induced apoptosis, suggesting that a cross talk between the pathways of autophagy and apoptosis.

1.4.2.4 Mitochondrial Survivin

Cancerous cell lines present a pool of biochemically localised survivin within the mitochondria, which cannot be detected in non-malignant cell lines (Dohi *et al* 2004). Until this study, survivin was not known to contain a mitochondrial targeting sequence, and so its mitochondrial import was linked to the action of mitochondrial chaperonal proteins. At least two mechanisms were suggested: survivin binds via its BIR domain to the mitochondrial chaperonal complex Hsp90 (Fortugno *et al* 2003); and survivin interacts with the cofactor aryl hydrocarbon receptor-interacting protein, facilitating its entry into the mitochondria via an interaction with its D142 residue (Kang *et al* 2011). It is also interesting to note that one splice variant of the human survivin gene, survivin-ΔEx3, contains a mitochondrial targeting sequence. However the expression of survivin isoforms during cancer is fairly low, so this may not be relevant (Wang *et al* 2002).

The function of mitochondrial survivin has not yet been characterised, however it is speculated that presence must perform a function within the organelle beneficial to the cancerous state. Apart from its role in energy production, the mitochondrion orchestrates the response to cell death stimuli through the intrinsic pathway of process apoptosis, spatially localised within the organelle. As an IAP, survivin plays a major cytoprotective role preventing the onset of cell death through the inactivation of caspases (Ambrosini *et al* 1997). Dohi *et al* 2004, aimed to investigate the potential benefit that this mitochondrial pool of survivin offers to cancer cells, particularly focusing on the anti-apoptotic function it provides. They found that mitochondrial survivin is released into the cytosol in response to pro-apoptotic stimuli, inhibiting apoptosis due to an interaction with caspase-9. Further investigation (Dohi *et al* 2007) found that the activity of the released mitochondrial survivin is dependent upon its phosphorylation status. Survivin is phosphorylated by protein kinase A (PKA) on serine 20, and is de-phosphorylated on the same residue by the broad-spectrum phosphatase PP2A, found in the mitochondria. Non-phosphorylated survivin released from the mitochondria was found to bind and stabilise the apoptosis inhibitor XIAP. These findings combined provide evidence for a cytoprotective role of mitochondrial survivin.

Mitochondrial survivin appears to also influence mitochondrial functions, both their dynamics and cellular metabolic output. Survivin has been linked to the mitochondrial fission protein Drp1, suggesting an influence over mitochondrial fragmentation (Hagenbuchner *et al* 2013). This alteration in mitochondrial dynamics was also linked to decreased mitochondrial respiration and increased aerobic glycolysis,

through inhibition of respiratory complex I. Generally, one would expect some alterations in both apoptosis and metabolism if survivin influenced mitochondrial integrity. In addition to this, survivin has been shown to support subcellular trafficking of mitochondria to the cortical cytoskeleton of tumour cells. This is associated with an increase in membrane ruffling and focal adhesion complex turnover, increasing the invasive properties of cancer cells (Rivadeneira *et al* 2015).

1.4.3 Regulation

Protein phosphorylation by serine/threonine kinases is one of the major post-translational mechanisms of altering protein activity, acting upon numerous cell-signalling pathways. The effect of phosphorylation upon differing residues of survivin appears to confer drastic changes to its function, allowing it to participate in a range of different pathways (Nogueira-Ferreira *et al* 2013). Phosphorylation of survivin controls its action during its time in the CPC, but also alters its interaction with its binding partners (see Figure 7).

During entry to mitosis, survivin is phosphorylated upon threonine 34 by cycle-dependent kinase 1 (Cdk1) (O'Connor *et al* 2000). T34 is located within survivins BIR domain and upon phosphorylation increases its stability, having distinct effects upon its mitotic and anti-apoptotic functions. A non-phosphorylatable mutant of T34 (threonine to alanine) increased cell growth rate but also induced apoptosis (O'Connor *et al* 2000). In comparison, the phospho-mimetic mutant (threonine to glutamic acid) has opposing effects, with a decreased cell growth rate and increased anti-apoptotic function, promoting cell survivial (Barrett *et al* 2009). The

T34E mutant also inhibited mitosis; suggesting that phosphorylation of this residue plays a role in the control of cell division. T34 phosphorylation therefore appears to preserve cell viability at the onset of cell division and represents a layer of control over mitosis passage.

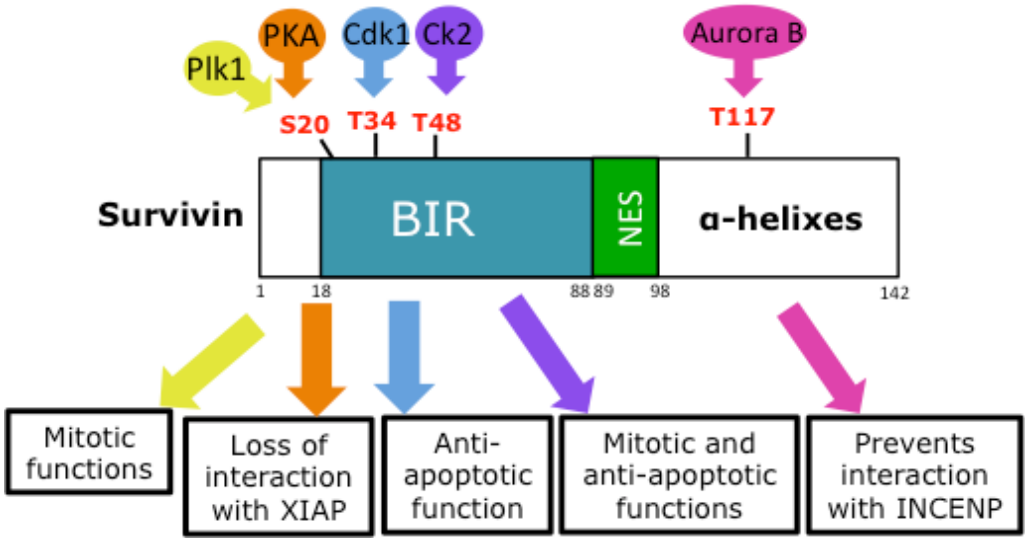


Figure 7. Schematic diagram illustrating the phosphorylation sites of survivin, with corresponding kinases (above) and effects (below). Survivin is phosphorylated by five kinases each with a distinct effect upon its function. Also illustrated is survivins nuclear exportation signal (NES), Baculovirus IAP repeat (BIR) and α-helix region.

Another key BIR domain residue that confers significant alterations in function upon phosphorylation is threonine 48, which is phosphorylated by Casein kinase 2 (Ck2). T48 phosphorylation also plays a role in both its mitotic and anti-apoptotic functions; T48E and T48A cell lines are unable to sustain cell growth or protect cells from TRAIL-induced apoptosis (Barrett *et al* 2011).

The serine 20 residue, located at the start of the BIR domain is phosphorylated by two different kinases. It is phosphorylated by cyclic AMP-dependent protein kinase A (PKA) in the cytoplasm causing a loss of interaction with XIAP,

however non-phosphorylated mitochondrial survivin released from the mitochondrion preserves this interaction. This suggests a compartmentalised pro-survivial function, linking mitochondrial survivin specifically to apoptosis inhibition (Dohi *et al* 2007). S20 is phosphorylated by Polo-like kinase 1 (Plk1), regulating its mitotic functions. Plk1 is implicated in the control over mitotic entry, centrosome separation, spindle assembly checkpoint and cytokinesis. Phosphorylation by Plk1 appears to exert no effect on survivins anti-apoptotic function, but influences the mitotic role in the CPC. A non-phosphorylatable mutant (S20A) is unable to correct incorrect kinetochore-spindle attachments and proceeds through anaphase prematurely. This emphasizes the importance of this phosphorylation event for proper activation of the SAC and for correct chromatid alignment (Colgnahi *et al* 2010).

Aurora-B kinase, the enzymatic protein of the CPC, phosphorylates survivin directly on threonine 117 (Wheatley *et al* 2003, 2007). Phosphorylation at this site regulates the dynamics of the CPC and therefore kinetochore-spindle microtubule attachments. A T117A mutation causes no alteration to the function of survivin, however the phosphomimetic mutation T117E localises incorrectly at every stage of mitosis, and prevents an interaction with the CPC member INCENP.

1.5.1 Mitochondrial structure and function

Described as the cellular powerhouse, the mitochondrion is an organelle present within all eukaryotic cells that produces the majority of the energy rich molecule adenine triphosphate (ATP) required for cellular growth and survival. Distinct in its structure, it is double membrane bound containing two enclosed spaces, the internal matrix space and the inter-membrane space (Figure 8). Infolding of the inner membrane produces the cristae, in which the proteins of the electron transport chain are housed required for oxidative phosphorylation (Palade, 1953). This distinctive structure of the mitochondrial membranes provides the organelle with properties that enable a wide range of metabolic processes, from fatty acid metabolism and the Krebs cycle to Haem biosynthesis (Pfanner *et al* 2000).

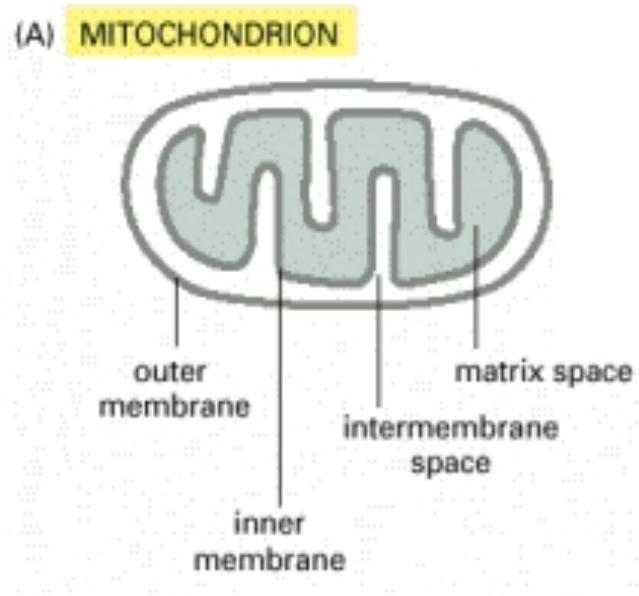


Figure 8. Diagram of a mitochondrion. Adapted from Molecular Biology of the Cell fourth edition

1.5.2 Protein transport into the Mitochondria

Separating specialised processes into compartments presents an issue when transporting newly synthesised proteins to the correct localisation. Only small selections of essential genes are encoded within the mitochondrial genome (Sickmann *et al* 2003), the rest are determined by the nucleus and must be inserted post-translationally into the correct region of the mitochondria (Hallermayer *et al* 1977). Most mitochondrial proteins therefore have an import signal peptide, which allows their transport across either the outer or both mitochondrial membranes. Generally, these targeting sequences are NH_2 terminal, positively charged amphipathic α-helixes, which are cleaved post-insertion (Vögtle *et al* 2009). With the help of chaperonal complexes, proteins can reach all the 4 regions of the mitochondria, docking and passing through receptor complexes at the outer membrane (TOM20 and TOM70) (Rapaport 2005), before being sorted through complexes into either the inner membrane (TIM22 and TIM23) or back to the outer membrane (SAM) (Pfanner *at al* 2001). The chaperonal complex Hsp70 in yeast, or Hsp90 in humans is largely responsible for ensuring that proteins are kept in an unfolded transport efficient state until import into the mitochondria (Wiech *et al* 1992, Young *et al* 2003).

1.6. Glycero-phospholipids

Recent research within the Wheatley Lab has identified the glycero-phospholipid conversion enzyme phosphatidylserine decarboxylase (PSD) to be a direct binding partner of the protein survivin. PSD is an inner mitochondrial membrane conversion enzyme responsible for the production of an essential membrane curving agent phosphatidylethanolamine (PE) within the mitochondria (Borkenhagen *et al* 1961) (Osman *et al* 2011). The function of this interaction is currently unknown, however if speculated that survivin manipulates the production of this phospholipid within the mitochondria, this could not only provide a new role of its role within cancer, but also extend its action to metabolic disorders. It is therefore essential to understand the complex field of glycero-phospholipid biosynthesis in order to elucidate the function of the interaction of survivin with this enzyme.

1.6.1 Introduction to Glycero-phospholipids

Lipids are a diverse group of molecules, which play key roles in an extensive network of biological processes. It is widely understood they comprise the major structural component of the lipid bi-layer, which acts as a semi-permeable barrier for both organelles and the outer plasma membrane, whilst providing a structural scaffold for proteins housed within them (Dowhan *et al* 1997). Glycero-phospholipids are amphipathic molecules whose structure is centred around a glycerol backbone containing two ester linked fatty acid tails at sn-1 and sn-2 positions, with a varying hydrophilic head group at the sn-3 position (Osman *et al* 2011) (9A).

Glycero-phospholipids are remarkably diverse molecules, varying their structure through the presence of differing head groups, including but not limited to choline, ethanolamine and serine. In mammalian cells the most abundant glycerol-phospholipid present in biological membranes (40-50%) is phosphatidylcholine (PC). The second (15-20%) is phosphatidylethanolamine (PE), followed by phosphatidylserine (PS) accounting for 5-10% of membrane composition (Osman *et al* 2011, Vance *et al* 2015). Each head group provides key structural differences to the phospholipid, generating a range of varying properties to be utilised.

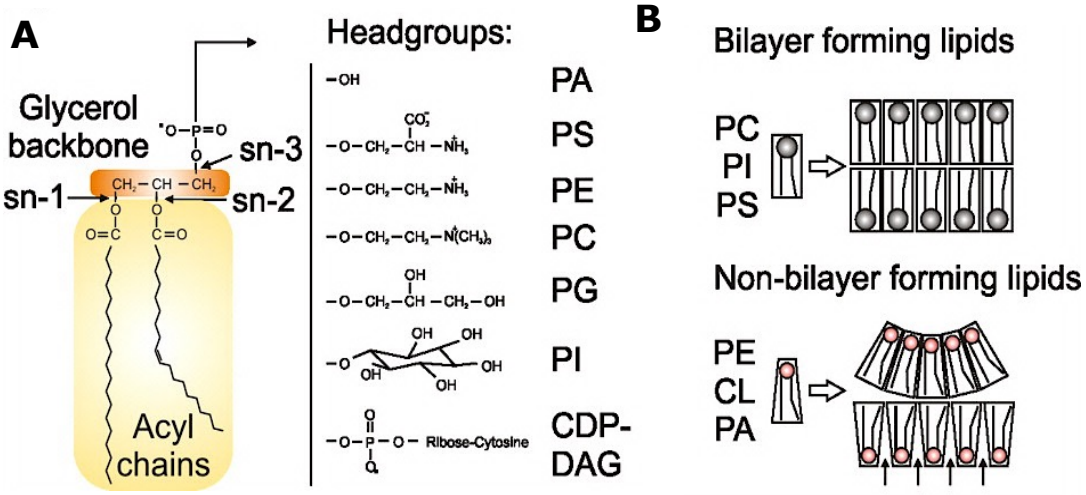


Figure 9. Schematic representation of glycero-phospholipid structure within bi-layers. A) The phospholipid structure is centred on a glycerol backbone, with acyl chains at the sn-1 and sn-2 positions, and a phosphate group/varying head domain at the sn-3 position. B) Influence of non-bilayer and bilayer phospholipids within biological membranes. Non-bilayer phospholipids and cardiolipin (CL) generate negative tension, curving the membrane. Adapted from Osman *et al* 2011.

As previously stated, glycero-phospholipids are key components of the lipid bi-layer, the structural backbone of the vast range of biological membranes. Thus, alterations to the phospholipid composite of lipid bi-layers can provide defining physical changes to membranes, which can be key in influencing spatially localised biological processes. Indeed

many membrane properties are governed by their lipid composition, including hydrophobicity, rigidity and thickness, and it has been demonstrated that these characteristics vary drastically between organelles (Vance *et al* 2015). The presence of 'non-bilayer forming' phospholipids within membranes can create these fore-mentioned changes to their structure. PE is a non bi-layer forming lipid; the presence of small hydrophilic ethanolamine head group gives it a conical shape, allowing the formation of hexagonal phases (Hitchcock *et al* 1974). This is in comparison to the cylindrical shape of PC; the perfect shape to form uniform lipid bi-layers (Figure 9 B). The presence of non bi-layer lipids (such as PE) within membranes generates negative tension, causing membrane curvature (Osman *et al* 2011). This is essential for many membrane bound processes that rely upon membrane bending for their action, such as mitochondrial membrane fusion/fission and protein-membrane insertion (van den Brink-van der Laan *et al* 2004).

However, classifying phospholipids as merely structural molecules within membranes is a grave understatement; their contribution to an extensive range of biological processes is becoming vastly more apparent. The conversion of one lipid to another involves a complex network of interactions that makes up the process of lipid biosynthesis. This process creates a wide range of biological intermediates that have roles in processes such as cell growth, survival, proliferation and apoptosis (Vance *et al* 2015). Alterations in lipid biosynthesis cannot only disrupt these processes but also alter membrane structure, integrity and permeability. This has been suggested to accelerate disease progression such as in cancer (Santos el al 2009), and also widely impact the structure and function of

the mitochondria having implications in both Alzheimer's and Parkinson's disease (Prasad *et al* 1998).

It is apparent that current research, particularly in the field of cancer, relies too heavily upon protein influence upon disease morphology with too little focus upon lipid biosynthesis alterations. As reviewed by Baenke *et al* 2013, the role of lipid biosynthesis in both cancer metabolism and tumour growth is becoming much more evident, with more research beginning to filter into the topic.

1.6.2 PS function and production

The synthesis of PS in mammalian cells occurs solely through calcium-dependent base-exchange reactions in which head groups of pre-existing phospholipids (PC or PE) are exchanged for L-serine (Vance *et al* 2013). This occurs through one of two PS synthases localised to a sub-fraction of the ER called mitochondrial-associated vesicles (MAMs) (Stone *et al* 2000). The synthases PSS1 or PSS2 catalyse PC and PE conversion respectively (Hübscher *et al* 1959).

Apart from its role in biological membranes, phosphatidylserine allows for the recognition of apoptotic cells by phagocytosis (Fadok *et al* 1992). It becomes exposed upon the external face of the plasma membrane during early apoptosis and acts as a signalling molecule to be recognised by macrophages. Interestingly, PS is also a membrane target for some proteins such as the tyrosine kinase Src, as well as Ras and Rho GTPases. Key positively charged sequences bind to the negatively charged PS head group, allowing their effective targeting and so also activation (Sigal *et al* 1994). PS also acts as a vital precursor for the formation of PE through

the decarboxylase pathway of Phosphatidylserine decarboxylase (PSD) (Voelker *et al* 1997).

1.6.3.1 PE production by Phosphatidylserine Decarboxylase

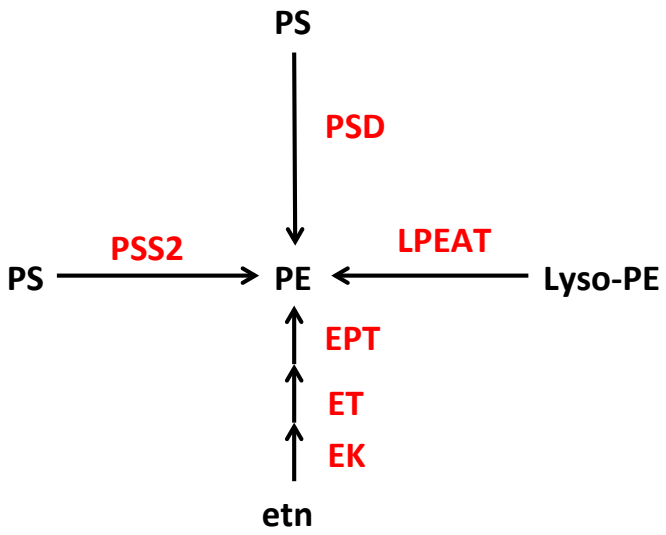


Figure 10. Schematic diagram illustrating the four mechanisms of Phosphatidylethanolamine production. Production can occur via PSS2 in mitochondrial-associated membranes, by phosphatidylserine decarboxylase (PSD) within the mitochondria, the ER localised Kennedy pathway or by lyso-PE acyltransferase (LPEAT)

In eukaryotic cells, PE is produced by one of four mechanisms; the exchange of choline to ethanolamine by PSS2 in mitochondria-associated membranes, PS decarboxylation by the mitochondrial enzyme phosphatidylserine decarboxylase (PSD), a series of reactions which make up the endoplasmic reticulum (ER)-localized CDP-ethanolamine (Kennedy) pathway or acylation of lyso-PE by lyso-PE acyltransferase (LPEAT) (Figure 10) (Vance *et al* 2013).

The main biosynthetic pathway that contributes to PE production is the enzyme PSD. Located on the external side of the inner mitochondrial membrane (IMM) it de-carboxylates PS to generate PE (Borkenhagen *et al* 1961) (Figure 11 A), using an unusual pyruvoyl prosthetic group for its activity (Zborowski *et al* 1983). PSD is produced as an inactive proto-

enzyme that is self-cleaved to create its mature form. Removal of its mitochondrial targeting sequence and inner membrane sorting sequence, before cleavage at a conserved C-terminal LGST sequence generates the final alpha and beta subunits (Schuiki *et al* 2009) (Figure 11 B).

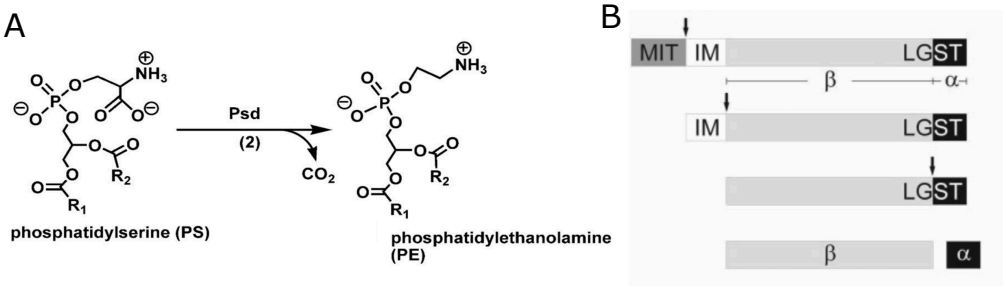


Figure 11 A) The mechanism of phosphatidylethanolamine (PE) production from phosphatidylserine (PS) by phosphatidylserine decarboxylase (PSD). PS is de-carboxylated by PSD producing CO_2 and PE. Adapted from Sohlenkamp *et al* 2016. B) Cleavage steps for the production of the mature PSD alpha and beta subunits. Adapted from Schuiki *et al* 2009.

1.6.3.2 Glycero-phospholipid transport

The production of PE requires the shuttling of its precursor phospholipid PS into the mitochondria from its production site in the endoplasmic reticulum (ER) and linked mitochondrial associated vesicles (MAMs). The shuttling of lipids between organelles occurs either through a direct interaction of their membranes at contact sites, or through a vesicular transport mechanism (Vance *et al* 2015). Evidence also suggests that cytosolic chaperone proteins such as VAT-1 may play a role in PS transport, however it seems more likely that direct contact facilitates the majority of PS transport as this mechanism avoids exposing hydrophobic PS to an aqueous environment (Junker *et al* 2015). On the other hand, transport of PE into the mitochondria seems to be restricted due to the preferential formation of mitochondrial localised PE by the enzyme PSD.

The two largest contributors to PE biosynthesis are compartmentalised in separate organelles, producing spatially different pools: PSD in the mitochondria and the Kennedy pathway in the ER. It has become evident that these pools are specific to their biosynthetic origin, emphasised through the lethality of mitochondrial PSD deletion in mice despite compensation by the fully functioning Kennedy pathway (Steenbergen *et al* 2005). It has been reported that PE transport from the ER to the mitochondria via the Kennedy pathway is not sufficient to meet the energetic demands of the cell (Trotter *et al* 1995). Most importantly, the Kennedy pathway does not appear to transport PE from the ER in a large enough quantity to the inner mitochondrial membrane where PE concentration is highest, only to the outer mitochondrial membrane (Bürgermeister *et al* 2011). This may be due to the presence of PSD within the IMM; a need for PE transport has never evolutionally arisen.

As the mitochondria have maintained the production of their own pool of PE rather than evolving a mechanism solely based upon transporting it from other sources, it is likely that this lipid is vital to normal mitochondrial functions. Evidence to support this theory is based upon multiple studies showing a reduction in mitochondrial PE has a negative effect on cell growth and mitochondrial morphology (Tasseva *et al* 2013, Böttinger *et al* 2012).

1.6.3.3 Roles of PE within the mitochondria

As previously discussed, the function of PE as a negative membrane-curving agent is particularly important within the mitochondria, where high concentrations are present within the IMM allowing for the formation of cristae (Osman *et al*

2011). This specialised structure within the mitochondria allows for efficient interactions between quaternary structures of respiratory chain complexes, called super-complexes (Cogliati *et al* 2013). The presence of PE determines the shape of the cristae therefore the assembly and efficiency of these super-complexes. PE also influences the maintenance, production and integrity of respiratory super-complexes within the mitochondria, either directly or indirectly through conservation of the cristae. A reduction in PE causes a decrease in CI and CIV respiratory complexes in the mitochondria, and shows defects in their organization into super-complexes (Tasseva *et al* 2013).

The cycle of mitochondrial fusion and fission events also relies highly upon membrane curvature and so the presence of PE for its effective completion, allowing for the production of a dynamic inter-connected chain of mitochondria (Frohman, 2015). The deletion of the PE biosynthesis enzyme PSD results in abnormal mitochondrial fusion and fission events, highlighting the need for mitochondrial PE in this process (Tasseva *et al* 2013). The resulted decrease in cellular PE also reduced liposome mixing with the mitochondria, suggesting the fusion defect could be directly explained by alterations in the physical properties of the inner mitochondrial membrane.

1.6.3.4 Alternate functions of cellular PE

Mitochondrial PE has a variety of cellular destinations: remaining within the mitochondria, transported to a different cellular location such as the plasma membrane (Vance *et al* 1991) or used as a precursor for the formation of PC. The role of PE as a membrane-curving agent means it is indispensible in processes where sharp membrane curvature is required. It

has also been implicated in other membrane bound processes such as vacuole fusion (SNARE proteins) in yeast (Zick *et at* 2014) and at the cleavage furrow in cytokinesis (Emoto *et al* 1996).

In addition to this, PE is a great source of ethanolamine donation for protein modification. It is utilised in the synthesis of glycosylphosphatidylinositol anchors on the plasma membrane, in which numerous signalling proteins attach to (Menon *et al* 1992). This function also relates to a previously mentioned role in autophagy; ethanolamine is conjugated with LC3-I and then inserted into the phagophore membrane promoting its curvature and phagosome formation (Glick *et al* 2010).

Lastly, like PS, PE is exposed to the external side of the plasma membrane in response to apoptosis, but unlike PS this response has not been correlated with a particular function (Emoto *et al* 1997).

1.7 Cellular-Src (c-Src)

It has been postulated within the Wheatley Lab that a second novel binding partner of the protein survivin is the tyrosine-kinase c-Src. This interaction was first hypothesised due to the recognition of the first ten amino acids of survivin as a PPII helix, combined with evidence suggesting alterations in the adhesive properties of HeLa cell lines expressing the NH_2 terminal of survivin (Dujuvanova *et al* 2016). PPII helixes bind with high affinity to SH3 domains such as present in c-Src, reasonably suggesting that the two proteins may interact (Adzhubei *et al* 2013). It is therefore essential to appreciate the complex network of process the tyrosine kinase c-Src functions within, to therefore understand the role of its interaction with survivin.

1.7.1 Introduction to c-Src

The proto-oncogene c-Src (cellular Src) is a non-receptor tyrosine kinase encoded in humans by the SRC gene, encompassed as part of the Src-family of tyrosine kinases (SRKs). First identified as the human cellular version of v-Src, the product of the avian tumour virus 'Rous sarcoma virus' (Rous, 1911) (Brown *et al* 1996), it is expressed in a ubiquitous manner across all tissue types. C-Src works downstream of various cell signals detected by a wide range of key cell surface receptors, forming a pivotal part of signal transduction pathways involved in cell proliferation, differentiation, migration and F-actin cytoskeletal arrangement (Thomas *et al* 1997).

1.7.2 Localisation

The SRKs (Src family kinases) are customarily found localised to the internal face of the plasma membrane, enabling an interaction with external cellular signals (Thomas *et al* 1997). However recent evidence has highlighted the importance of the SRK family in numerous subcellular compartments such as the mitochondria and nucleus. Ogura *et al* 2012, reported that depletion of Src activity or targeting of a kinase-defective Src into the mitochondria caused defects in respiratory chain proteins, and that c-Src was essential for oxidative phosphorylation through the phosphorylation of the respiratory chain proteins. It is also apparent that c-Src and other SFKs are implicated in chromatin re-organisation in the nucleus (Takahashi *et al* 2009).

1.7.3 c-Src Structure

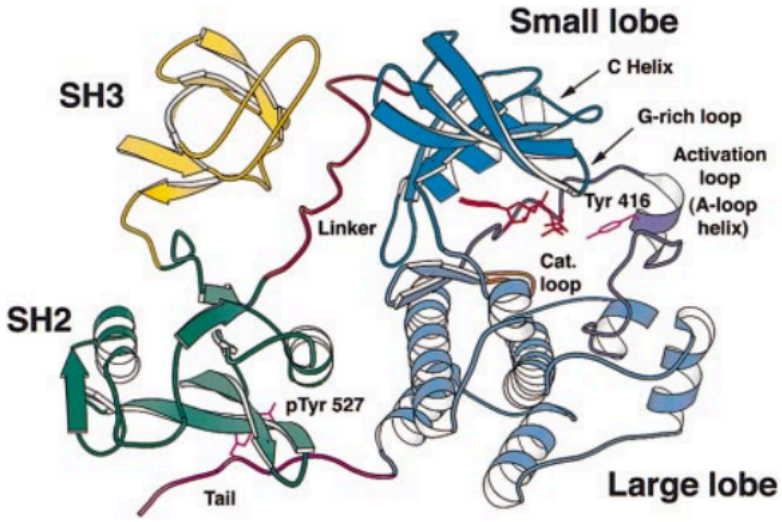


Figure 12. Ribbon diagram illustrating c-Src structure, showing SH2 and SH3 domains, activating residue Tyrosine 416, and inactivating residue Tyrosine 527. Taken from Xu *et al* 1999.

The structure of Src comprises of: from N- to C- terminal, an NH_2-terminal unique domain, an SH3 domain, SH2 domain, a protein-tyrosine kinase domain and a C-terminal regulatory

tail (Xu *et al* 1999) (Figure 12). The SH2 and SH3 domains are responsible for the majority of its protein-protein interactions; SH3 domains bind to left-handed helical sequences normally rich in prolines (Adzhubei *et al* 2013). The kinase domain contains the catalytic unit of the protein, phosphorylating tyrosine substrates associated with its corresponding targets to signalling pathways.

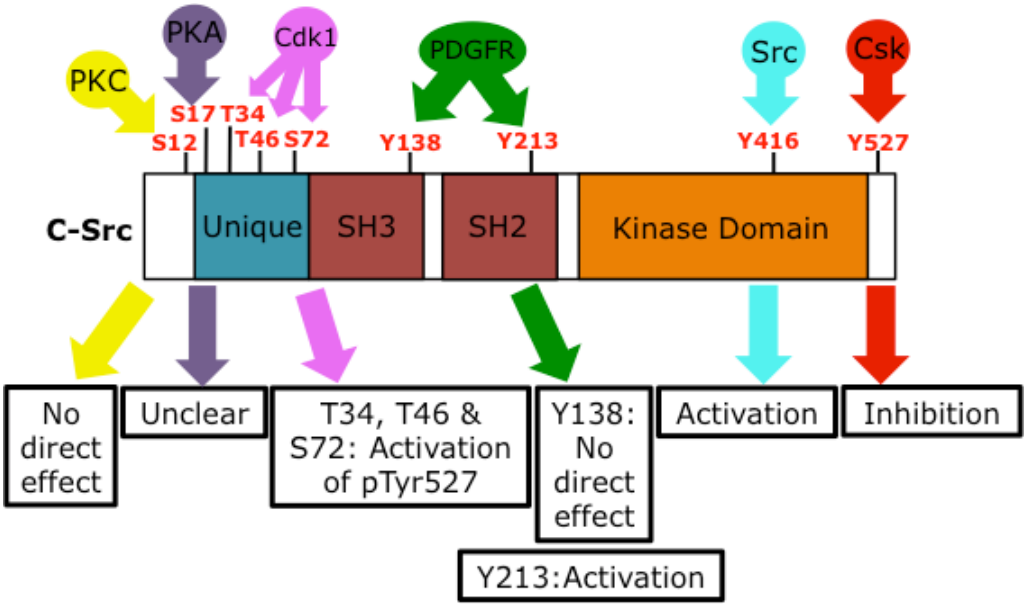


Figure 13. Schematic diagram illustrating c-Src structure, key domains, phosphorylation sites, kinases responsible (above) and effects (below). Protein Kinase C phosphorylates serine 12, Protein kinase A serine 17, Cyclin-dependent kinase 1 threonine 34, threonine 46 and serine 72, platelet-derived growth factor receptor tyrosine 138 and tyrosine 213, c-Src auto-phosphorylates tyrosine 416 and CDK-activating kinase tyrosine 527. Domains also listed include the N-terminal unique domain, SH2 and SH3 domains and the protein kinase domain. Adapted from Roskoski 2005.

C-Src is phosphorylated at two sites allowing for its activation or inactivation, tyrosine 416 or tyrosine 527 respectively (Roskoski *et al* 2004) (Figure 13). It is produced in the cytoplasm and under normal circumstances held in an inactive state through binding of two intramolecular regions: the C-terminal CSK-phosphorylated tyrosine (Tyr527) with the SH2 domain, and an interaction of a PPII (polyproline type II)

helical motif within the SH2 domain to the SH3 domain (Cowan-Jacob *et al* 2005).

1.7.4 c-Src and Cancer

It is widely understood that Src is both over-expressed and its activation increased in a wide range of human cancers. Not only this, but its activation has been implicated in both the oncogenic transformation and the metastatic properties of some cancerous cells (Irby *et al* 1999, Boyer *et al* 2002). The quantity of c-Src expression and activation also appears to correlate with the stage of cancer progression (Sweeney *et al* 2008).

There are various possible mechanisms implicated in the activation of c-Src, however how each contributes to its deregulation normally observed in cancer is debated. Disruptions in its phosphorylation or the intra-molecular binding interactions of the SH2/SH3 domains disrupts the fine balance of control present within the c-Src structure, resulting in its activation thus stimulating its associated pro-survival pathways (Roskoski *et al* 2004). Phosphatase activity upon Tyr527 acts as an activating stimulus, and phosphatases implicated in this process include PTP-α and PTP1B (Zheng *et al* 2000, Zhu *et al* 2007). Disruption of the kinase Csk responsible for Tyr527 phosphorylation can also have a similar effect. Ligand based interactions, which disrupt the SH2 and SH3 domain intramolecular bindings also act to activate c-Src, and proteins implicated in this mechanism include platelet derived growth factor receptor (PDGFR) and focal adhesion kinase (FAK) (Kypta *et al* 1990, Thomas *et al* 1998). In addition to this, an activating mutation of c-Src has been identified in late stage human colon cancers (Src-531),

contributing to the oncogenic theory of c-Src. The mutation was shown to induce oncogenic transformation of fibroblasts and increase their metastatic potential (Irby *et al* 1999).

The extent to which mechanism of c-Src activation pre-dominates is unclear, however the consequences of its over-expression and activation has wide implications for the numerous signal transduction pathways it participates in. The biological targets of membrane receptor activated c-Src tyrosine kinase are vast and numerous, causing extensive effects in processes such as cell adhesion, angiogenesis, tumour growth and apoptosis (Thomas *et al* 1997). For example, c-Src has been linked to focal adhesion complex turnover with evidence suggesting dominant-inhibitory Src proteins cause enlarged cellular adhesions and lack migration properties (Thomas *et al* 1998). The contribution of c-Src to cellular adhesion suggests that its de-regulation in cancer may contribute to cells migratory properties, and hence the metastatic tendency of some cancer cells (Summy *et al* 2003). C-Src has also been implicated in the effective completion of G1 phase and the G2/M transition of the cell cycle. It is generally accepted that de-regulation of Src in cancer stimulates cell growth through the opposition of negative cell growth regulators (Frame *et al* 2002). The vast array of pro-oncogenic properties Src over-stimulation displays therefore ultimately makes it an attractive target for cancer therapy.

1.8 Aims

The aims of this thesis are to investigate two novel binding partners of survivin, the lipid conversion enzyme phosphatidylserine decarboxylase (PSD) and the non-receptor tyrosine kinase c-Src. We aim not only to investigate the

mechanism in which survivin interacts with these proteins, but also determine the effects of this interaction with their associated functions. We hypothesise that survivin interacts with both PSD and c-Src in two new advantageous mechanisms that cancer cells can exploit. These novel molecular insights could suggest that the multi-functional protein survivin may be even more diverse in its action than previously known.

2: Methods

2.1 Molecular Biology

2.1.1 Constructs

In this study we used a range of gene constructs to analyse the effect of both the N-terminal of survivin and the phosphorylation status of its threonine 34 residue (Figure 14). Constructs were previously used to produce stable HeLa cell lines, or used to transiently transfect HEK293T cells.

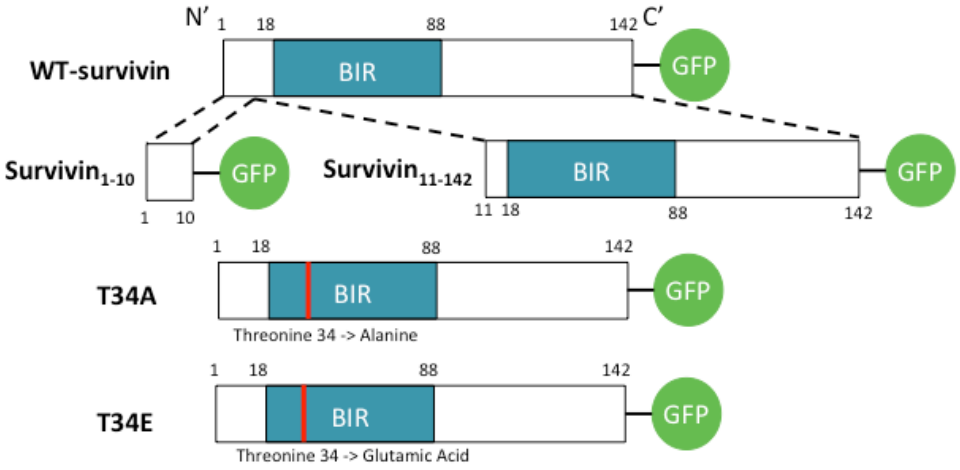


Figure 14. GFP tagged constructs used within the following thesis.

Construct	**Vector**	**Reference**
GFP	pcDNA3.1	Carvalho *et al* 2003
Survivin-GFP	pcDNA3.1	Carvalho *et al* 2003
$Survivin_{1-10}$-GFP	pcDNA3.1	Wheatley Lab
$Survivin_{11-142}$-GFP	pcDNA3.1	Wheatley Lab
Survivin-T34A-GFP	pcDNA3.1	Barrett *et al* 2009
Survivin-T34E-GFP	pcDNA3.1	Barrett *et al* 2009
PSD isoform I	pcDNA3.1	Wheatley Lab

2.1.2 Transformation of *E.coli* DH5α competent cells

1µg of DNA plasmid was added to 100µl competent *E.coli* DH5α and incubated for 30 minutes on ice. Cells were heat-shocked for 50 seconds at 42°C and then returned to ice for 2 minutes before the addition of 900µl of sterile Lysogeny broth (LB) and incubation at 37°C with shaking at 200rpm for 1 hour. Cells were pelleted at 4,000 g for 10 minutes and 2/3 of the media removed. Cells were spread onto LB agar plates containing appropriate antibiotic (Kanamycin 30µg/ml, Ampicillin 100µg/ml, Sigma) and incubated overnight at 37°C.

2.1.3 Purification of DNA plasmid

A single colony was picked and grown shaking at 200rpm in 2ml sterile LB media with appropriate antibiotic overnight at 37°C. DNA plasmid was purified from bacteria using a QIAprep Spin Miniprep purification kit (Qiagen) according to the manufacturer's instructions. DNA was eluted into 30µl of sterile water and concentration determined by measuring absorbance at 260/280nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.2 Mammalian Cell Culture Techniques

2.2.1 Cell culture

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen) with 2 mM L-glutamine (Sigma), 10% Fetal Calf Serum (FCS, Thermo Scientific), 10µg/ml penicillin/streptomycin and 2.5µg/ml fungizone (Sigma). HeLa cell lines expressing GFP, survivin-GFP, survivin$_{1\text{-}10}$-GFP, survivin-$_{11\text{-}142}$-GFP, survivin-T34E-GFP and survivin-T34A-GFP were maintained under the selection pressure of 500µg/ml G418 (Fisher). Cells were incubated in 5% CO_2 at 37°C and

passaged appropriately when reaching near confluence in tissue culture treated plates (Thermo Fisher Nunc).

2.2.2 DNA transfections

3.0 x 10^5 HEK293T cells were seeded per well into a 6 well plate containing antibiotic free media, and incubated for 12 hours prior to transfection or until 50-80% confluency was reached. Torpedo transfection reagent (Ibidi) and DNA were diluted into reduced serum media OptiMem (Gibco, Invitrogen) in a 3:1 ratio of Torpedo (µl): DNA (µg). Diluted solutions were gently mixed then incubated for 15-20 minutes at RT. The transfection reagent-DNA complex was then added drop-wise to cells and incubated at 37°C for a minimum of 12 hours and a maximum of 48 hours before use.

2.3 Immunoprecipitation

Per immunoprecipitation reaction 1 x 10^6 – 10^7 HeLa cell lines expressing GFP-tagged proteins of interest were harvested through washing and scraped into ice cold 1 x PBS (8g/l NaCl, 0.2g/l KCl, 1.15g/l HO_4PNa_2, 0.2g/l KH_2PO_4, pH 7.3 (+/-0.2) at 25°C, Gibco ThermoFisher). Cells were spun at 500 g for 5 minutes at 4°C, pellets washed with ice-cold PBS and then re-suspended in ice-cold lysis buffer (10 mM Tris/HCl pH 7.5 (Sigma), 150mM NaCl (Fisher), 0.5mM EDTA (Sigma), 0.5% NP-40 (Sigma)) supplemented with 100µM PMSF (Phenylmethylsulfonyl fluoride), 1 µg/ml CLAP (Chymostatin, Leupeptin, Antipain, Pepstatin A), 2 mM β-glycerphosphatase, 0.2 U/ml benzonase (Sigma) and 2 mM $MgCl_2$ (BDH). The sample was placed on ice for 30 minutes with interim re-suspension, centrifuged at 20,000 g for 2 minutes at 4°C and then supernatant diluted with dilution buffer (10mM Tris/HCl pH7.5, 150mM NaCl, 0.5mM EDTA). 25µl of GFP-trap_A beads

(Chromotek) were used per sample. Note that bead quantity was optimised for the expression of GFP-tagged protein of interest; 10µl of GFP-trap bead slurry binds 3-4 µg of protein. GFP Beads were washed with dilution buffer and centrifuged at 2,500 g for 2 minutes at 4^{o}C. Diluted cell lysate was then added to beads and incubated for 1 hour at 4^{o}C before a second centrifugation.

Pelleted beads were washed twice with ice-cold dilution buffer, and finally suspended in 30 µl dilution buffer and 10µl 5x SDS sample buffer (5% SDS (w/v, Fisher), 250mM Tris-HCl, 50% Glycerol (v/v Fisher), 0.05% Bromophenol blue (v/v, Sigma), 250 mM β-mercaptoethanol (v/v, Sigma)) and boiled at 95^{o}C for 10 minutes. Beads were centrifuged at 2,500 g for 2 minutes at 4^{o}C and supernatant loaded for analysis on a SDS-page gel (See 2.6).

2.4 Subcellular fractionation

Cells grown to confluence in 15 cm^2 petri dishes were washed and scraped into ice cold PBS. Cells were pelleted at 300 g for 3 minutes at 4°C, supernatant removed and cells re-suspended in 2ml homogenisation buffer (200 mM Mannitol (Sigma), 70 mM Sucrose (Sigma), 1 mM EGTA (Sigma), 10 mM HEPES (Sigma), pH 7.5 supplemented with 100 µM PMSF (Phenylmethylsulfonyl fluoride), 1µg/ml CLAP (Chymostatin, Leupeptin, Antipain, Pepstatin A), 2 mM β-glycerphosphatase, 0.2 Um/ml benzonase and 2 mM $MgCl_2$). Suspension was homogenised in a glass homogeniser (Teflon) and lysed cells spun at 1000 g, 4°C for 5 minutes. The supernatant was transferred to a fresh tube (mitochondrial/cytoplasmic fraction) and process repeated upon the cellular pellet. Supernatants and pellets were combined respectively and

pellet washed with buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl (Fisher), 0.5 mM DTT (Melford), 0.3% NP-40) before centrifugation. Pellet was re-suspended in 200µl of homogenisation buffer, sonicated, then spun at 17,200 g, 4°C for 10 minutes. Protein concentration was determined using a Bradford assay, where sample absorbance was measured at 595nm and a standard curve determined using 0, 1, 1.5, 2, 2.5, 5 and 10 µg/ml of BSA. The sample was then boiled in 5x SDS sample buffer before loading onto SDS page gel.

Mitochondrial/cytoplasmic supernatant was centrifuged at 1000 g, 4°C for 5 minutes and supernatant transferred to a fresh tube before centrifugation at 10,000 g, 4°C for 15 minutes to pellet mitochondria. Pellet was re-suspended in 1ml homogenisation buffer and spun again to increase mitochondrial purity. Protein concentration was established and the fraction boiled in 5x SDS sample buffer in 5:1 ratio (See Figure 15).

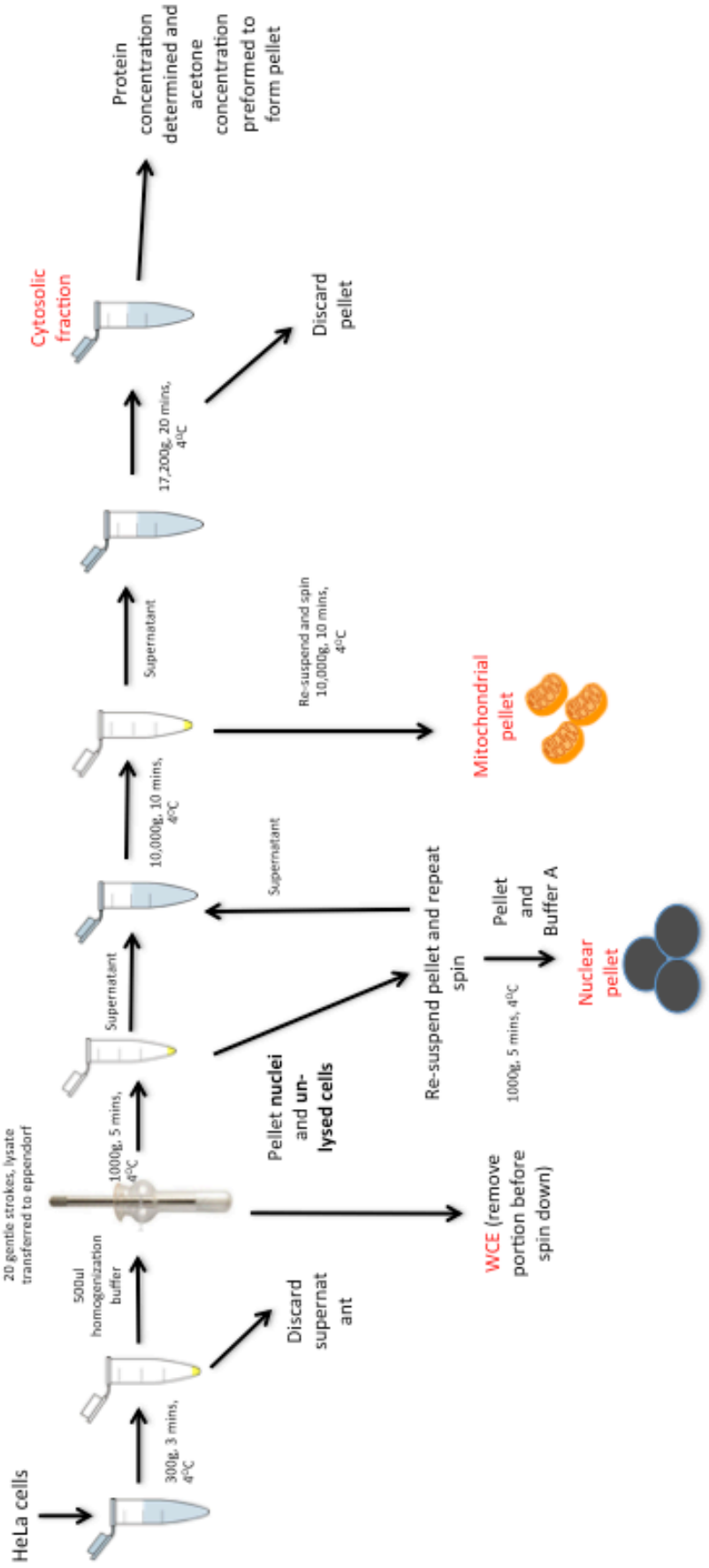
Supernatant was transferred to a fresh tube and spun at 17,200 g, 4°C for 20 minutes to remove debris. Protein concentration was then measured and supernatant concentrated by acetone precipitation (see 2.5). Sample boiled with 5x SDS sample buffer. Protein quantities required of each fraction: 20µg whole cell extract (WCE), nuclear and cytosolic and 4µg mitochondrial.

2.5 Acetone precipitation

Samples were prepared in an acetone compatible eppendorf tube (Sarstedt 72690), four times the sample volume of ice-cold acetone (Sigma) was added, vortexed and incubated at -20°C overnight. Samples were centrifuged for 10 minutes at 13,000 g, supernatants disposed and pellet left to dry.

Samples were re-suspended in appropriate volume of buffer to concentrate.

Figure 15. Subcellular fractionation schematic diagram



2.6 SDS polyacrylamide gel electrophoresis and immunoblotting

2.6.1 SDS-page

Protein samples were prepared with 5x SDS sample buffer, boiled for 3 minutes at 90°C and loaded onto 12% acrylamide SDS-PAGE gels. Gels were run at 200V for 1 hour, in 1x SDS running buffer (25 mM Tris, 192 mM glycine (Sigma), 0.1% (w/v) SDS).

2.6.2 Immunoblotting

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, 0.05% SDS, 10% Methanol (Sigma)). The transfer was carried out at 350 mA for 1.5 hours and the membrane blocked with 5% non-fat milk (Marvel, in PBS +0.1% Tween 20 (PBST) (Sigma)) for either 1-2 hours at RT or over night at 4°C. Membranes were incubated with primary antibody (see 2.7) diluted in 5% milk for 1 hour at RT or overnight at 4°C, followed by four 10 minute washes using PBST. The membranes were then incubated in the appropriate horseradish peroxidase conjugated secondary antibody (see 2.7) in 5% non-fat milk for 1 hour at RT and four 10 minute washes were then performed with PBST. Membranes were incubated with Z-ECL chemilluminescence detection reagents (Geneflow) before exposure to detection film (Roche).

2.7 Antibody specifications and sourcing's.

Antibody Specificity	Host Species	Dilution WB	Media	Source
β-Actin	Mouse	1/1000	5% Milk PBST	Sigma A5315
B512 (Tubulin)	Mouse	1/2000	5% Milk PBST	Sigma T5168
c-Src	Rabbit	1/200	5% Milk PBST	Santa Cruz SC-18
Duramycin	Rabbit	1/100	1%PBS/BSA/ Azide	Novus NB110-91362
GFP	Rabbit	1/500	5% Milk PBST	Wheatley Lab
GFP	Mouse	1/5000	5% Milk PBST	Sigma G1546
P-Src (Tyr416)	Rabbit	1/1000	5% BSA TBST	Cell Signalling D49G4
PSD	Rabbit	1/1000	5% Milk PBST	Sigma HPA031090
Survivin	Rabbit	1/1000	5% Milk PBST, Secondary PBST	Novus AB469
TATA binding protein (TBP)	Rabbit	1/1000	5% Milk PBST	Cell Signalling D5G7Y

Voltage dependent anion channel (VDAC)	Rabbit	1/1000	5% Milk PBST	Cell Signalling D73D12
Rabbit HRP conjugated	Goat	1/2000	5% Milk PBST	Dako PO448
Mouse HRP conjugated	Rabbit	1/2000	5% Milk PBST	Dako PO161
Anti-Rb Texas Red	Goat	1/1000	1%BSA/PBS/ Azide	Sigma

2.8 Duramycin sensitivity assay

HeLa cells ($5x10^3$) were seeded into 96 well plates, left for 3 hours to adhere and medium changed to DMEM supplemented with 10% FCS, 1% glutamine and 1% G418 containing either 0 μM, 1.25 μM, 2.5 μM, 5 μM or 10 μM of duramycin (Acros Organic). Three repeats of each condition and cell line were measured with samples left for either 24, 48, 72 or 96 hours. Day 0 time point was immediately measured after incubation for 1h at 37°C in DMEM media containing 10μg/ml resazurin (Sigma). Absorption measured spectrophotometrically (FLUOstar Galaxy, BMG Labtechnologies) with excitation and emission spectra at 530/590 nm.

2.9 Microscopy

2.9.1 Live Cell Fluorescence imaging

To visualise active mitochondria, HeLa cells were grown overnight in Ibidi 8-chambered micro-slides. Cells were stained for mitochondria using 100 nM MitoTrackerRed CMXRos (Invitrogen), and nucblue to visualise DNA (Thermo Fischer Scientific) in CO_2 independent media (DMEM, Invitrogen) supplemented with 2 mM L-glutamine and 10% Fetal Calf Serum, for 15 minutes at 37°C. Imaging was performed using an inverted (DMRIB Olympus, Delta Vision Elite) microscope with a 40x (NA1.2, oil) or 60x (NA1.4, oil) objective. Stacks of 0.3 μm slices were taken for each channel, images de-convolved and saved as TIFF files.

2.9.2 Fixed Cell Fluorescence imaging

To visualise exposed extra-cellular PE, $4x10^5$ HeLa cells expressing a relevant GFP tagged construct were cultured overnight in ethanol washed coverslips within 6 well plates,

containing a sub-lethal dose of 1 μM duramycin. Coverslips were then washed twice using PBS and fixed with 4% formaldehyde/PBS (Sigma) for 5 minutes at RT. Coverslips were washed three times with PBS and blocked for 15 minutes using 1% BSA/PBS/Azide (PAA, Sigma). Coverslips are then inverted onto 200μl of primary antibody on a clean sheet of plastic (α-duramycin), and then placed in the dark for 1 hour at room temperature (RT). Coverslips are washed with PBS, and the process repeated for the secondary antibody. Coverslips were then dried for 1 hour and mounted onto glass slides using Mowiol (Sigma Aldrich).

2.10 Lipid Techniques

2.10.1 Lipid Extraction

Lipids were extracted from $1x10^6$ HeLa cells as described by (Folch *et al* 1957). Cells were washed with PBS and harvested by trypsinization before centrifugation at 300 g for 3 minutes to pellet. 2ul of cell suspension was taken for protein quantification using a Bradford Assay. Lipids were extracted from pellets by addition of 200μl chloroform/methanol mix (Sigma Aldrich) in a 2:1 ratio with a brief vortex. Suspension was incubated for 20 minutes with constant shaking at room temperature, when 40 μl of 0.9% NaCl was then added. Suspension was thoroughly vortexed and centrifuged for 10 minutes, RT, at 500 g to separate upper aqueous and lower organic phases. The lower organic phase was transferred to a fresh eppendorf and vacuum evaporated using an Eppendorf concentrator 5301.

2.10.2 Thin Layer Chromatography (TLC)

Extracted lipid samples were re-suspended in 20µl chloroform with a brief vortex. Samples were separated upon TLC silica gel 60 plates (Merck) in 50:30:8:3 (v/v) ratio of chloroform/methanol/acetic acid/water alongside authentic PS, PC (Avanti) and PE (Sigma) standards. Plates were run approximately for 1 hour until sufficiently separated, left to dry and visualized by exposure to iodine vapour (Sigma).

2.10.3 Lipid Quantification

Silica spots identified as desired phospholipid were scraped from TLC plates into chromic acid (Sigma) cleaned Pyrex tubes. Samples were digested with 80µl 70% perchloric acid at 170°C for 1 hour to release inorganic phosphate. Phosphate release is quantified by malachite green method described by Itaya *et al* 1966. Samples were diluted with 400µl H_2O and span down to pellet silica gel. Standard samples were prepared using 1mM solution KH_2PO_4 to obtain 0, 1, 2, 4, 8, 12, 16, 32, 40 nmol of phosphate in each tube (duplicate). These were heated to 170°C with 80µl 70% perchloric acid and diluted with 400 µl H_2O. Malachite Green reagent was prepared by mixing 0.2% malachite green solution (w/v in ddH_2O, filtered with 2 x Whatmann filter paper) (Sigma), 4% ammonium heptamolybdate (w/v 5M HCl) (Sigma) and 0.5% Tween (w/v ddH_2O) in a ratio of 15:5:1. The mixture was stirred for at least 30 minutes at 4°C. 250 µl of each sample and standards were transferred to fresh eppendorfs and mixed with 1ml of reagent for 30 minutes at RT. Absorbance was measured spectrophotometrically using a SpectraMax 340PC plate reader (Molecular Devices) at 660 nm and results expressed as nmol/mg (using Bradford data).

2.11 Statistical Tests

Lipid quantification data and duramycin growth data was analyzed using a Multiple comparison one-way ANOVA, where $p<0.05$ is considered significant. All statistical analysis was performed using Prism 7 (GraphPad) software.

RESULTS

3: Survivin is a novel regulator of phosphatidylserine decarboxylase

3.1 Introduction

3.1.1 Phosphatidylethanolamine synthesis by phosphatidylserine decarboxylase

The non-bilayer forming glycero-phospholipid phosphatidylethanolamine (PE) plays a crucial structural role within cellular membranes, through the generation of negative tension sufficient to promote membrane curvature (van den Brink-van der Laan *et al* 2004). Particularly important within the mitochondria, this facilitates the formation of cristae within the inner mitochondrial membrane, allowing for the process of oxidative phosphorylation (Cogliati *et al* 2013, Tasseva *et al* 2013).

The majority of PE produced in mammalian cells is sourced from one of two biosynthetic pathways; the mitochondrial inner membrane protein phosphatidylserine decarboxylase (PSD) (Borkenhagen *et al* 1961, Schuiki *et al* 2009) and the Kennedy pathway located in the endoplasmic reticulum (Vance *et al* 2013). The mitochondrial enzyme PSD decarboxylates phosphatidylserine (PS) into phosphatidylethanolamine (PE) and is the focus of the following experiments (Schuiki *et al* 2009). PSD is not only the major contributor to PE content within the mitochondria, having previously been established that mitochondrial PE cannot be supplemented from other pathways after PSD disruption (Steenbergen *et al* 2005), but is also a source of PE supplied to other cellular pools such as the plasma membrane (Vance *et al* 1991).

3.1.2 Survivin and PSD

We identified via a Mass Spectrometry screen of a GST-survivin pull down (Wheatley Lab, unpublished) that the enzyme PSD was a binding partner of survivin. To determine if the binding interaction was direct, a reciprocal pull-down assay was performed with GST-PSD/GST-survivin and ^{35}S-labelled IVT PSD/SVN. Positive interactions were seen between the full-length proteins and subsequent analysis using protein truncations showed an interaction between survivins N-terminal 90 amino acids (BIR domain) and the decarboxylase domain of PSD (Figure 16). It was also established that survivin binds to the active form of PSD after its self-cleavage to form the mature enzyme (Schuiki *et al* 2009). This was performed through the generation of a mutant in the PSD cleavage domain, a C-terminal LGST motif, where serine is substituted with alanine. No interaction was observed between survivin and the PSD cleavage mutant (unpublished, Wheatley Lab).

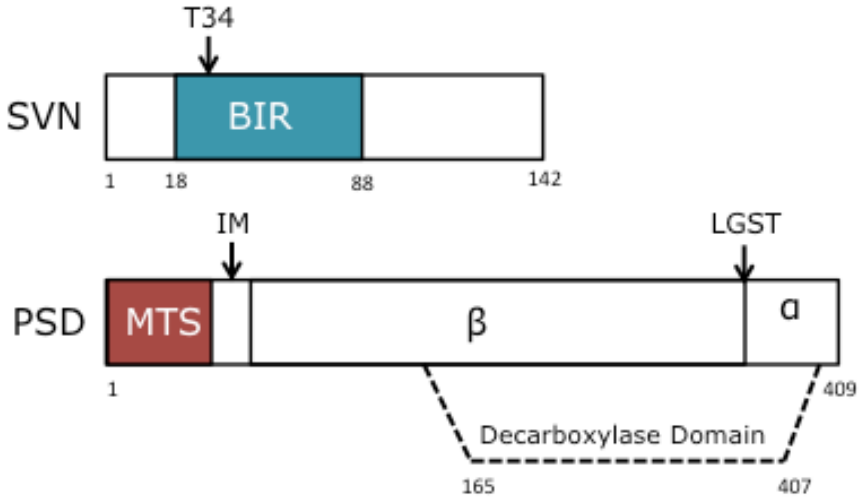


Figure 16. Schematic diagram of binding regions of survivin and PSD. Survivins BIR domain binds to the decarboxylase domain of PSD. Survivins threonine 34 residue is highlighted, theorised to be key in this interaction.

The function of this interaction and the mechanism by which it occurs is currently unknown, however we theorise a known phosphorylation site within survivin, threonine 34, may be responsible. Phosphorylation mutant constructs of this residue have previously displayed remarkably different growth rates, sensitivity to irradiation (Barrett *et al* 2009), and have noteworthy structural changes to their mitochondria visualised under electron microscopy (Wheatley Lab, unpublished).

3.1.3 Aims

The aims of this chapter were to investigate the influence of the phosphorylation status of the survivin BIR domain residue threonine 34 upon the interaction of survivin with PSD, whilst also observing the effects upon cellular PE production. This was achieved through experimentation using a phosphomimetic (T34E-GFP) or non-phosphorylatable mutant (T34A-GFP). We hypothesised that the changes observed to mitochondrial structure by the phosphorylation of the threonine 34 residue is due to the regulation of PSD by survivin.

3.2 T34E-GFP and T34A-GFP are mitochondrial and provide changes to mitochondrial morphology

In order to determine the localisation and confirm expression of both the survivin BIR domain constructs T34E-GFP and T34A-GFP, HeLa cells expressing the relevant GFP tagged construct (green) were visualised using fluorescence microscopy and stained with mitotracker (Red) and nucblue (blue) to visualise mitochondria and DNA respectively. This experiment also allowed us to confirm previous observations (Wheatley Lab, unpublished) of altered mitochondrial structures shown in both the cell lines.

All cell lines were confirmed to express the relevant GFP tagged construct (Figure 17 A) and images showed changes to mitochondrial structures consistent with previous observations. T34E-GFP cells showed truncated, small mitochondria lacking the normal integrated, connected mitochondrion observed in the GFP expressing cell lines (Figure 17 B). On the other hand, T34A-GFP cells maintained the normal mitochondrial morphology but were visually numerically sparser in comparison. We also observed that the mainly cytoplasmic localisation of survivin was maintained in both the T34E-GFP and T34A-GFP cell lines.

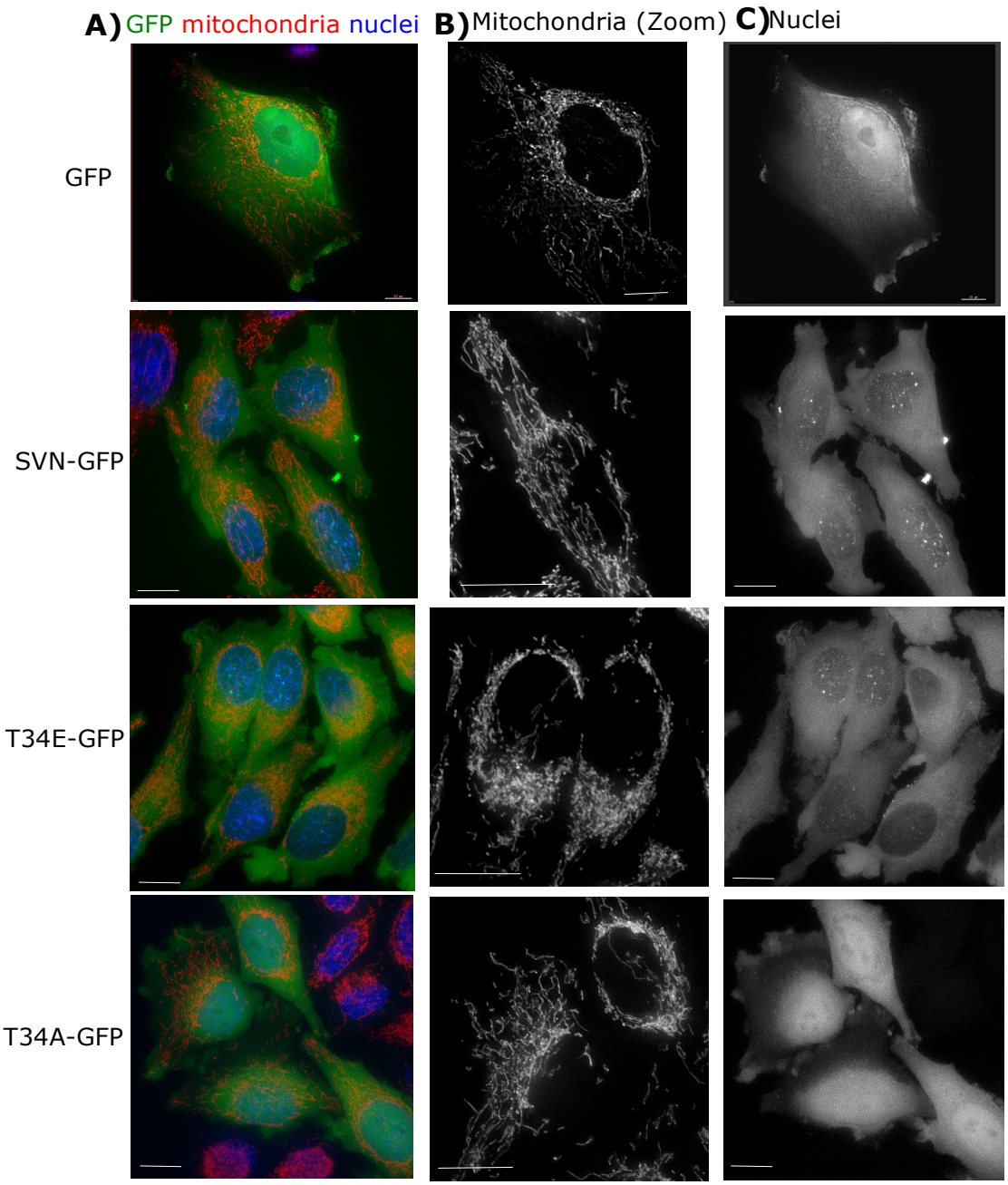


Figure 17. HeLa cells expressing GFP, SVN-GFP, T34A-GFP and T34E-GFP were visualised using fluorescence microscopy to confirm expression and localisation of the construct (green) (C) and mitochondrial morphology (mitotracker red) (B). DNA is visualised using nucblue (blue), and channel merge is shown in (A). Scale bar represents 10μM.

To establish whether the survivin BIR domain mutants T34A-GFP and T34E-GFP maintain the mitochondrial localisation of survivin, their distribution was investigated biochemically using a subcellular fractionation experiment. Mitochondrial survivin is only visualised using this technique and cannot be observed under fluorescence microscopy due to the abundance of the cytoplasmic pool. To address this HeLa cell lines expressing both constructs were compared to those expressing either survivin-GFP or a GFP control; organelle extracts were obtained through a subcellular fractionation experiment and ran upon a 12% SDS-page gel before transferring to a nitrocellulose membrane. VDAC was used as a mitochondrial marker, TBP as a nuclear marker and tubulin to assess the purity of the mitochondrial fraction obtained. T34E-GFP extracts were also run on a separate gel to allow for appropriate visualisation due to the low expression level of this

As shown in Figure 18, both T34E-GFP and T34A-GFP maintain the mitochondrial localisation of survivin. It is also important to note that GFP has not biochemically localised to the nucleus, as previously observed in Figure 17. It has been documented that a small proportion of GFP localises to nucleus due to passive diffusion, so in this instance it appears only a small quantity of GFP has reached that region, in comparison to the larger quantity observed in Figure 17.

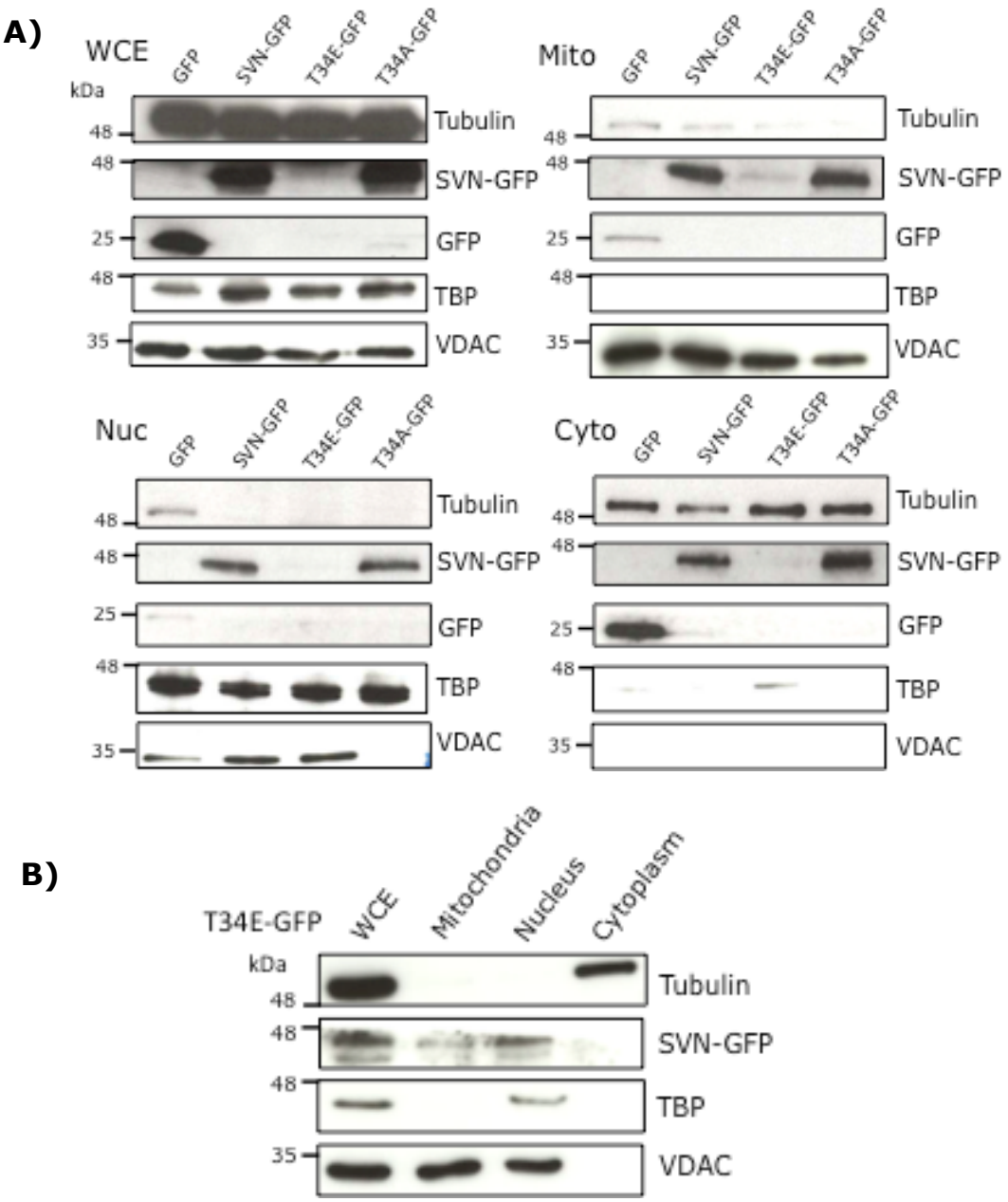


Figure 18 A) Subcellular fractionation of HeLa cells expressing T34E-GFP and T34A-GFP cells, showing mitochondrial localisation of both constructs. VDAC was used as a mitochondrial marker, with tubulin used to identify possible cellular contamination. B) T34E-GFP fractions are shown individually due to low expression of HeLa stable line.

3.3.1 Phospholipid composition is significantly altered in HeLa cells expressing T34E-GFP and T34A-GFP

To determine if the characteristics observed in HeLa cell lines expressing the T34 phosphorylation mutants were due to an altered interaction of survivin with PSD influencing cellular PE content, HeLa cells expressing survivin-GFP, T34E-GFP and T34A-GFP, were analysed for their phospholipid composition using a malachite green assay and compared to GFP control (Figure 19). The lipid concentration of at least three independent samples of cell extracts were measured, and expressed as nmol (lipid) per mg (protein) to normalise lipid concentration with protein quantity accounting for discrepancies in sample loading. Results are expressed as the ratio of PS:PE, where 2 represents 1PS:2PE. Data was analysed by a multiple comparison one-way ANOVA test,

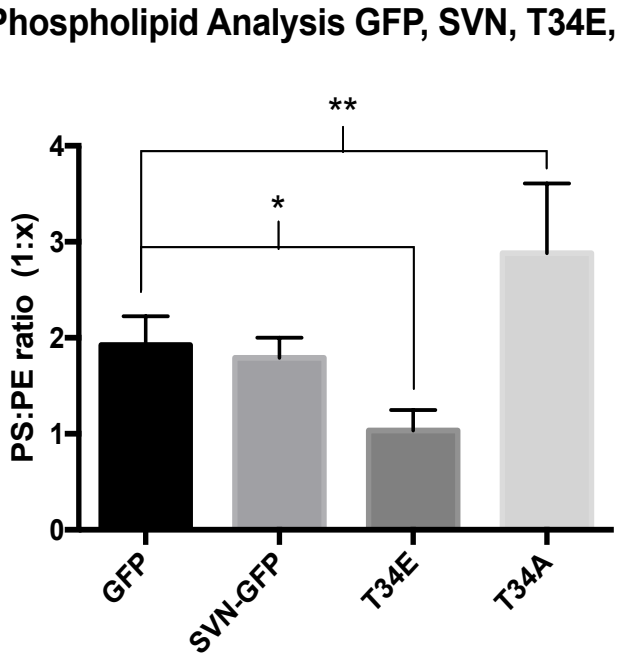


Figure 19. PS:PE ratio of HeLa cells expressing GFP, SVN-GFP, T34E-GFP and T34A-GFP, where T34E-GFP cell lines showed a reduced PE content, and T34A-GFP cells an increased PE content. A multiple analysis one way ANOVA test was performed to analyse the statistical significance of data, where '*' represents $p<0.05$, and '**' $p<0.005$. In comparison to the control, T34E-GFP $p=0.0115$, degrees of freedom (DF)=19, T34A-GFP $p= 0.0039$, DF=19.

where data is classified significantly different when $p < 0.05$.

As shown in Figure 19, control cell lines had a 1:1.8 ratio of PS:PE with no significant alteration upon survivin-GFP expression, with a 1:1.9 ratio. However expression of the non-phosphorylatable, fast growing T34A-GFP significantly increased the PS:PE ratio of HeLa cells to 1:2.9 (adjusted p value=0.0039). In contrast, expression of the slow growing, phosphomimetic T34E-GFP significantly decreased cellular PE, with a ratio of 1:1 confirming their PE deficit (adjusted p value=0.0115). Over-expression of survivin-GFP does not significantly alter cellular PE levels, however this may be due to the presence of both phosphorylated and non-phosphorylated threonine 34 within the survivin pool.

To further investigate the altered PE content of the survivin-T34 mutants, the sensitivities of HeLa cells expressing the constructs were analysed in response to the lantiobiotic (lanthionine antibiotic) duramycin (Figure 20). HeLa cells expressing the fore-mentioned constructs were grown over a 5-day period under a range of concentrations of duramycin: 0μM, 1.25μM, 2.5μM and 5μM. Duramycin binds to PE exposed upon the external cellular plasma membrane in a 1:1 ratio, lysing cells in a dose dependent manner. Cell lines containing greater amounts of cellular PE will therefore be more sensitive to duramycin treatment. The greatest differences in growth observed by the cell lines were at the end of the 5 day growth period (Day 4). The data is presented as a percentage of growth in comparison to no treatment (0μM) represented at 100%.

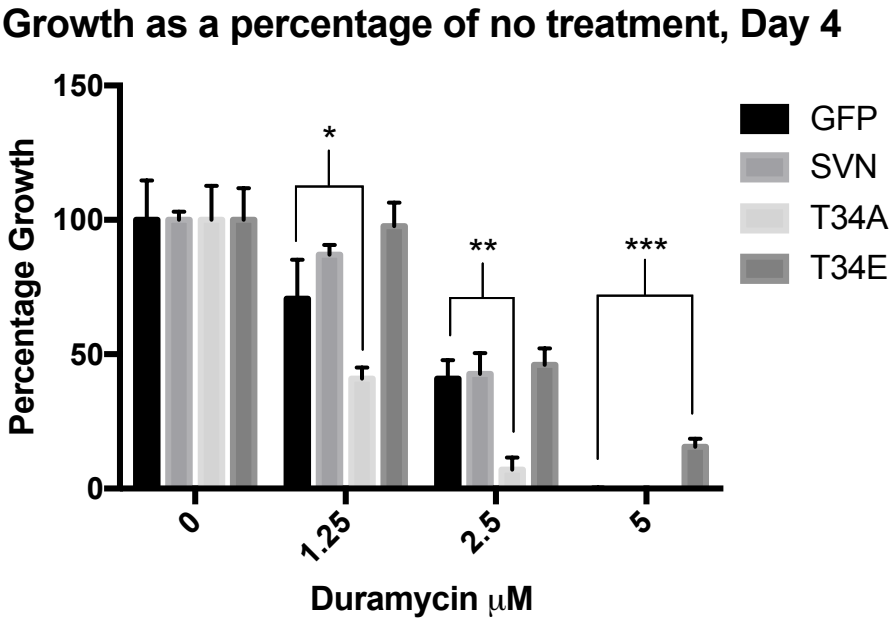


Figure 20. Percentage growth of HeLa cells expressing GFP, SVN-GFP, T34E-GFP and T34A-GFP incubated in growth media containing 0, 1.25, 2.5 or 5μM of duramycin, shown at day 4 time point. A multiple analysis one way ANOVA was performed to analyse the statistical significance of the data, where $p<0.05$ is represented in the graph by '*', $p<0.005$ by '**' and $p<0.0005$ by '***' .In comparison to control significant growth differences were seen by T34A-GFP (1.25μM) p=0.0089 DF=8, (2.5μM) p=0.0019 DF=8, and T34E-GFP (5μM) p=0.0003 DF=8. Overall T34E-GFP cell lines showed a decreased sensitivity to duramycin treatment; with T34A-GFP cell lines showing an increased sensitivity.

The results obtained in Figure 20 further highlight the differences observed in the lipid quantification data, growth was statistically slower for the T34A-GFP cell line at 1.25 and 2.5μM duramycin treatment (P value=0.0089, 0.0019), confirming a greater quantity of exposed PE. Growth was statistically higher for the T34E-GFP cell line at the 5μM duramycin treatment (P value =0.0003), confirming a lower amount of exposed PE.

3.3.2 T34E-GFP and T34A-GFP cells have a visibly altered externalised PE content

To further clarify the glycero-phospholipid content data provided in Figures 19 and 20, a microscopy experiment was designed in which the external membrane PE content of HeLa cells expressing GFP, survivin-GFP, T34E-GFP or T34A-GFP was visualised. Differences in cellular PE content allow us to observe different sensitivities of HeLa cells expressing survivin BIR domain mutants to duramycin treatment (Fig 20). Therefore it is reasonable to suggest that this altered PE content may be visualised exposed upon the external plasma membrane. In order to achieve this cells were seeded upon coverslips in a 6 well plate, incubated overnight with a sub-lethal dose of duramycin, fixed and then visualised through immunostaining with an anti-duramycin antibody and a corresponding fluorescently conjugated secondary antibody. Duramycin (and externalised PE) was visualised in red, GFP tagged constructs in green and DNA through nucblue staining (blue). The cleavage furrow of replicating cells were observed in order to easily identify differences in external PE content, as this a known area in which PE is externalised. Due to the varying time frames in which PE is exposed upon the membrane and the lack of knowledge of why this occurs, it was determined that this would be the most accurate method in establishing changes in cellular PE content.

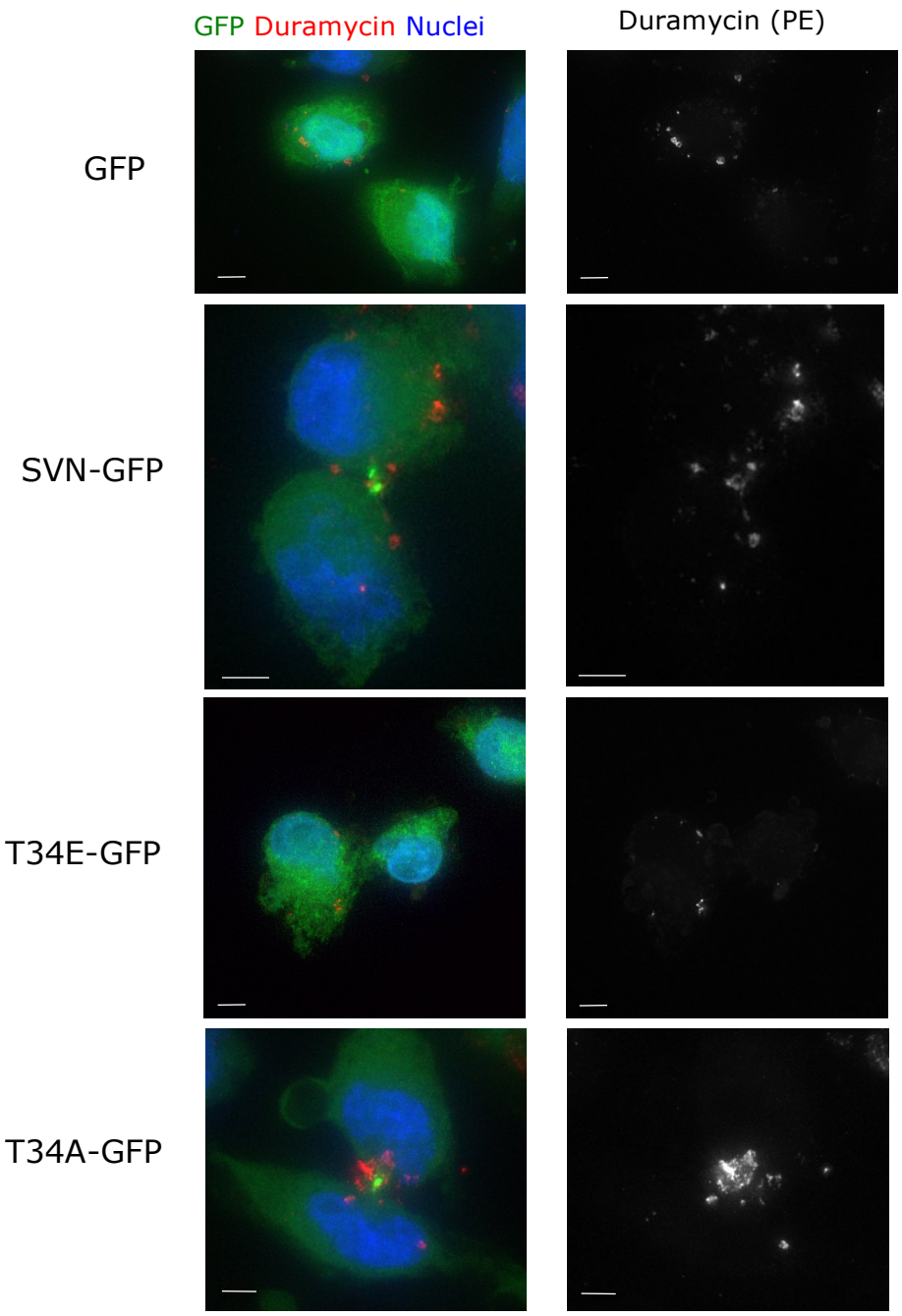


Figure 21. Duramycin treated HeLa cells expressing GFP, SVN-GFP, T34E-GFP and T34A-GFP stained for duramycin to visualise exposed phosphatidylethanolamine (PE). T34E-GFP cells consistently show decreased levels of externalised PE, with the majority of T34A cell lines showing increased PE membrane content. Anti-duramycin stained by appropriate Texas red secondary antibody (red), nucblue for DNA (blue) and GFP tagged construct (Green). Scale bar represents 10μM.

We can visually confirm (Figure 21) that HeLa cells expressing the T34E-GFP construct show a decreased PE content at the

cleavage furrow, validating both the lipid quantification and duramycin treated growth data (Figure 19 and 20), with T34A-GFP cells showing the converse. Signal intensity quantification is required to prove the data is statistically valid, however this has proved problematic due to the nature of PE exposure and low signal intensities observed.

3.4 Phosphorylation status of threonine 34 does not alter the binding interaction of survivin with PSD.

We have previously demonstrated that survivin binds to the decarboxylase domain of PSD directly via its BIR domain (unpublished, Wheatley Lab). To establish how the phosphorylation status of the survivin threonine 34 residue alters the interaction of survivin with PSD, a co-immunoprecipitation experiment was performed. Due to the variable growth rates and expression levels shown by the stable HeLa T34E-GFP cell line, attempts to perform a co-immunoprecipitation experiment were initially inconclusive. To circumvent this problem GFP, survivin-GFP, T34E-GFP and T34A-GFP constructs were transiently expressed in HEK-293T cells alongside PSD I, and an immuno-precipitation experiment performed using the GFP trap method after 48 hours to assess the extent of their interaction. Whole cell extracts were obtained and exposed to GFP-trap beads to pull down tagged protein of interest and interacting proteins. Sample was then boiled off beads and ran upon a 12% SDS-page gel, transferred to a nitrocellulose membrane and probed for GFP, PSD and Tubulin as a loading control.

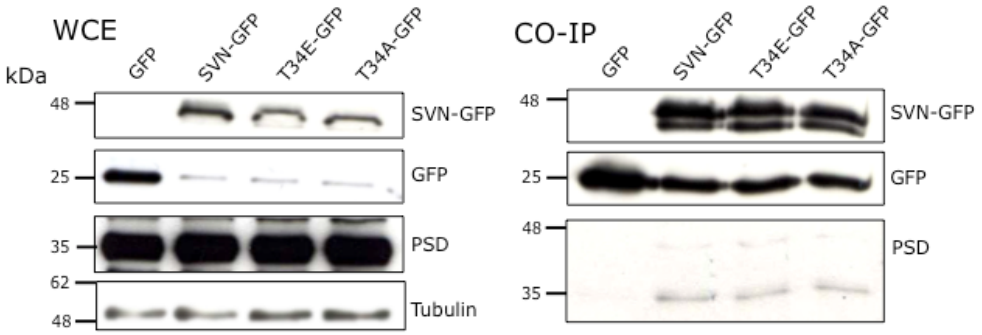


Figure 22. Co-IP of HEK293 cells expressing GFP, SVN-GFP, T34E-GFP and T34A-GFP. Whole cell extracts (left) confirms relevant construct expression and PSD over-expression. Co-IP samples (right) demonstrate a positive interaction of survivin-GFP, T34E-GFP and T34A-GFP cells with PSD. Tubulin is used as loading control.

We can therefore conclude that survivin-GFP, T34E-GFP and T34A-GFP all showed a positive interaction with PSD (both an unprocessed and processed form at 35 and 46.2 kDa (Figure 22). There is no remarkable difference observed in band thickness between the three survivin cell lines, suggesting that threonine 34 phosphorylation status does not alter the binding interaction of survivin with PSD. All four cell lines were to some extent expressing GFP due to the presence of an alternate start codon before the GFP fragment of the survivin-GFP constructs. This does not affect the results of this pull down as a negative interaction was observed between GFP and PSD.

4: NH_2 terminal of survivin provides its mitochondrial targeting and interaction with c-Src

4.1 Introduction

4.1.1 Mitochondrial transport of survivin

Cancerous cells display a small pool of survivin within the mitochondria, that is not present in non-malignant cells (Dohi *et al* 2004). How this pool of survivin is transported to the mitochondria and its function therein is currently unknown, however it is reasonable to speculate that this pool is exercising a function within the organelle beneficial to cancer cells. As previously mentioned, to date no mitochondrial targeting sequence has been identified for survivin, and evidence mostly suggests its import occurs through an interaction with a mitochondrial chaperonal protein. Current opinion suggests either the chaperonal complex Hsp90 (Fortugno *et al* 2003) or the aryl hydrocarbon receptor-interacting protein facilitate its entry into the mitochondria (Kang *et al* 2011).

In this study it was noted that the first 10 amino acids of the NH_2 terminus of survivin (MGAPTLPPAW, Uniprot) contains a remarkably high presence of prolines. Current crystal structures provided of survivin do not show its N-terminal, which maybe a result of this high concentration of prolines (Verdecia *et al* 2000). Modelling predicted sequences of $survivin_{1\text{-}10}$ has led us to hypothesise it to be a so-called poly-l-proline type II (PPII) helix (Adzhubei *et al* 2013), a binding domain that plays a major role in protein-protein interactions. We hypothesise this sequence allows for the targeting of survivin into the mitochondria, either acting as a direct

targeting sequence or through protein-protein interactions it provides with mitochondrial chaperones.

4.1.2 c-Src and survivin

An interaction between the N-terminal of survivin and the tyrosine-kinase c-Src was hypothesised due to the recognition of the first ten amino acids of survivin as a PPII helix, combined with evidence suggesting alterations in the adhesive properties of HeLa cells lines expressing the NH_2 terminal of survivin (Dujuvanova *et al* 2016). PPII helixes bind with high affinity to SH3 domains such as present in c-Src, reasonably suggesting that the two proteins may interact (Adzhubei *et al* 2013).

4.1.3 Aims

The aim of this chapter was to test the hypothesis that the NH_2 terminus first 10 amino acids of survivin acts as a mitochondrial targeting sequence in cancerous cells, and also interacts with the tyrosine kinase c-Src. In order to achieve this experimentation was performed using HeLa cells expressing survivin$_{1\text{-}10}$-GFP and survivin$_{11\text{-}142}$-GFP to show the effects of both the N- terminal of survivin, and full length survivin lacking this region.

4.2 The NH_2 terminus 1-10 amino acids of survivin is a mitochondrial targeting sequence

To test the hypothesis that the NH_2 terminal of survivin is a mitochondrial targeting sequence, a subcellular fractionation of GFP tagged survivin, survivin$_{1\text{-}10}$ and survivin $_{11\text{-}142}$ expressing HeLa cell lines was performed using GFP as a negative control. Mitochondrial, nuclear and cytoplasmic fractions were obtained (see Figure 15), samples ran upon a 12% SDS-page gel, transferred to a nitrocellulose membrane and probed for VDAC as a mitochondrial marker, TBP as a nuclear marker and tubulin to identify to cytosol (Figure 23).

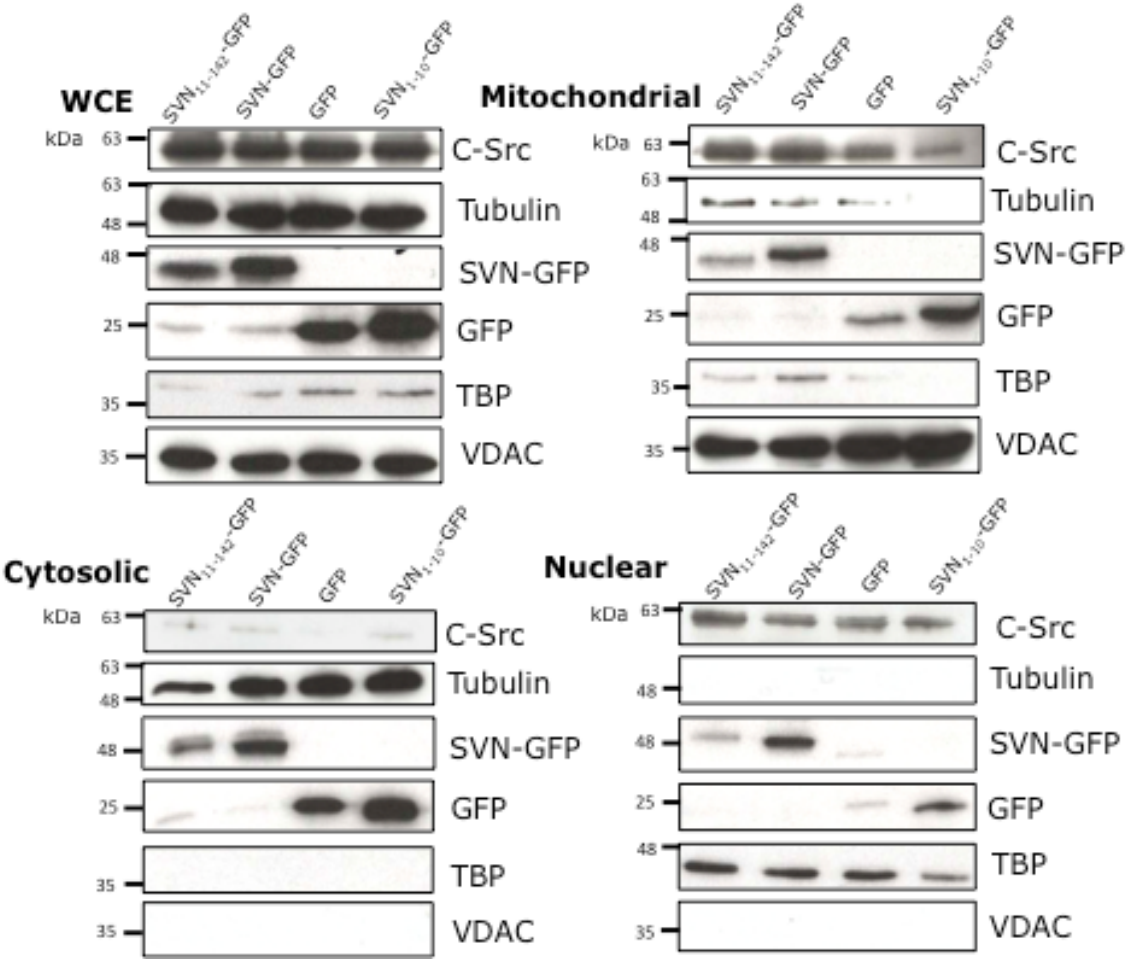


Figure 23. Subcellular fractionation of HeLa cells expressing GFP, survivin-GFP, survivin$_{1\text{-}10}$-GFP and survivin$_{11\text{-}142}$-GFP. Expression of survivin$_{1\text{-}10}$ is sufficient to transport GFP into the mitochondria.

All three GFP tagged constructs; survivin-GFP, survivin$_{1-10}$-GFP and survivin$_{11-142}$-GFP were transported in varying degrees into the mitochondria, with survivin$_{1-10}$-GFP found to a much larger extent in the mitochondria than both the GFP control and survivin$_{11-142}$-GFP, suggesting that survivin$_{1-10}$ can act as a bona fide mitochondrial targeting sequence (Figure 23). The amount of survivin$_{11-142}$-GFP detected biochemically within the mitochondria is at an indistinguishable concentration to that of the GFP control, suggesting that over-expression of these proteins plays a factor in their mitochondrial localisation.

4.3.1 Survivin$_{1-10}$ is essential for the interaction of survivin and c-Src

To test whether an interaction occurs between survivin and endogenous c-Src and at which region of survivin this interaction is located, survivin-GFP, survivin$_{1-10}$-GFP and survivin$_{11-142}$-GFP were expressed in HeLa cells and an immuno-precipitation experiment performed using the GFP trap method, using GFP as a negative control (Figure 24). Pulled down proteins were boiled off beads and loaded onto a 12% SDS-page gel, transferred to a nitrocellulose membrane and probed for GFP, c-Src and β-actin as a loading control.

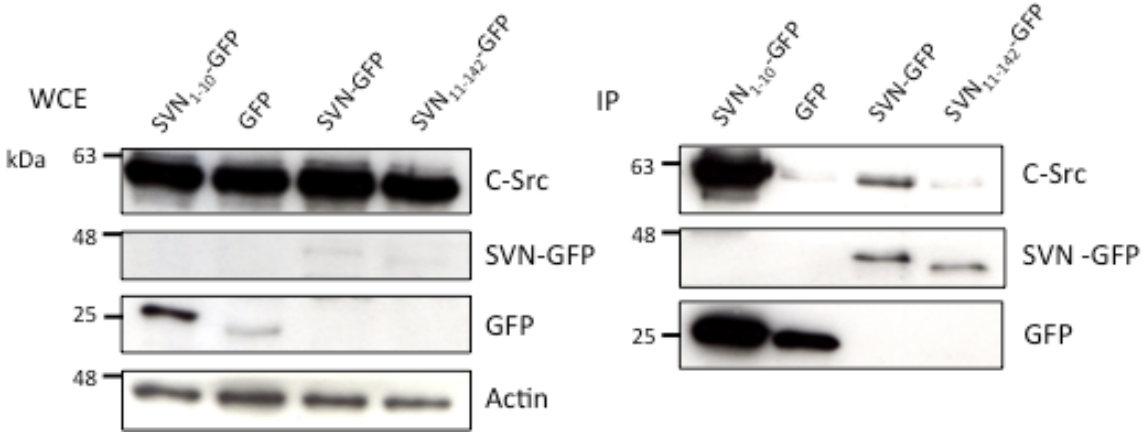


Figure 24. Co-IP of Hela cells expressing GFP, survivin-GFP, survivin$_{1-10}$-GFP and survivin$_{11-142}$-GFP. Whole cell extracts (left) A positive pull down occurs between survivin-GFP and survivin$_{1-10}$-GFP with c-Src. B- actin was used as a loading control.

A positive interaction was observed between survivin-GFP and survivin$_{1-10}$-GFP with endogenous c-Src, suggesting a binding interaction occurs through the NH_2 terminus of survivin to c-Src. This interaction was further confirmed due to the absence of binding between endogenous c-Src and survivin$_{11-142}$-GFP. All four cell lines were pulled down with the GFP trap method successfully, and the discrepancy in band size appears to be

due to differing construct expression levels rather than issues with sample loading.

4.3.2 Src auto-phosphorylation is elevated in HeLa cells expressing survivin$_{11-142}$-GFP

After establishing that the first ten amino acids of the survivin NH_2 terminus binds to c-Src, we aimed to investigate whether HeLa cells expressing the afore-mentioned constructs caused an alteration in the level of activated c-Src (phosphorylated at Tyrosine 416). To achieve this HeLa cells expressing GFP, survivin-GFP, survivin$_{1-10}$-GFP and survivin$_{11-142}$-GFP were harvested, lysed, samples ran on a 12% SDS page gel and then transferred to a nitrocellulose membrane before probing for c-Src, P-Src (Tyr416), GFP to confirm expression, and tubulin for a loading control.

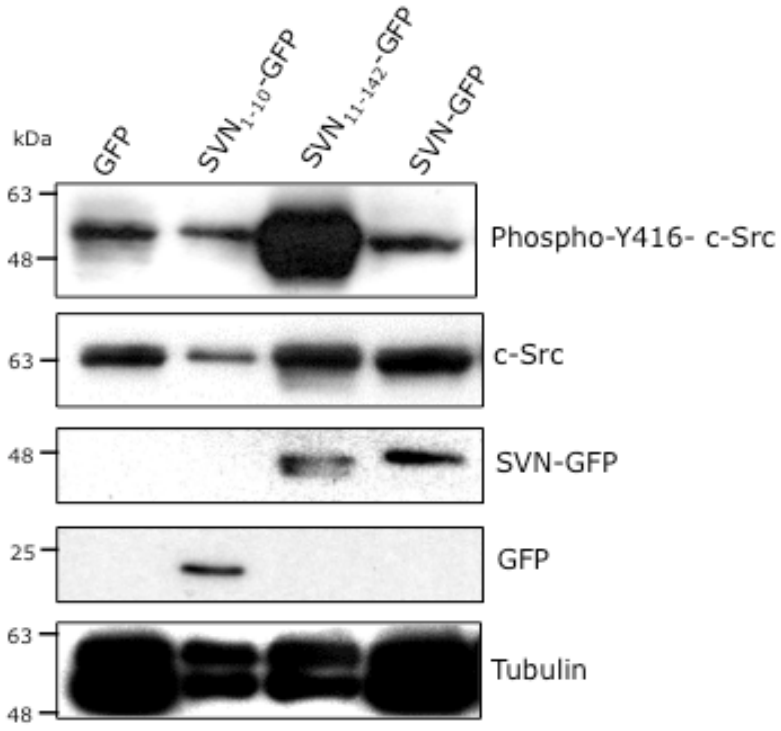


Figure 25. Whole cell extracts of HeLa cells expressing GFP, survivin-GFP, survivin$_{1-10}$-GFP and survivin$_{11-142}$-GFP were probed to detect elevated c-Src activation at Tyr416. C-Src activation is elevated within the HeLa cell line expressing survivin$_{11-142}$-GFP. Tubulin is used as a loading control.

As seen in Figure 25, Src phosphorylation at tyrosine 416 was remarkably increased in HeLa cells expressing survivin$_{11-142}$-GFP, with no difference observed between survivin$_{1-10}$-GFP and the GFP control. Unfortunately the GFP control HeLa stable cell line appeared to no longer be expressing the relevant construct (GFP), yet due to a negative binding result observed between GFP and c-Src this still allows us to reason that the difference observed in P-Src is due to survivin$_{11-142}$ expression and not due to the GFP tag.

Discussion

5: Survivin is a novel regulator of phosphatidylserine decarboxylase

5.1 Survivin inhibits PE production upon threonine 34 phosphorylation

We have previously presented data suggesting that survivin interacts with the decarboxylase domain of PSD directly via its BIR domain (Wheatley Lab, unpublished). In this report we now highlight the importance of the BIR domain residue threonine 34 in the interaction of survivin and PSD. Previous experimentation to observe the effects of threonine 34 phosphorylation was achieved using either the mutant construct T34E-GFP (phosphomimetic), or T34A-GFP (non-phosphorylatable). HeLa cells expressing these constructs displayed alterations to growth rates and to their mitochondrial architectures (Barrett *et al* 2009**,** Wheatley Lab unpublished). T34E-GFP cells were slow growing, insensitive to irradiation and when visualised under electron microscopy had large, swollen mitochondria lacking cristae. In contrast T34A-GFP cells were fast growing, sensitive to irradiation and had fewer but inter-connected mitochondria. Due to the drastic alterations observed in mitochondrial structure combined with the knowledge that survivin interacts with PSD, a hypothesis was generated; 'Phosphorylation of threonine 34 plays a role in the regulation of survivin and PSD'. If proven this could explain the observed alterations in mitochondrial architecture; changes in the abundance of the membrane-curving agent PE causes structural modifications to the inner mitochondrial membrane (Tasseva *et al* 2013).

Firstly, we have found that both T34E-GFP and T34A-GFP maintain the mitochondrial localisation of survivin, thus confirming that both forms can still gain access to PSD and thus regulate its activity. Secondly, statistical differences in cellular PE levels were determined using a malachite green assay. HeLa cells expressing T34E-GFP showed an appreciably reduced amount of PE, with a lower PS:PE ratio, whereas T34A-GFP expressing cells had an increased cellular PE content and an increased PS:PE ratio. Thus we hypothesise that phosphorylation of T34 allows survivin to inhibit PSD and to reduce cellular PE levels.

The data obtained from phospholipid quantification was further confirmed by the different sensitivities of cell lines exposed to the lantibiotic duramycin, which binds to exposed PE on the external membrane leaflet in a 1:1 ratio, causing cell lysis in a dose dependent manner (Navarro *et al* 1985). The mechanism in which duramycin triggers cell death has not been fully established, however an alteration in membrane permeability is known to play a role (Iwamoto *et al* 2007). In normal cell lines both PS and PE are asymmetrically distributed across the plasma membrane, found mostly upon the inner face. Cancer cells however, lose their ability to maintain the gradient of the phospholipid PS upon the inner membrane leaflet, allowing for their decoration upon the external surface. As both PS and PE are co-regulated by the same transporters, it therefore likely to suggest that cancer cells also show exposed PE upon their plasma membrane (Marconescu *et al* 2008, Stafford *et al* 2011). These data demonstrate that T34A-GFP cell lines had an increased sensitivity to duramycin treatment, with T34E-GFP cell lines showing a decreased sensitivity to the lantibiotic. These data alone show that the level of exposed PE was

statistically altered within these cell lines. Collectively coupled with knowledge of the lipid quantification assay, we can conclude that an alteration of the cellular PE content also influences the quantity of PE exposed upon the external plasma membrane i.e. T34E-GFP cells had a reduced cellular PE content, less exposed PE upon the plasma membrane and therefore were less sensitive to duramycin compared to control cells. Conversely, T34A-GFP had an increased cellular PE content, with more exposed PE upon the plasma membrane resulting in an increased sensitivity to duramycin in comparison to the control cell line.

In order to visualise the exposed PE content of the stable cell lines afore-mentioned, cells were incubated with a sub-lethal dose of duramycin, fixed and then stained using an anti-duramycin antibody and a fluorescently conjugated secondary antibody. The results obtained visually confirmed that T34E-GFP cells had reduced external PE and T34A-GFP cells an increased amount of PE. Even though this experiment is visually conclusive, quantification of exposed PE intensity has proven problematic. Current knowledge does not fully explain what causes PE externalisation in cancer cells and in what particular regions, only descriptions of its use at the cleavage furrow and during apoptosis (Emoto *et al* 1996, Emoto *et al* 1997). If combined with various treatments, such as those triggering apoptosis or other means of cell death thus increasing the intensity of PE, it may be possible to more accurately quantitate differences between cell lines. For example Marconescu *et al* 2008, showed that irradiation increased PE exposure that they were able to therefore quantitate.

However, these data combined suggests that the phosphorylation of the survivin threonine 34 residue alters the interaction of survivin and PSD to a significant degree to restrict the cellular PE content. Moreover this data can act to explain the characteristics observed by T34-GFP mutant cell lines; the alterations to mitochondrial structure under electron microscopy (Wheatley Lab, unpublished) and within this thesis under fluorescence microscopy. Deformities to the inner mitochondrial membrane are observed due to a lack of the essential membrane-curving agent PE (Tasseva *et al* 2013), T34E-GFP cells therefore might have deformities to their mitochondria due to their reduced PE content.

5.2 Survivin and mitochondrial health

If survivin inhibits PSD this would not only result in a reduced cellular PE content, but would more importantly restrict the amount of mitochondrial PE, having drastic consequences upon inner mitochondrial membrane architecture (van den Brink-van der Laan *et al* 2004, Tasseva *et al* 2013). Such alterations to the mitochondria allows us to suggest that normal mitochondrial processes might be compromised due to disruptions in mitochondrial inner membrane integrity. More specifically, a deficiency in PE has already been shown to cause deformities of the cristae, which severely impacts the process of oxidative phosphorylation. Tasseva *et al* 2013 demonstrated that a reduction of even less than 30% of mitochondrial PE levels alters not only mitochondrial architecture but its function and therefore cellular growth capabilities. Furthermore they identified that sufficient mitochondrial PE levels were not only essential for the function of the electron transport chain complexes I and CIV, but also enabled their formation. X-ray crystallography has also

demonstrated that PE is strongly bound to bovine heart mitochondrial CIV complex, illustrating its presence is required for complex function (Shinzawa-Itoh *et al* 2007). The data presented within this thesis therefore explains the growth alterations observed by HeLa cells expressing T34E-GFP and T34A-GFP (Barrett *et al* 2009); decreasing or increasing cellular PE levels could result in corresponding growth rate changes due to alterations in oxidative phosphorylation functionality.

While over-expression of T34E-GFP exacerbates the effects of survivin threonine 34 phosphorylation, it highlights that phosphorylation of this residue contributes to the reduction of oxidative phosphorylation observed in cancer. Previous studies of survivin over-expression mirror that of reduced mitochondrial PE, causing a decrease in mitochondrial respiration and furthermore an increase in aerobic glycolysis due to survivin inhibition of oxidative complex I (Hagenbuchner *et al* 2013).

5.3 Survivin contributes to the Warburg effect

Because survivin is only mitochondrial in cancer cell lines and combined with the knowledge that it interacts with the inner mitochondrial membrane protein PSD, we therefore suggest this interaction must occur because it is beneficial to cancer cells. We hypothesise that the destabilisation of oxidative phosphorylation by threonine 34 phosphorylation contributes to the phenomenon known as the Warburg effect (Warburg, 1956). The evidence presented by this theory describes how cancer cells use aerobic glycolysis, present in the cytoplasm, to a greater extent than oxidative phosphorylation for the majority of their energy production. Even though they have an

apparently intact oxidative phosphorylation pathway, the more inefficient pathway of aerobic glycolysis dominates. Normally cells alter the ratio of glycolysis and mitochondrial oxidative phosphorylation depending upon the cellular energetic demands and their present environment. Cancer cells can rapidly change their metabolic processes depending upon a variety of micro-environmental factors, allowing for them to capitalise upon situations that could be presented as a growth disadvantage (Zheng *et al* 2012). It is already apparent that organisms such as *E.coli* and *S.cerevisiae* can regulate oxidative phosphorylation to compensate for external resources; it is therefore possible that cancer cells could apply this mechanism for their own benefit. Not only this but it seems apparent that different cancerous cell lines also have very different metabolic processes, Suganuma *et al* 2010, analysed the amount of glycolysis in four leukaemia cell lines using the glycolysis inhibitor 2-deoxy-D-glucose (2-DG) and oxidative phosphorylation inhibitor oligomycin. Some cell lines were particularly sensitive to 2-DG, whilst others resistant to 2-DG and sensitive to oligomycin, suggesting they had varying levels of metabolic processes. Therefore the metabolic adaptability cancer cells portray provides flexibility for a wide range of tumour bioenergetics demands (Jose *et al* 2011).

As long as the overall balance of ATP production remains stable, cells can trade between methods of low ATP yield with a high rate (aerobic glycolysis) over mechanisms of a high ATP yield with a much lower rate (oxidative phosphorylation) (Pfeffier *et al* 2001). It also appears that using a lower yield mechanism of ATP production does not limit cancer cell growth due to the over abundance of resources available to them within the body. Even though aerobic glycolysis has a slower

rate of ATP production than oxidative phosphorylation, it has a wide range of advantages perfect for cancer cells (Vander Heiden *et al* 2009). Firstly, it produces significantly less ROS in comparison to oxidative phosphorylation and is useful in conditions of low oxygen levels where oxidative phosphorylation is inefficient. Normally cells source 79% of ATP from oxidative phosphorylation, however in conditions of hypoxia, for example such as deep within a tumour cell, aerobic glycolysis increases to compensate for the reduced rate of energy production by oxidative phosphorylation (30%) (Rodríguez-Enríquez *et al* 2010). Secondly, it provides advantages to cells with particularly high ATP demand, such as muscle cells and also cancer cells, which have a characteristically high growth rate as illustrated in Figure 26 (Pfeiffer *et al* 2001). Aerobic glycolysis produces a wide range

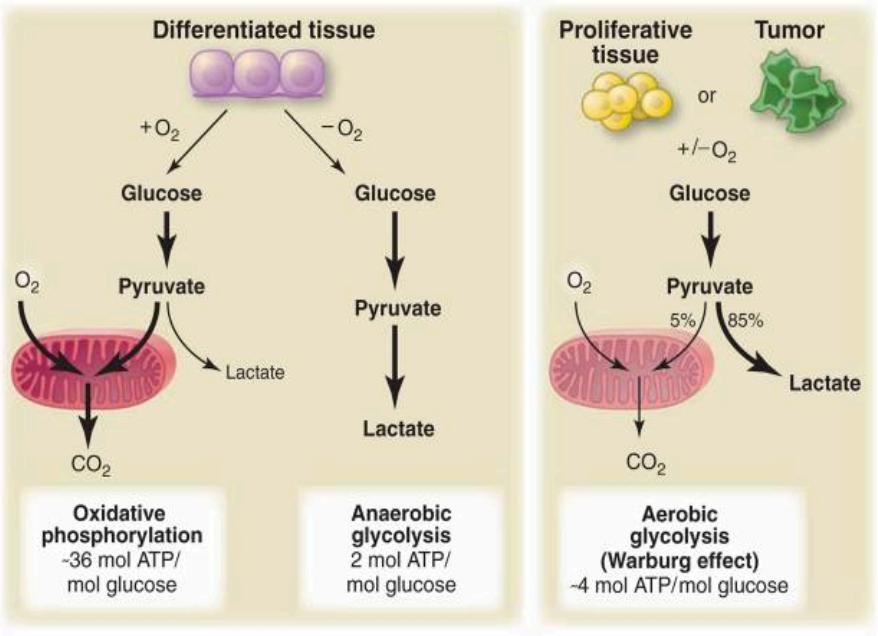


Figure 26. Schematic diagram illustrating energy production by the processes of oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis. Differentiated cells tend to favour oxidative phosphorylation in normoxia, and aneraboic glycolysis in hypoxia, whereas higher proliferating cell lines such as cancer cells under both conditions favours aerobic glycolysis. Adapted from Vander Heiden *et al* 2009.

of intermediate molecules needed for amino acid and DNA production, required in a high quantity to allow for cancer cells higher growth rate, which can be lacking if too much glucose is funnelled through the oxidative phosphorylation pathway (Zheng *et al* 2012).

We therefore hypothesise that survivin acts to manipulate the efficiency of oxidative phosphorylation through the regulation of PSD and destabilisation of membrane curvature within the inner mitochondrial membrane, partially accounting for cancer cells metabolic versatility. If survivin inhibits PSD when phosphorylated at T34, this could control how much oxidative phosphorylation the cell carries out. Therefore in a microenvironment in which oxidative phosphorylation is inefficient this mechanism would allow for the versatility of cancer cells metabolism. In order to further establish this theory, one would need to analyse the level of metabolic pathways present in HeLa cells expressing both the survivin phosphorylation mutant constructs T34E-GFP and T34A-GFP. This is achieved through analysing the metabolic parameters of both aerobic glycolysis and oxidative phosphorylation through the investigation of live cell growth media and the response of cells to metabolic inhibitors such as 2-DG (glycolysis) and oligomycin (oxidative phosphorylation) (Suganuma *et al* 2010).

5.4 Survivin influences mitochondrial fragmentation

Mitochondrial fusion and fission is a dynamic process that determines mitochondrial length due to conditions both within the mitochondria, and within the external cellular environment. Mitochondrial fusion is important for the maintenance of healthy, functioning mitochondria through the

mixing of mitochondrial contents. This is essential in cells with high respiration levels, and so a network of inter-connected mitochondria is linked to a high-energy demand (Chen *et al* 2005). The process of mitochondrial fission is essential for the removal of damaged mitochondria that are disposed by autophagy, as well as the inheritance of organelles during cell division (Twig *et al* 2008). Henceforth, mitochondrial fission is also associated with a lower demand for cellular energy production.

The phospholipid PE is crucial in the process of mitochondrial fusion as its presence generates negative membrane curvature, thus reducing the activation energy required for membrane fusion (Frohman, 2015). PE is also required in yeast for the correct processing of an essential inner mitochondrial membrane fusion protein s-Mgm1 (Chan *et al* 2012), suggesting a further level of control over mitochondrial fusion. The human homologue of Mgm1 was determined as OPA1, the gene responsible for the neurodegenerative eye disease Dominant Optic Atrophy (DOA). It is believed that OPA1 is also targeted to the mitochondria, influencing mitochondrial biogenesis and mitochondrial membrane architecture (Alexander *et al* 2000). It has been documented that under conditions of mitochondrial PE deficiency and normal or up-regulated mitochondrial fusion proteins, mitochondrial fragmentation is exceedingly observed suggesting a major influence of PE upon mitochondrial fragmentation and fusion (Tasseva *et al* 2013).

Therefore, the data provided within this thesis demonstrates that survivin upon threonine 34 phosphorylation inhibits the production of PE. Survivin over-expression also mirrors the effect of PE deficiency on mitochondrial fragmentation.

Increased fragmentation is observed coupled with an increase in Drp1 (Hagenbucher *et al* 2013), the protein responsible for mitochondrial fission (Smirnova *et al* 2001). It is now therefore known that the characteristics observed through survivin over-expression could be partially due to a collaboration of survivin and PSD, restricting PE production and therefore increasing mitochondrial fission. Furthermore through the limitation of PE by survivin, this mechanism may also contribute to the reduction in oxidative phosphorylation observed in cancer cells described by the Warburg effect (Warburg, 1956).

5.5 Mechanistic basis of PSD inhibition by survivin

We therefore need to address how threonine 34 phosphorylation mechanistically alters the interaction of survivin and PSD to inhibit PE production. Through the results provided within this thesis we can eliminate three potential mechanisms responsible for the inhibition of PSD. Firstly, phosphorylation could alter the binding strength of survivin and PSD so that upon binding inhibition occurs. We have established that the phosphorylation status of this residue does not alter the binding interaction strength of survivin to PSD. Secondly, survivin could impede the processing of the mature enzyme resulting a reduced quantity of functioning PSD and the observed reduction in PE content. PSD is processed by various self-cleavage steps to form the mature active enzyme, removing the mitochondrial and inner mitochondrial membrane targeting sequences, and well as processing into alpha and beta subunits (Schuiki *et al* 2009), see Figure 11 B. We have also found that an equal interaction occurs between T34E-GFP and both the processed and unprocessed forms of PSD at 35kDa and 46.2kDa (Kuge *et al*

1994). This suggests that phosphorylation at this residue does not inhibit the processing of the mature enzyme, and that the interaction occurring between the proteins is present in both processed forms of PSD. Thirdly, inhibition of PSD could occur through a removal or addition blockage of one of its essential co-factors. PSD is known to contain an essential pyruvoyl prosthetic group for its activity which is covalently bonded onto the enzyme during its processing into its mature form (Zborowski *et al* 1983, Choi *et al* 2015). If T34E-GFP inhibited the addition of this prosthetic group it would seem likely that this would prevent the formation of the mature enzyme; to which T34E-GFP binds.

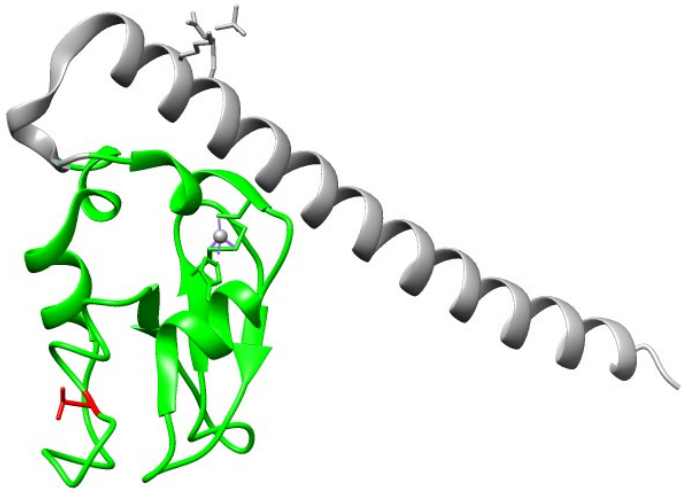


Figure 26. Ribbon diagram illustrating survivin structure. The externally facing threonine 34 residue (red) neighbours a crevice that upon phosphorylation could provide drastic structural changes to the protein.

It therefore seems more likely that phosphorylation of threonine 34 alters the activity of PSD through another mechanism than the three previously mentioned. As survivin binds to the decarboxylase domain of PSD (Wheatley Lab, unpublished), the binding of the substrate PS could be inhibited. Phosphorylation of threonine 34 could also alter the functionality of survivin rather than PSD. The T34 residue is

outwardly facing upon the crystal structure of survivin positioned neighbouring a crevice that upon phosphorylation could act to provide drastic conformational alterations to the protein changing its functionality (Figure 26). Understanding how the mechanism occurs would be greatly facilitated if the crystal structure of both proteins when bound could be obtained. The location of the survivin T34 residue within the structure of PSD could provide an insight into what regions it may interact with.

5.6 Survivin threonine 34 phosphorylation within the mitochondrion

The last question to address is how survivin becomes phosphorylated within the mitochondria to allow for this level of metabolic control. Over recent years it has become apparent that a wide array of kinases and phosphatases localise to the mitochondria, acting as part of the numerous signalling pathways that divulge there (Lim *et al* 2016). Potential mitochondrial serine/threonine kinases and phosphatases that could phosphorylate mitochondrial survivin at threonine 34 include the kinases protein kinase A (PKA), protein kinase C (PKC), ERK1/2, p18 MAPK, and phosphatases PP2C and PP2A (Arciuch *et al* 2009, Dohi *et al* 2004). Using kinase sequence predictors (KinasePhos, PPSP) we can identify the most probable kinase to be p18 MAPK. MAPK is serine/threonine kinase classified within the same kinase grouping as cyclin-dependent kinases (Chen *et al* 2007) of which one, cdk1, has already been described to phosphorylate threonine 34 (O'Connor *et al* 2000). The function of MAPK within the mitochondria is currently unknown and further experimentation is needed to determine its interaction with survivin (Figure 27).

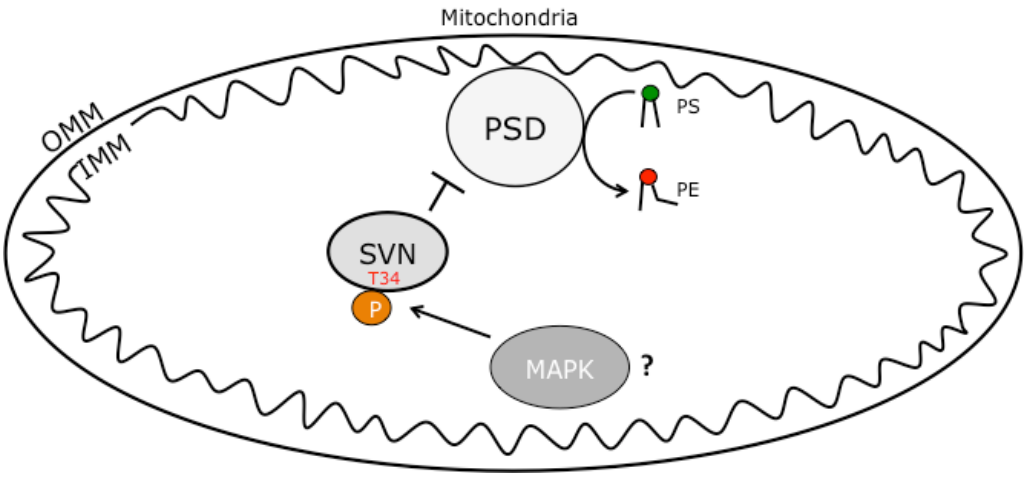


Figure 27. Schematic diagram illustrating the inhibition of phosphatidylserine decarboxylase (PSD) by survivin. Survivin inhibits the conversion of phosphatidylserine to phosphatidylethanolamine, through the inhibition of PSD upon phosphorylation of threonine 34. This is hypothesised to occur through the action of the inner mitochondrial membrane kinase p13 MAPK.

5.7 Conclusions

These data support the hypothesis that mitochondrial survivin inhibits phosphatidylserine decarboxylase when phosphorylated at the BIR domain residue threonine 34. This inhibitory action restricts PE production thus implicating survivin in the regulation of mitochondrial membrane architecture. This could also contribute to cancer cells versatility through the alteration of their oxidative phosphorylation capabilities.

This interaction has unveiled a novel function of survivin within a fundamental organelle, governing membrane integrity and metabolism through the manipulation of PE availability. This discovery also offers a new perspective to the multi-functional roles of survivin, offering a new outlook in its contribution to not only cancer but also to metabolic disorders.

6: Survivin$_{1-10}$ is a mitochondrial targeting sequence and c-Src regulator

6.1 Survivin$_{1-10}$ acts as a bona fide mitochondrial targeting sequence

The presence of mitochondrial survivin in cancer cells has currently been unexplained, both in its mechanism of transport and function. Our data demonstrate that the NH_2 terminal 10 amino acids of survivin are sufficient to transport GFP into the mitochondria. Mitochondrial targeting sequences (MTS) tend to be NH_2 terminal, positively charged amphipathic α-helixes, which are cleaved post-insertion (Vögtle *et al* 2009). Modelling has hence been performed using a hydropathy plot to investigate if the NH_2 terminal of survivin has a helical structure and amphipathic properties matching that described. As shown in Figure 28, a helical structure can be modelled

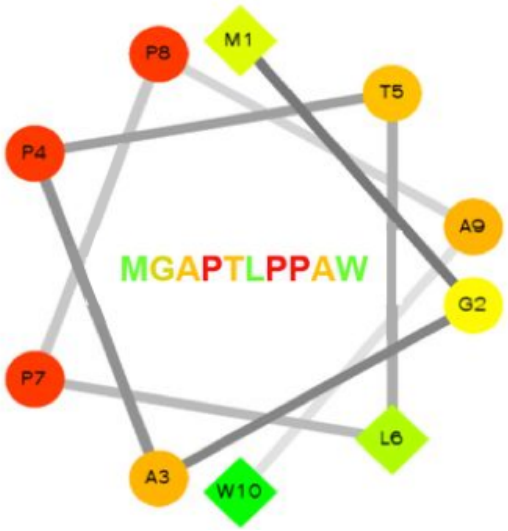


Figure 28. Hydropathy plot of survivin$_{1-10}$ helical structure. Hydrophobic residues are shown in red, with hydrophilic increasing towards the green scale. Residue types are therefore shown as circles for hydrophobic and diamonds for hydrophilic. Hydrophobic residues are concentrated on one side of the helix. Adapted from Dunajová *et al* 2016.

with clustered hydrophobic residues and more hydrophilic residues opposing, confirming its amphipathic nature. In an attempt to disrupt the defining properties of this helix, a mutated construct of the survivin$_{1\text{-}10}$ sequence was generated in which all prolines were replaced by alanines (survivin$_{1\text{-}10\Delta P}$-GFP). Upon expression of this sequence in HeLa cells, mitochondrial targeting of GFP was no longer observed, demonstrating the importance of this structure in the mitochondrial targeting of survivin (Dunajová *et al* 2016).

The action of mitochondrial chaperones has previously been used to describe the mitochondrial targeting of survivin. Mitochondrial chaperones act at a pre-import stage of protein targeting, delivering mitochondrial proteins to outer membrane import receptors such as the 'translocase of outer membrane receptor complex' or TOM70 (Fan *et al* 2006). Survivin has previously been linked to the chaperone Hsp90 (Fortugno *et al* 2003, Karagöz *et al* 2015); could it therefore be that this sequence allows for the targeting of the mature folded enzyme instead of the pre-protein form. Many intra-organelle localised proteins are not limited to one mechanism of intracellular transport, and this could also be the case for survivin.

6.2.1 The NH_2 terminal survivin$_{1\text{-}10}$ sequence is a polyproline-II helix and c-Src binding partner

We hypothesised that the growth and adhesion characteristics observed by HeLa cells expressing both survivin$_{1\text{-}10}$ and survivin$_{11\text{-}142}$ were due to a link between survivin and c-Src. Our data suggest that survivin$_{1\text{-}10}$ positively interacts with c-Src, and we hypothesise this is due to its identification as a 'polyproline-II helix'. The PPII helix is a binding domain that

directly interacts and binds to SH3 domains, such as present within c-Src (Yu *et al* 1994, Adzhubei *et al* 2013).

As seen in Figure 29, the Src SH3 domain contains two canonical 'XP binding regions' which bind with high specificity to a PPII domain containing a 'φPXφP' sequence; two hydrophobic residues (φ) flanked by two prolines, separated by one amino acid (Aitito *et al* 2010). The PPII helix is described as an extended left-handed helix which lacks cysteine residues, and contains two specific prolines that act as a direct binding site for the SH3 domain, with a neighbouring proline to stabilise the helical structure (Yu *et al* 1994). All of the previous descriptions therefore match the first ten amino acids of survivin with a great similarity (MGAPTLPPAW, Uniprot). Adzhubei *et al* 2013, demonstrated that PPII helixes ideally have three residues per left-handed helical turn, resulting in a triangular prism structure. Due to

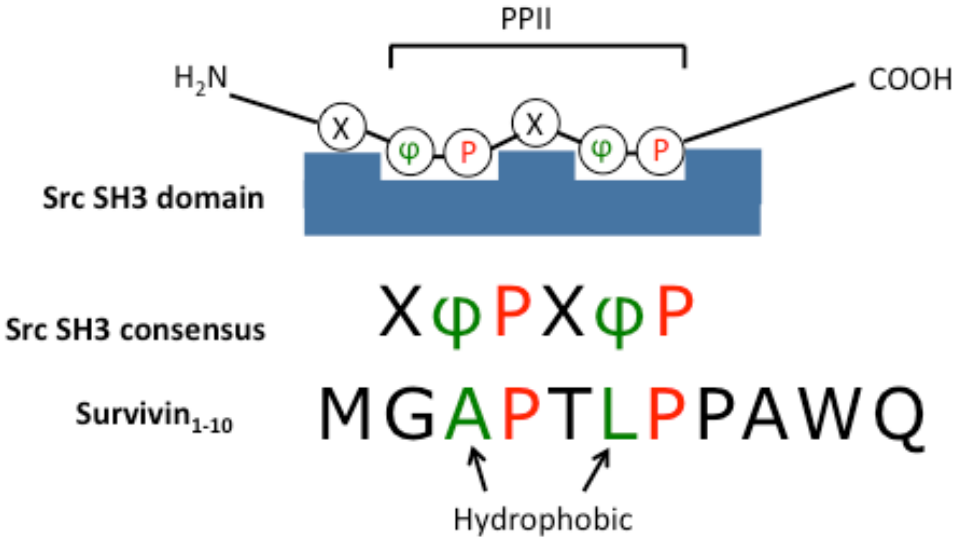


Figure 29. Schematic diagram illustrating a comparison of the Src SH3 domain consensus binding sequence with the NH_2 terminal of survivin. $Survivin_{1-10}$ provides a match to the documented PPII sequence that binds with great affinity to SH3 domains. Hydrophobic amino acids (φ, green) flank neighbouring prolines (red), binding to two canonical 'XP' binding pockets. Adapted from Aitito *et a*l 2010.

the large angle sizes between residues within the PPII helix, they tend to provide the flexibility of structures in regions linking alpha helixes, beta sheets and domain linker regions. Even though the predicted helical structure of survivin appears to have more residues per turn (Figure 27), a degree of elasticity is always observed in the PPII helical structure.

Due to the nature of the GFP trap experiment, it does not solely allow us to determine if a direct binding interaction occurs between survivin$_{1-10}$ and c-Src. However we hypothesise that survivin$_{1-10}$ is a genuine PPII helix due to the great similarity seen between the Src SH3 domain consensus binding sequence and survivin$_{1-10}$. This allows us to hypothesise that a direct interaction must occur between c-Src and survivin$_{1-10}$, due to the close association of PPII helixes and SH3 domains (Yu *et al* 1994).

The fact that survivin$_{1-10}$ is not only a mitochondrial targeting sequence but also a PPII helix, allows us to hypothesise that it performs a multi-functional role. As previously mentioned PPII helixes bind with great affinity to SH3 domains, thus this sequence not only allows for mitochondrial transport of survivin but may also facilitate the transport of other SH3 domain containing proteins. Evidence already demonstrates that this mechanism is plausible; p13, a mitochondrial accessory protein binds to the Src family kinases via its PPII helix assisting their mitochondrial transport (Tibaldi *et al* 2011). We therefore hypothesise that survivin$_{1-10}$ interacts with c-Src facilitating its transport into the mitochondria. It was reported that the mitochondrial transport of Src occurs through A-kinase anchor protein (AKAP121) binding to protein tyrosine phosphatase (PTPD1) (Alessandra Livingi *et al* 2006). Even though a c-Src mitochondrial transport mechanism has

therefore been determined, as with many other mitochondrial proteins including survivin, its conveyance may not be limited to one chaperonal complex.

Evidence suggests that members of the Src kinase family are present within the mitochondria and are key for the regulation of mitochondrial function (Salvi *et al* 2002). Miyazaki et al 2003, demonstrated that Src activity in the mitochondria affects energy metabolism through the phosphorylation and activation of the cytochrome-c oxidase subunit II. Mitochondrial targeting of Src has also been shown to increase the phosphorylation of mitochondrial substrates, increasing ATP production and mitochondrial outer membrane potential (Alessandra Livingi *et al* 2006). These data combined suggest that the mitochondrial targeting of c-Src could allow for distinct tyrosine kinase signalling, possibly shifting the focus of away from the plasma membrane to specific distal organelles (Livigni *et al* 2006).

Alternatively, survivin may interact with Src once inside of the organelle, causing the alterations to the oxidative phosphorylation machinery observed through their experimentation (Miyazaki *et al* 2013). It has previously been demonstrated that survivin over-expression decreases the process of oxidative phosphorylation due to inhibition of oxidative complex I (Hagenbuchner *et al* 2013). It is therefore important to consider the interaction of c-Src and survivin when observing changes to mitochondrial metabolism associated with the two proteins.

6.2.2 Survivin$_{1\text{-}10}$ inhibits Src activation

However the binding mechanism survivin and c-Src may occur, it is clear that this interaction has an influence over

pathways regulated by c-Src activation. It has typically been reported that c-Src receptor tyrosine-kinase activation and signalling orchestrates an extensive network of pathways that contributes to both tumour propagation and the metastatic state, through a reduction in cell adhesion properties (Ogura *et al* 2012). We recently reported (Dunajová *et al* 2016) that HeLa cells expressing survivin$_{11\text{-}142}$-GFP (lacking the NH_2 terminal) in culture displayed a greater quantity of focal adhesions and F-actin fibres. In comparison, HeLa cells expressing survivin$_{1\text{-}10}$-GFP showed a reduction in adhesion during handling and displayed a significantly higher growth rate, suggesting an involvement in this sequence with the complex network of signalling pathways stimulated by Src activation. Upon expressing survivin$_{1\text{-}10\Delta P}$-GFP in HeLa cells, focal adhesions and F-actin assembly were restored in comparison to survivin$_{1\text{-}10}$-GFP expression.

Contradictorily, we have found that P-Src is elevated in HeLa cells expressing survivin$_{11\text{-}142}$-GFP, which is coupled with increased adhesion properties typically associated with c-Src inactivation. Src is inactivated either when phosphorylated at an inactivating tyrosine 527 residue, preventing tyrosine 416 auto-phosphorylation and thus enzyme activation. Tyr527 phosphorylation prevents an interaction of itself with the SH2 domain, causing a conformational change resulting in c-Src to be in a closed position (Cowan-Jacob *et al* 2005). Phosphatase activity at this residue allows Src to be in an 'open' position, allowing for Tyr416 auto-phosphorylation and hence activation and substrate recognition (Zheng *et al* 2000).

Paradoxically it has been reported that a prolonged increase in activated c-Src acts to stimulate metastasis through increasing cancer cells migratory properties, but this is also correlated

with a rise in integrin-dependent adhesions (Jones *et al* 2002). Therefore it is possible that the NH_2 terminal of survivin plays a role in the inactivation of Src, explaining the increase in P-Src Tyr416 in HeLa cells expressing survivin$_{11\text{-}142}$-GFP, which when prolonged displays the observed adhesion characteristics by the cell line. It is already well established that the network of signalling pathways orchestrated by c-Src activation is exceedingly complex, and thus exactly how survivin contributes to its activation and its downstream targets is ultimately hard to determine.

6.3 Conclusions

We present the novel findings that the NH_2 terminus of survivin acts as a mitochondrial targeting sequence, and interacts with c-Src via its properties as polyproline-II helical structure, thus altering c-Src activation. We therefore suggest a new role of survivin in collaboration with c-Src, altering adhesion dynamics and further expanding its contribution in cancer.

7: References

Adzhubei, A. A. Sternberg, M. J. E. & Makarov, A. A. 2013. Polyproline-II Helix in Proteins: Structure and Function, *J Mol Biol,* **425(12)**:2100-2132, DOI:10.1016/j.jmb.2013.03.018

Aitio, O. Hellman, M. & Kazlauskas, A. *et al* 2010. Recognition of tandem PxxP motifs as a unique Src homology 3-binding mode triggers pathogen-driven actin assembly, *Proc Natl Acad Sci U S A*, **107(50)**:21743-21748. DOI: 1.1073/pnas.1010243107.

Alberts, B. Johnson, A. Lewis, J. *et al* 2002. Molecular Biology of the Cell, 4th edition.

Alessandra Livingni, A. Scorziello, A. & Agnese, S. *et al* 2005. Mitochondrial AKAp121 Links cAMP and src Signaling to Oxidative Metabolism, *MBC,* **17(1)**:263-271, DOI:10.1091/mbc.E05-09-0827.

Alexander, C. Votruba, M. & Pesch, U. *et al* 2000. OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28, *Nat Genet*, **26(2)**:211-215. DOI:10.1038/79944.

Altieri, D. C. 2003. Validating Survivin as a cancer therapeutic target, *Nat Rev Cancer,* **3**:46-54, DOI:10.1038/nrc968.

Altieri, D. C. 2006. The case for survivin as a regulator of microtubule dynamics and cell-death decisions, *Curr Opin Cell Biol,* **18(6)**:609-615, DOI: 10.1016/j.ceb.2006.08.015

Altieri, D. C. 2008. Survivin, cancer networks and pathway-directed drug discovery, *Nat Rev Cancer,* **8(1)**:61-70. DOI:10.1038/nrc2293.

Ambrosini, G. Adida, C. & Altieri, D. C. 1997. A novel antiapoptosis gene, Survivin, expressed in cancer and lymphoma, *Nat Med* **3**: 917-921, DOI: 10.1038/nm0897-917.

Andersson, K. M. E. Brisslert, & M. Cavallini, N. F. 2015. Survivin co-ordinates formation of follicular T-cells acting in synergy with Bcl-6, *Oncotarget* **6(24)**:20043–20057, DOI: 10.18632/oncotarget.4994.

Arciuch, V. Aliipe, Y. & Carreras, M. *et al* 2009. Mitochondrial kinases in cell signalling: Facts and perspectives, *Adv Drug Deliv Rev,* **61(14)**:1234-1249. DOI:10.1016/j.addr.2009.04.025.

Ashkenazi, A. Dixit, V. & Steller, H. *et al* 1998. Death receptors: signalling and modulation, *Science*, **281(5381)**:1305-1308. DOI:10.1126/science.281.538.1305.

Baenke, F. Peck, B. & Miess, H. *et al* 2013. Hooked on fat: the role of lipid synthesis in cancer metabolism and tumour development, *Dis Model Mech,* **6(6)**:1353-1363. DOI:10.1242/dmm.011338.

Barnum, K. O'Connell, M. 2014. Cell cycle regulation by checkpoints, *Meth Mol Biol,* **1170**:29-40. DOI:10.1007/978-1-4939-0888-2_2.

Barrett, R. M. A. Colnaghi, R. & Wheatley, S. P. 2011. Threonine 48 in the BIR domain of survivin is critical to its mitotic and anti-apoptotic activities and can be phosphorylated by CK2 in vitro, *Cell Cycle*, **10:3**:538-548, DOI: 10.4161/cc.10.3.14758.

Barrett, R. M. A. Osborne, T. P. & Wheatley, S. P. 2009. Phosphorylation of survivin at threonine 34 inhibits its mitotic

function and enhances its cytoprotective activity, *Cell Cycle* **8(2)**:278-283, DOI: 10.4161/cc.8.2.7587.

Blanc-brude, O. P. Mesri, M. & Wall, N. R. 2003. Therapeutic Targeting of the Survivin Pathway in Cancer -Initiation of Mitochondrial Apoptosis and Suppression of Tumor-associated Angiogenesis, *Clin Cancer Res* **9**:2683.

Bolton, M. Lan, W. & Powers, S. *et al* 2002. Aurora B kinase exists as a complex with survivin and INCENP and its kinase activity is stimulated by survivin binding and phosphorylation, *Mol Biol Cell*, **13(9)**:3064-3077. DOI:10.1091/mbc.E02-02-0092.

Borkenhagen, L. F. Kennedy, & E. P. Fielding, L. 1961. Enzymatic formation and decarboxylation of phosphatidylserine, *J Biol Chem*, **236**:PC28–PC32.

Böttinger, L. Horvath, S. E. & Kleinschroth, T. *et al* 2012. Phosphotidylethanolamine and cadiolipin differentially affect the stability of mitochondrial respiratory chain supercomplexes, *J Mol Bio,* **423(5)**:677-686, DOI:10.1016/j.jmb.2012.09.001.

Boyer, B. Bourgeois, Y. & Poupon, M. 2002. Src kinase contributes to the metastatic spead of carcinoma cells, *Oncogene*, **21(15)**:2347-2356, DOI:10.1038/sj.onc.1205298.

Brown, M. T. & Cooper, J. A. 1996. Regulation, substrates and functions of src, *BBA-Rev Cancer,* **1287(2-3)**:121-149. DOI:10.1016/0304-419X(96)00003-0.

Bürgermeister, M. Birner-Grünberger, R. & Nebauer, R. *et al* 2011. Contribution of different pathways to the supply of phosphatidylethanolamine and phosphatidylcholine to mitochondrial membranes of the yeast *Saccharomyces*

cerevisiae, BBA-Mol Cell Biol L, **1686(1-2)**:161-168, DOI:10.1016/j.bbalip.2004.09.007.

Cai, J. Yang, J. & Jones, D. 1998, Mitochondrial control of apoptosis: the role of cytochrome c, *BBA-Bioenergetics*, **1366(1)**:139-149, DOI:10.1016/S0005-2728(98)00109-1.

Carmena, M. Wheelock, M. & Funabiki, H. *et al* 2012. The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis, *Nat Rev Mol Cell Biol,* **13**:789-803, DOI:10.1038/nrm3474.

Carvalho, A. Carmena, M. & Sambade, C. *et al* 2003. Survivin is required for stable checkpoint activation in taxol-treated HeLa cells, *J Cell Sci,* **116(Pt14)**:2987-2998. DOI:10.1242/jcs.00612.

Chan, E. Y. L. & McQuibban, G. A. 2012. Phosphatidylserine Decarboxylase 1 (Psd1) Promotes Mitochondrial Fusion by Regulating the Biophysical Properties of the Mitochondrial Membrane and Alternative Topogenesis of Mitochondrial Genome Maintenance Protein 1 (Mgm1), *J Biol Chem,* **287**:40131-40139, DOI: 10.1074/jbc.M112.399428.

Cheeseman, I. A. & Desai, A. 2008. Molecular archintecture of the kinetochore-microtubule interface, *Nat Rev Mol Cell Bio,* **9**:33-46, DOI:10.1038/nrm2310.

Chen, H. Choymn, A. & Chan, D. 2005. Disruption of Fusion results in Mitochondrial Heterogeneity and Dysfunction, *J Biol Chem,* **280(28)**:26185-26192. DOI:10.1074/jbc.M503062200.

Chen, R. & Thorner, J. 2007. Function and regulation in MAPK signalling pathways: Lessons learned from the yeast

Saccharomyces cerevisiae, *Biochim Biophys Acta,* **1773(8)**:1311-1340. DOI: 10.1016/j.bbamcr.2007.05.003.

Chiu, H. Ho, Y. & Wang, Y. *et al* 2011. Arsenic trioxide induces autophagy and apoptosis in human glioma cells in vitro and in vivo through downregulation of survivin, *J Mol Med,* **89(9)**:927-941, DOI: 10.1007/s00109-011-0763-1.

Choi, J. Y. Duraisingh, M. T. & Marti, M. *et al* 2015. From Protease to Decarboxylase: THE MOLECULAR METAMORPHOSIS OF PHOSPHATIDYLSEINE DECARBOXYLASE, *J Biol Chem,* **290(17)**:10972-10980. DOI:10.1074/jbc.M115.642413.

Cogliati, S. Frezza, C. & Soriano, M. E. *et al* 2013. Mitochondrial Cristae Shape Determines Respiratory Chain Supercomplexes Assembly and Respiratory Efficiency, *Cell,* **155(1)**:160–171, DOI:10.1016/j.cell.2013.08.032

Colnaghi, R. Connell, C. M. & Barrett, R. M. *et al* 2006. Separating the anti-apoptotic and mitotic roles of survivin, *J Biol Chem,* **281(44)**:33450-33456, DOI:10.1074/jbc.C600164200.

Colnaghi, R. & Wheatley, S. P. 2010. Liaisons between Survivin and Plk1 during Cell Division and Cell Death, *J Biol Chem*, **285(29)**:22592–22604, DOI:10.1074/jbc.M109.065003.

Connell, C. Colnaghi, R. & Wheatley, S. 2008. Nuclear Survivin Has Reduced Stability and Is Not Cytoprotective, *J Biol Chem,* **283(6)**:3289-3296. DOI:10.1074/jbc.M704461200.

Cory, S. & Adams, J. 2002. The bcl2 family: regulators of the cellular life-or-death switch, *Nat Rev Cancer*, **2(9)**:647-656. DOI:10.1038/nrc883.

Cowan-Jacob, S. Fendrich, G. & Manley, P. *et al* 2005. The Crystal Structure of a c-Src Complex in and Active Conformation Suggests Possible Steps in c-Src Activation, *Structure,* **13(6)**:861-871. DOI:10.1016/j.str.2005.03.012.

Cryns, V. & Yuan, J. 1998. Proteases to die for, *Genes & Dev*, **12**:1551-1570, DOI:10.1101/gad.12.11.1551.

Debnath, J. Baehrecke, E. & Kroemer, G. 2005. Does Autophagy Contribute to Cell Death? *Autophagy,* **1(2)**:66-74. DOI:10.4161/auto.1.2.1738.

Denton, D. Nicolson, S. & Kumar, S. 2012. Cell death by autophagy: facts and apparent artefacts, *Cell Death Differ,* **19(1)**:87–95, 10.1038/cdd.2011.146.

Deveraux, Q. & Reed, J. 1999. IAP family proteins-suppressors of apoptosis, *Gene Dev*, **13(3)**:239-252.

Dohi, T. Beltrami, E. & Wall, N. R. *et al* 2004. Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis, *J Clin Invest,* **114(8)**:1117-1127, DOI:10.1172/JCLI22222.

Dohi, T. Xia, F. & Altieri, D. C. 2007. Compartmentalized Phosphorylation of IAP by Protein Kinase A Regulates Cytoprotection, *Mol Cell,* **27(1)**:17-28, DOI:10.1016/j.molcel.2007.06.004.

Dowhan, W. 1997. Molecular basis for membrane phospholipid diversity: Why are there so many lipids?, *Annu Rev Biochem,* **66**:199-232, DOI:10.1146/annurev.biochem.66.1.199.

Dunai, Z. Bauer, P. & Mihalik, R. 2011, Necroptosis: Biochemical, Physiological and Pathological Aspects, *Pathol Oncol Res*, **17(4)**:791-800, DOI:10.1007/s12253-011-9433-4.

Duvajová, L. Cash, & E. Markus, R. *et al* 2016. The N-terminus of survivin is a mitochondrial-targeting sequence and Src regulator, *J Cell Sci,* **129(4)**:2702-2712. DOI:10.1242/jcs.183277.

Elmore, S. 2007. Apoptosis: A Review of Programmed Cell Death, *Toxicol Pathol,* **35(4)**:495–516, DOI:10.1080/01926230701320337.

Emoto, K. Kobayashi, T. & Yamaii, A. *et al* 1996. Redistribution of phosphatidylethanolamine at the cleavage furrow of dividing cells during cytokinesis, *Proc Natl Acad Sci USA,* **93(23)**:12867-12872.

Emoto, K. Toyama-Sorimachi, N. & Karasuyama, H. *et al* 1997. Exposure of phosphatidylethanolamine on the surface of apoptotic cells, *Exp Cell Res*, **232**:430–434. DOI:10.1006/excr.1997.3521.

Escuín, D. & Rosell, R. 1999. The Anti-apoptosis Survivin Gene and its Role in Human Cancer: An Overview, *Clin Lung Cancer,* **1(2)**:138-143, DOI:10.3816/CLC.1999.n.011.

Fadok, V. A. Voelker, D. R. & Campbell, P. A. *et al* 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages, *J Immunol,* **148(7)**:2207-2216.

Fan, A. Bhangoo, M. & Young, J. 2006. Hsp90 functions in the targeting and outer membrane translocation steps of Tom70-mediated mitochondrial import, *J Biol Chem,* **281(44)**:33313-33324. DOI:10.1074/jbc.M605250200.

Fleming, A. Noda, T. & Yoshimori, T *et al* 2011. Chemical Modulators of autophagy as biological probes and potential

theraputics, *Nat Chem Biol,* **7**:9-17, doi:10.1038/nchembio.500.

Folch, J. Lees, M. & Sloane Stanley, G. H. 1957. A simple method for the isolation and purification of total lipids from animal tissues, *J Biol Chem,* **226**:497-509.

Fortugno, P. Beltrami, E. & Plescia, J. 2003. Regulation of survivin function by Hsp90, *Proc Natl Acad Sci USA,* **100(24)**:12791-12796, DOI:10.1073/pnas.2434345100.

Frame, M. C. 2002. Src in cancer: deregulation and consequences for cell behaviour, *Biochim Biophys Acta,* **1602**:114-130. DOI:10.1016/S0304-419X(02)00040-9.

Frohman, M. A. 2015. Role of mitochondrial lipids in guiding fission and fusion, *J Mol Med (Berl),* **93(3)**:263-269. DOI: 10.1007/s00109-014-1237-z.

Fuchs, Y. & Steller, H. 2015. *Nat Rev Mol Cell Bio,* **16(6)**:329-344. DOI:10.1038/nrm3999.

Fukunda, S. & Pelus, L. M. 2006. Survivin, a cancer target with an emerging role in normal adult tissues, *Mol Cancer Ther,* **5**:1087, DOI: 10.1158/1535-7163.MCT-05-0375.

Funderburk, S. Wang, Q. & Yue, Z. 2010. The Beclin 1-VPS34 complex – at the crossroads of autophagy and beyond, *Trends Cell Biol*, **20(6)**:355-362. DOI:10.1016/j.tcb.2010.03.002.

Gerben Vader, S. Kauw, J. J. W. & Medema, R. H. *et al* 2006. Survivin mediates targeting of the chromosomal passenger complex to the centromere and midbody, *EMBO R,* **7**:85-92, DOI:10.1038/sj.embor.7400562.

Glick, D. Barth, S. & Macleod, K. F. 2010. Autophagy: cellular and molecular mechanisms, *J Pathol*, **221(1)**:3–12, DOI:10.1002/path.2697.

Glover, D. M. Leibowitz, M. H. & McLean, D. A. *et al* 1995. Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles, *Cell,* **81(1)**:95-105, DOI: 10.1016/0092-8674(95)90374-7.

Gross, A. Jockel, J. & Wei, M. C. *et al* 1998. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis, *EMBO J,* **12**:3878-3885, DOI:10.1093/emboj/17.14.3878.

Hagenbuchner, J. Kuznetsov, A. V. & Obexer, P. *et al* 2013. BIRC5/Survivin enhances aerobic glycolysis and drug resistance by altered regulation of the mitochondrial fusion/fission machinery, *Oncogene,* **32**:4748–4757, DOI:10.1038/onc.2012.500.

Hallermayer, G. Zimmermann, R. & Neupert, W. 1977. Kinetic studies on the transport of cytoplasmically synthesized proteins into the mitochondria in intact cells of Neurospora crassa, *Eur J Biochem,* **81(3)**:523-532, DOI: 10.1111/j.1432-1033.1977.tb11978.

Hayashi-Nishino, M. Fujita, N. & Noda, T. *et al* 2009. A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation, *Nat Cell Biol,* **11(12)***:1433-1437,* DOI: 10.1038/ncb1991.

Hitchcock, P. Mason, R. & Thomas, K. *et al* 1974. Structural chemistry of 1,2dilauroyl-DL-phosphatidylethanolamine: molecular conformation and intermolecular packing of phospholipids, *Proc Natl Acad Sci U.S.A.,* **71(8)**:3036-3040.

Hübscher, H. G. Dils, R. R. & Pover, W. F. R. 1959. Studies on the biosynthesis of phosphatidylserine, *Biochim Biophys Acta,* **36:**518-525.

Igney, F. & Krammer, P. 2002, Death and Anti-death: Tumour resistance to apoptosis. *Nat Rev Cancer,* **2(4)**:277-288. DOI:10.1038/nrc776.

Irby, R. B. Mao, W. & Coppola, D. *et al* 1999. Activating SRC mutation in a subset of advanced human colon cancers, *Nat Genet,* **21(2)**:187-90. DOI:10.1038/5971.

Itaya, K. & Ui, M 1966. A new micromethod for the colormetric determination of inorganic phosphate, *Clinica Chimica Acta,* **14(3)**:361-365. DOI:10.1016/0009-8981(66)90114-8.

Iwamoto, K. Hayakawa, T. & Murate, M *et al* 2007. Curvature-dependent recognition of ethanolamine phospholipids by duramycin and cinnamycin, *Biophys J*, **93**:1608–1619. DOI:10.1529/biophysj.106.101584.

Jacobson, M. Weil, M. & Raff, M. 1997. Programmed Cell Death in Animal Development, *Cell*, **88(3)**:347-354. DOI:10.1016/S0092-8674(00)81873-5.

Jeyaprakash, A. A. Basquin, C. Jayachandran, U. *et al* 2011. Structural basis for the recognition of phosphorylated histone h3 by the survivin subunit of the chromosomal passenger complex, *Structure,* **19(11)**:1625-34, DOI: 10.1016/j.str.2011.09.002.

Jeyaprakash, A. A. Klein, U. R. Linder, D. *et al* 2007. Structure of a Survivin-Borealin-INCENP core complex reveals how chromosomal passengers travel together, *Cell,* **131(2)**:271-85, DOI:10.1016/j.cell.2007.07.045.

Jones, R. J. Avizienyte, E. & Wyke, A. W. *et al* 2002. Elevated c-Src is linked to altered cell – matrix adhesion rather than proliferation in KM12C human colorectal cancer cells, *Br J Cancer,* **87(10)**:1128-1135. DOI:10.1038/sj.bjc.6600594.

Jose, C. Bellance, N. & Rossignol, R. 2011. Choosing between glycolysis and oxidative phosphorylation: A tumor's dilemma?, *Biochim Biophys Acta Bioenergetics,* **1807(6)**:552-561. DOI:10.1016/j.bbabio.2010.10.012.

Junker, M. & Rapoport, T. A. 2015. Involvement of VAT-1 in Phosphatidylserine Transfer from the Endoplasmic Reticulum to Mitochondria, *Traffic,* **16(12)**:1306-1317, DOI: 10.1111/tra.12336.

Kang, B. H. Fang, X. & Pop, R. 2011. Developmental Control of Apoptosis by the Immunophilin Aryl Hydrocarbon Receptor-interacting Protein (AIP) Involves Mitochondrial Import of the Survivin Protein, *J Biol Chem,* **286**:16758-16767, DOI:10.1074/jbc.M110.210120.

Karagöz, G. Rüdiger, S. & Daggett, V. *et al* 2015. Hsp90 interacts with clients, *Trend Bioch Sci,* **40(2)**:117-125. DOI:10/1016/j.tibs.2014.12.002.

Kerr, J. Wyllie, A. & Currie, A. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics, *BJC*, **26(4)**:239-257.

Kim, H. Fernandes, G. & Lee, C. 2016, Protein Phosphatases Involved in Regulating Mitosis: Facts and Hypotheses. *Mol Cell,* **39(9)**:654-662, DOI: 10.14348/molcells.2016.0214.

Kitagawa, M. & Lee, S. H. 2015. The chromosomal passenger complex (CPC) as a key orchestrator of orderly mitotic exit

and cytokinesis, *Front Cell Dev Biol,* **3**:14, DOI:10.3389/fcell.2015.00014.

Kuge, O. Saito, K. & Kojima, M. *et al* 1994. Post-translational processing of the phosphatidylserine decarboxylase gene product in Chinese hamster ovary cells, *Biochem J,* **319**:33-38, DOI:10.1042/bj3190033.

Kypta, R. Goldberg, Y. & Ulug, E. *et al* 1990. Association between the PDGF receptor and members of the src family of tyrosine kinases, *Cell*, **62(3)**:481-492. DOI:10.1016/0092-8674(90)90013-5.

Levine, B. & Kroemer, G. 2008. Autophagy in the pathogenesis of disease, *Cell*, **132(1)**:27-42. DOI:10.1016/j.cell.2007.12.018.

Li, F. Ackermann, E. J. & Bennett, C. F *et al* 1999. Pleiotropic cell-division defects and apoptosis induced by interference with survivin function, *Nat Cell Biol,* **1**:461-466, DOI:10.1038/70242.

Li, L. Okura, M. & Imamoto, A. *et al* 2002. Focal Adhesions Require Catalytic Activity of Src Family Kinases To Mediate Integrin-Matrix Adhesion, *Mol Cell Biol,* **22(4)**:1203-1217, DOI:10.1128/MCB.22.4.1203-1217.2002.

Lim, S. Smith, K. R. & Lim, S-T. S. *et al* 2016. Regulation of mitochondrial functions by protein phosphorylation and dephosphorylation, *Cell Biosci,* **6**:25. DOI:10.1186/s13578-016-0089-3.

Linkermann, A. & Green, D. Necroptosis, *N Engl J Med,* **370(5)**:455-465, DOI:10.1056/NEJMra1310050.

Liu, T. Brouha, B. & Grossmann, D. 2004. Rapid induction of mitochondrial events and caspase-independent apoptosis in Survivin-targetined melanoma cells, *Oncogene,* **23(1)**:39-48. DOI:10.1038/sj.onc.1206978.

Lockshin, R. A. & Beaulaton, J. 1974. Programmed Cell Death, *Life Sci,* **15**:1549-1565. DOI:10.1016/0024-3205(74)90321-X.

Locksley, R. Killeen, N. & Lenardo, M. 2001. The TNF and TNF receptor superfamilies: Integrating Mammalian Biology, *Cell,* **104(4)**:487-501. DOI:10.1016/S0092-8674(01)00237-9.

Mahotka, C. Wenzel, M. & Springer, E. *et al* 1999. Survivin-ΔEx3 and Survivin-2B: Two Novel Splice Variants of the Apoptosis Inhibitor Survivin with Different Antiapoptotic Properties, *Cancer Res* **59**:6097. DOI:10.1146/annurev.med.48.1.267.

Marconescu, A. & Thorpe, P. E. 2008. Coincident exposure of phosphatidylethanolamine and anionic phospholipids on the surface of irradiated cells, *Biochim Biophys Acta,* **1778(10)**2217-2224. DOI:10.1016/j.bbamem.2008.05.006.

Marusawa, H. Matsuzawa, S. & Welsh, K. *et al* 2003. HBXIP functions as a cofactor of survivin in apoptosis suppression, *EMBO J,* **22**:2729-2740*,* DOI 10.1093/emboj/cdg263.

Matsuura, A. Tsukada, M. & Wada, Y. *et al* 1997. Apg1p, a novel protein kinase required for the autophagic process in Saccharomyces cerevisiae, *Gene*, **192(2)**:245-250. DOI:10.1016/S0378-1119(97)00084-X.

Menon, A. K. & Stevens, V. L. 1992. Phosphatidylethanolamine Is the Donor of the Ethanolamine Residue Linking a

Glycosylphosphatidylinositol Anchor to Protein, *J Biol Chem,* **267(22)**:15277-15280.

Mizushuma, N. Sugita, H. & Yoshimori, T. *et al* 1998. A New Protein Conjugation System in Human. THE COUNTERPART OF THE YEAST Apg12p CONJUGATION SYSTEM ESSENTIAL FOR AUTOPHAGY, *J Biol Chem,* **273(51)**:33889-33892. DOI:10.1074/jbc.273.51.33889.

Miyazaki,T. Neff, L. & Tanaka, S. *et al* 2003. Regulation of cytochrome *c* oxidase activity by c-Src in osteoclasts, *JCB,* **160(5)**:709-718. DOI:10.1083/jcb.200209098.

Musacchio, A. 2015. The Molecular Biology of Spindle Assembly Checkpoint Signaling Dynamics, *Curr Biol*, **25(22)**:3017, DOI:10.1016/j.cub.2015.08.051.

Navarro, J. Chabot, J. & Sherrill, K. *et al* 1985. Interaction of duramycin with artificial and natural membranes, *Biochemistry,* **24(17)**:4645-4650, DOI:10.1021/bi0033a025.

Neganova, I. & Lako, M. 2008, G1 to S phase cell transition in somatic and embryonic stem cells, *J Anat,* **213(1)**:30-44, DOI:10.111/j.1469-7580.2008.00931

Niedzialkowska, E. Wang, F. & Porebski, P. J. *et al* 2011. Molecular basis for phosphospecific recognition of histone H3 tails by Survivin paralogues at inner centromeres, **23(8)**:1457-1466, DOI:10.1091/mbc.E11-11-0904.

Niu, T. Cheng, Y. & Ren, X. *et al* 2010. Interaction of Beclin 1 with Survivin Regulates Sensitivity of Human Glioma Cells to TRAIL-induced Apoptosis, *FEBS Lett,* **584(16)**:3519-3524, DOI: 10.1016/j.febslet.2010.07.018.

Nogueira-Ferreira, R. Vitorino, R. & Ferreira-Pinto, M. J. *et al* 2013. Exploring the role of post-translational modifications on protein–protein interactions with survivin, *Arch Biochem Biophys,* **538(2)**:64-70, DOI:10.1016/j.abb.2013.07.027.

O'Connor, D. S. Grossman, D. & Plescia, J. 2000. Regulation of apoptosis at cell division by $p34^{cdc2}$ phosphorylation of survivin, *Proc Natl Acad Sci* **97(24)**:13103–13107, DOI: 10.1073/pnas.240390697.

Ogura, M. Yamaki, J. & Homma M. K. *et al* 2012. Mitochondrial c-Src regulates cell survivial through phosphorylation of respiratory chain components, *Biochem J,* **447(2)**:281-289, DOI:10.1042/BJ20120509.

Okada, H. Bakal, C. & Shahinian, A. *et al* 2004. Survivin Loss in Thymocytes Triggers p53-mediated Growth Arrest and p53-independent Cell Death, *J Exp Med* **199(3)**:399-410, DOI: 10.1084/jem.20032092.

Osman, C. Voelker, D. R. & Langer, T. 2011. Making heads or tails of phospholipids in mitochondria, *J Cell Biol,* **192(1)**:7-16, DOI:10.1083/jcb.201006159.

Ott, M. Gogvadze, V. & Orrenius, S. *et al* 2007, Mitochondria, oxidative stress and cell death, *Apoptosis*, **12**:913-922, DOI: 10.1007/s10495-007-0756-2.

Palade, G. 1953. An electron microscope study of the Mitochondrial Structure, *J Histochem Cytochem,* **1(4)**:188-211. DOI:10.1177/1.4.188.

Pan, G. O'Rourke, K. & Dixit, V. M. 1998. Caspase-9, Bcl-X_L, and Apaf-1 Form a Ternary Complex, *J Biol Chem,* **273**:5841-5845, DOI:10.1074/jbc.273.10.5841.

Parzych, K. R. & Klionsky, D. J. 2014. An Overview of Autophagy: Morphology, Mechanism, and Regulation, *Antioxid Redox Signal*, **20(3)**:460–473, DOI: 10.1089/ars.2013.5371.

Pfanner, N. & Geissler, A. 2001. Versatility Of The Mitochondrial Protein Import Machinery, *Nat Rev Mol Cell Bio,* **2**:339-349, DOI:10.1038/35073006.

Pfeiffer, T. Schuster, F. & Bonhoeffer, S. 2001. Co-operation and Competition in the Evolution of ATP-Producing Pathways, *Science,* **292(5516)**:504-507, DOI: 10.1126/science.1058079.

Prasad, M. Lovell, M. & Yatin, M. *et al* 1998. Regional Membrane Phospholipid Alterations in Alzheimer's Disease, *Neurochem Res*, **23(1)**:81-88. DOI:10.1023/A:1022457605436.

Rapaport, D. 2005. How does the TOM complex mediate insertion of precursor proteins into the mitochondrial outer membrane?, *JCB*, **171(1)**:419, DOI: 10.1083/jcb.200507147.

Rauch, A. Henning, D. & Schäfer, C. 2014. Survivin and YM155: How faithful is the liaison?, *BBA-Rev Cancer,* **1845(2)**:202-220, DOI:10.1016/j.bbcan.2014.01.003.

Riedl, S. J. Renatus, M. & Schwarzenbacher, R. *et al* 2001. Structural Basis for the Inhibition of Caspase-3 by XIAP, *Cell* **104(5)**:791-800, DOI: 10.1016/S0092-8674(01)00274-4.

Rivadeneira, D. B. Caino, M. C. & Seo, J. H. *et al* 2015. Survivin promotes oxidative phosphorylation, subcellular mitochondrial repositioning, and tumor cell invasion, *Sci Signal,* **8(389)**:80, DOI: 10.1126/scisignal.aab1624.

Rodríguez-Enríquez, S. Carreno-Feuntes, L. & Gallardo-Perez, J. *et al* 2010, Oxidative Phosphorylation is impaired by prolonged hypoxia in breast and possibly cervix carcinoma, *Int J Biochem Cell Biol,* **42(10)**:1744-1751. DOI:10.1016/j.biocel.2010,07.010.

Roskoski Jr, R. 2004. Src protein–tyrosine kinase structure and regulation, *Biochem Bioph Res Co,* **324(1)**:1155-1164, DOI:10.1016/j.bbrc.2004.09.171.

Roskoski Jr, R. 2005, Src kinase regulation by phosphorylation and dephosphorylation, *Biochem Bioph Res Co,* **331(1)**:1-14, DOI:10.1016/j.bbrc.2005.03.0120006291X.

Roy, S. & Nicholson, D. W. 2000. Cross-Talk in Cell Death Signaling, *J Exp Med,* **192(8)**:f21-f26.

Rous, P. 1911. *J. Exp. Med,* **13**:397–411.

Rubinsztein, D. Shpilka, T. & Elazar, Z. 2012. Mechanisms of Autophagosome Biogenesis, *Curr Biol*, **22(1)**:R29-R34. DOI: 10.1016/j.cub.2011.11.034.

Ruchaud, S. Carmena, M. & Earnshaw, W. C. 2007. Chromosomal passengers: conducting cell division, *Nat Rev Mol Cell Bio,* **8**:798-812, DOI:10.1038/nrm2257.

Saelens, X. Festjens, N. & Walle, L. V. *et al* 2004. Toxic proteins released from mitochondria in cell death, *Oncogene,* **23**:2861–2874, DOI:10.1038/sj.onc.1207523.

Salvi, M. Brunati, A. M. & Bordin, L. *et al* 2002. Characterization and location of Src-dependent tyrosine phosphorylation in rat brain mitochondria, *Biochim Biophys Acta,* **1589**:181-195, DOI:10.1016/S0167-4889(02)00174-X.

Santos, C. R. & Schulze, A. 2012. Lipid metabolism in cancer, *FEBS J,* **279(15)**:2610-2623, **DOI:**10.1111/j.1742-4658.2012.08644.

Schuiki, I. & Daum, G. 2009. Phosphatidylserine decarboxylases, Key Enzymes of Lipid Metabolism, *Life,* **61(2)**:151-162. DOI: 10.1002/iub.159.

Shin, S. Sung, B. & Cho, Y. *et al* 2001. An Anti-apoptotic Protein Human Survivin Is a Direct Inhibitor of Caspase-3 and -7, *Biochemistry*, **40(4)**:1117–1123, DOI:10.1021/bi001603q.

Shinzawa-Itoh, K. Aoyama, H. & Muramoto, K. *et al* 2007. Structures and physiological roles of 13 integral lipids of bovine heart cytochrome c oxidase, *EMBO J,* **26(6)**:1713-1725. DOI: 10.1038/sj.emboj.7601618.

Sickmann, A. Reinders, J. & Wagner, Y. *et al* 2003. The proteome of *Saccharomyces cerevisiae* mitochondria, *Proc Natl Acad Sci USA,* **100**:13207-13212, DOI:10.1073/pnas.2135385100.

Sigal, C. T. Zhou, W. & Buser, C. A. *et al* 1994. Amino-terminal basic residues of Src mediate membrane binding through electrostatic interaction with acidic phospholipids, *Proc Natl Acad Sci USA,* **91(25)**:12253-12257.

Smirnova, E. Griparic, L. & Shurland, D. *et al* 2001. Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells, *Mol Biol Cell,* **12(8)**:2245-2256.

Sohlenkamp, C. Geiger, O. & Abbanat, D. *et al* 2016. Bacterial membrane lipids: diversity in structures and pathways, *FEMS Microbiol Rev,* **40(1)**:133-159. DOI:10.1093/femsre/fuv008.

Stafford, J. H. & Thorpe, P. E. 2011. Increased Exposure of Phosphatidylethanolamine on the Surface of Tumor Vascular Endothelium, *Neoplasia,* **13(4)**:299-308.

Stauber, R. Mann, W. & Knauer, S. 2007. Nuclear and cytoplasmic survivin: molecular mechanism, prognostic, and therapeutic potential, *Cancer Res,* **67(13)**:5999-6002. DOI:10.1158/0008-5472.CAN-07-0494.

Stauber, R. H. Rabenhorst, U. & Rekik, A. *et al* 2006. Nucleocytoplasmic shuttling and the biological activity of mouse survivin are regulated by an active nuclear export signal, *Traffic,* **7(11)**:1461-1472, DOI: 10.1111/j.1600-0854.2006.00486.

Steenbergen, R. Nanowski, T. S. & Beigneux, A. *et al* 2005. Disruption of the Phosphatidylserine Decarboxylase Gene in Mice Causes Embryonic Lethality and Mitochondrial Defects, *J Biol Chem,* **280**:40032-40042, DOI: 10.1074/jbc.M506510200.

Stone, S. J. & Vance, J. E. 2000. Phosphatidylserine synthase-1 and -2 are localized to mitochondria-associated membranes. *J Biol Chem*, **275**:34534–34540. DOI:10.1074/jbc.M002865200.

Straight, A. Cheung, A. & Limouze, J. *et al* 2003, Dissecting temporal and spatial control of cytokinesis with a myosin II inhibitor, *Science,* **299(5613)**:1743-1747. DOI:10.1126/science.1081412.

Stuart, R. Cyr, D. & Craig, E. *et al* 1994. Mitochondrial molecular chaperones: their role in protein translocation, *Trends Biochem Sci,* **19(2)**:87-92. DOI:10.1016/0968-0004(94)90041-8.

Suganuma, K. Miwa, H. & Imai, N. *et al* 2010. Energy metabolism of leukemia cells: glycolysis versus oxidative phosphorylation, *Leuk Lymphoma,* **51(11)**:2112-2119, DOI:10.3109/10428194.2010.512966.

Summy, J. & Gallick, G. 2003, Src family kinases in tumor progression and metastasis, *Cancer Metastasis Rev,* **22(4)**:337-258. DOI:10.1023/A:1023772912750.

Sweeney, W. von Vinger, R. Frost, P. *et al* 2002. Src inhibition Amerliorates Polycystic Kidney Disease, *J Am Soc Nephrol,* **19(7)**:1331-1341. DOI:10.1681/ASN.2007060665.

Takahashi, A. Obata, Y. & Fukumoto, Y. *et al* 2009. Nuclear localization of Src-family tyrosine kinases is required for growth factor-induced euchromatinization, *Exp Cell Res,* **315(7)**:1117-1141, DOI:10.1016/j.yexcr.2009.02.010.

Tanida, I. Ueno, T. & Kominami, E. 2004. LC3 conjugation system in mammalian autophagy, *Int J Biochem Cell Biol,* **36(12)**:2503-2518. DOI:10.1016/j.biocel.2004.05.009.

Tasseva, G. Bai, H. D. & Davidescu, M. *et al* 2013. Phosphatidylethanolamine Deficiency in Mammalian Mitochondria Impairs Oxidative Phosphorylation and Alters Mitochondrial Morphology, *J Biol Chem,* **288**:4158-4173, DOI:10.1074/jbc.M112.434183.

Thomas, J. Ellis, B. & Boerner, R. *et al* 1998. SH2- and SH3-mediated Interactions between Focal Adhesion Kinase and Src, *J Biol Chem,* **273(1)**:577-583. DOI:10.1074/jbc.273.1.577.

Thomas, S. M. & Brugge, J. S. 1997. Cellular Functions Regulated By Src Family Kinases, *Annu Rev Cell Dev Bi,* **13**:513-609. DOI:10.1146/annurev.cellbio.13.1.513.

Thornberry, N. Lazebnik, Y. & Kerr, J. *et al* 1998. Caspases: enemies within, *Science,* **281(5381)**:1312-1316. DOI:10.1126/science.281.5381.1312.

Tibaldi, E. Venerando, A. & Zonta, F. *et al* 2011. Interaction between the SH3 domain of Src family kinases and the proline-rich motif of HTLV-1 p13:a novel mechanism underlying delivery of Src family kinases to mitochondria, *Biochem J,* **439(3)**:505-516, DOI:10.1042/BJ20101650.

Tice, D. A. Biscardi, J. S. & Nickles, A. L. *et al* 1999. Mechanism of biological synergy between cellular Src and epidermal growth factor receptor, *Proc Natl Sci U S A,* **96(4)**:1415-20.

Tirrò, E. Consoli, M. & Massimino, M. *et al* 2006. Altered expression of c-IAP1, survivin and Smac contributes to chemotherapy resistance in thyroid cancer cells, *Cancer Res,* **66(8)**:4263-4272. DOI:10.1158/0008-5472.CAN-02-3248.

Trotter, P. J. & Voelker, D. R. 1995. Identification of a Non-mitochondrial Phosphatidylserine Decarboxylase Activity (PSD2) in the Yeast *Saccharomyces cerevisiae, J Biol Chem, **270(11)**:6062-6070,* DOI:10.1074/jbc.270.11.6062.

Twig, G. Hyde, B. & Shirihai, O. *et al* 2008. Mitochondrial fusion, fission and autophagy as a quality control axis: the bioenergetics view, *Biochim Biophys Acta,* **1777(9)**:1092-1097. DOI:10.1016/j.bbabio.2008.05.001.

Van den Brink-van der, L. E. Killian, J. A. & de Kruijff, B. 2004. Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure profile, *Biochim Biophys Acta*, **1666(1-2)**:275-88, DOI: 10.1016/j.bbamem.2004.06.010.

Vance, J. E, 2015. Phospholipid synthesis and transport in mammalian cells, *Traffic,* **16(1)**:1-18, DOI:10.1111/tra.12230.

Vance, J. E. & Tasseva, G. 2013. Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells, *BBA-Mol Cell Biol L*, **1831(3)**:543-554, DOI:10.1016/j.bbalip.2012.08.016.

Vance, J. E. Aasman, E. J. & Szakra, E. 1991. Brefeldin A Does Not Inhibit the Movement of Phosphatidylethanolamine from Its Sites of Synthesis to the Cell Surface, *J Cell Biol,* **266(13)**:8241-8247.

Vander Heiden, M. G. Cantley, L. C. & Thompson, C. B. 2009. Understanding the Warburg effect: The Metabolic requirements of Cell Proliferation, *Science,* **324(5930)**:1029-1033, DOI: 10.1126/science.1160809.

Velculescu, V. E. Madden, S. L. & Zhang, L. *et al* 1999. Analysis of human transciptomes, *Nature genetics,* **23(4)**:387-388, DOI:10.1038/70487.

Verdecia, M. A. Huang, H. Dutil, E. *et al* 2000. Structure of the human anti-apoptotic protein survivin reveals a dimeric arrangement, *Nat Struct Biol* **7**:602 – 608, DOI: 10.1038/76838

Voelker, D. 1997. Phosphatidylserine decarboxylase, *BBA,* **1348(1-2)**:236-244. DOI:10.1016/S0005-2760(97)00101-X.

Vögtle, F. N. Wortelkamp, S. & Zahedi, R. P. *et al* 2009. Global Analysis of the Mitochondrial N-Proteome Identifies a Processing Peptidase Critical for Protein Stability, *Cell*, **139(2)**:428-439, DOI:10.1016/j.cell.2009.07.045.

Vong, Q. P. Cao, K. & Li, H. Y. *et al* 2005. Chromosome Alignment and Segregation Regulated by Ubiquitination of Survivin, *Science,* **310(5753)**:1499-1504, DOI:10.1126/science.1120160.

Wajant, H. Krammer, P. & Siegel, R. *et al* 2002. The Fas signalling pathway: more than a paradigm, *Science,* **296(5573)**:1635-1636. DOI:10.1126/science.1071553.

Wang, H. Sharp, T. V. 7 Koumi, A. *et al* 2002. Characterization of an anti-apoptotic glycoprotein encoded by Kaposi's sarcoma-associated herpesvirus which resembles a spliced variant of human survivin, *EMBO J*, **21(11)**:2602–2615, DOI: 10.1093/emboj/21.11.2602.

Wang, Q. Chen, Z. & Diao, X. *et al* 2011. Induction of autophagy-dependent apoptosis by the survivin suppressant YM155 in prostate cancer cells, *Cancer Lett,* **302(1)**:29-36, DOI:10.1016/j.canlet.2010.12.007.

Warburg, O. 1956. On the origin of Cancer Cells, *Science,* **123(3191)**:309-314, DOI: 10.1126/science.123.3191.309.

Wei, M. C. Lindsten, V. K. & Mootha, S. *et al* 2000. tBID, a membrane-targeted death ligand, oliomerizes BAK to release cytochrome c, *Genes Dev,* **14**:2060-2071, DOI:10.1101/gad.14.16.2060.

Wei, M. C. Zong, W. & Cheng, E. *et al* 2001. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death, *Science,* **292(5517)**:727-730. DOI:10.1126/science.1059108.

Westermann, S. & Schleiffer, A. 2013. Family matters: structural and functional conservation of centromere-

associated proteins from yeast to humans, *Trends Cell Biol,* **23(6)**:260-269, DOI:10.1016/j.tcb.2013.01.010

Wheatley, S. P. Barrett, R. & Andrews, P. *et al* 2007. Phosphorylation by Aurora- B Negatively Regulates Survivin Function During Mitosis, *Cell cycle,* **6(10)**:1220-1230. DOI:10.4161/cc.6.10.4179.

Wheatley, S. P. Carvalho, A. & Vagnarelli, P. 2001. INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis, *Curr Biol,* **11(11)**:886-890, DOI:10.1016/S0960-9822(01)00238-X.

Wheatley, S. P. Henzing, A. J. & Dodson, H. *et al* 2003. Aurora-B Phosphorylation *in Vitro* Identifies a Residue of Survivin That Is Essential for Its Localization and Binding to Inner Centromere Protein (INCENP) *in Vivo, J Biol Chem,* ***79****:*5655-5660, DOI: 0.1074/jbc.M311299200.

Wheatley, S. P. Carvalho, A. & Vagnarelli, P. 2001. INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis, *Curr Biol,* **11(11)**:886-890, DOI:10.1016/S0960-9822(01)00238-X.

Wiech, H. Buchner, J. & Zimmermann, R. *et al* 1992. Hsp90 chaperones protein folding *in vitro*, *Nature,* **358**:169-170, DOI:10.1038/358169a0.

Wirth, K. G. Wutz, G. & Kudo, N. R. *et al* 2006. Separase: a universal trigger for sister chromatid disjunction but not chromosome cycle progression, *J Cell Biol* **172 (6)**: 847, DOI: 10.1083/jcb.200506119.

Xie, Z. & Klionsky, D. 2007. Autophagosome formation: core machinery and adaptations, *Nat Cell Biol,* **9(10)**:1102-1109. DOI:10.1038/ncb1007-1102.

Xu, W. Doshi, A. & Lei, M. *et al* 1999. Crystal Structure of c-Src Reveal Features of its Autoinhibitory Mechanism, *Mol Cell*, **3(5)**:629-638, DOI: 10.1016/S1097-2765(00)80356-1.

Yang, Z. & Klionsky, D. J. 2010. Eaten alive: a history of macroautophagy, *Nat Cell Bio,* **12(9)**:814-822. DOI:10.1038/ncb0910-814.

Yonekawa, T. & Thorburn, A. 2014, Autophagy and Cell Death, *Essays Biochem*, **55**:105-117, DOI:10.1042/bse0550105.

Young, J. C. Hoogenraad, N. J. & Hartl, F. U. 2003. Molecular chaperones Hsp70 and Hsp90 deliver Preproteins to the Mitochondrial import receptor Tom70, *Cell,* **112(1)**:41-50, DOI:10.1016/S0092-8674(02)01250-3.

Yu, H. Chen, J. K. & Feng, S. *et al* 1994. Structural Basis for the Binding of Proline-Rich Peptides to SH3 Domains, *Cell,* **76**:933-945, DOI: 10.1016/0092-8674(94)90367-0.

Zborowski, J. Dygas, A. & Wojtczak, L. 1989. Phosphotidylserine decarboxylase is located on the external side of the inner mitochondrial membrane, *FEBS Lett,* **157(1)**:179-182, DOI:10.1016/001405793(83)81141-7.

Zheng, J. 2012. Energy Metabolism of cancer: Glycolysis versus oxidative phosphorylation (Review), *Oncol Lett,* **4(6)**:1551-1557, DOI:10.3892/ol.2012.928.

Zheng, X. Resnick, R. J. & Shalloway, D. 2000. A phosphotyrosine displacement mechanism for activation of Src by PTPα, *EMBO J,* **19**:964-978. DOI:10.1093.emboj/19.5.964.

Zheng, X. Resnick, R. J. & Shalloway, D. 2000. A phosphotyrosine displacement mechanism for activation of Src by PTPα, *EMBO J,* **19**:964-978. DOI:10/1093/emboj/19.5.964.

Zhu, S. Bjorge, J. D. & Fujita, D. J. 2007. PTP1B contributes to the oncogenic properties of colon cancer cells through Src activation, *Cancer Res,* **67(21)**:10129-10137. DOI:10.1158/0008-5472.CAN-06-4338.

Zick, M. Stroupe, C. & Orr, A. *et al* 2014. Membranes linked by *trans*-SNARE complexes require lipids prone to non-bilayer structure for progression to fusion, *eLife,* **3**, DOI:10.7554/eLife.01879.