Examining the importance of survivin phosphorylation at Threonine 5 through its relationship with the kinase, TBK1, and the substrate, c-Src

Jamie Lishak

School of Life Sciences

Abstract

As a protein that is significantly involved in many processes of cellular life and death, survivin has garnered much interest as a cancer-associated protein. It is the fourth most upregulated transcript in human cancers and it cooperates with several other proteins to aid oncogenesis. One of these is the non-receptor tyrosine kinase c-Src, which is regulated by the N-terminus of survivin. Here, we have shown that this interaction is dependent on the phosphorylation of survivin at Threonine 5. The kinase that induces this phosphorylation is thought to be the immune protein TANK-Binding Kinase 1 (TBK1) and consequently, the effects of TBK1 on survivin were also studied. TBK1 was found to reduce survivin expression in HeLa cells that had been transfected with GFP and survivin-GFP constructs, however no such effect was found on non-transfected HeLa cells (i.e. endogenous survivin) or HeLa cells transfected with survivin$_{1\text{-}10}$-GFP. The experiments detailed here provide some insight into the relationships between survivin, c-Src and TBK1, with potential implications for the study of cell adhesion, apoptosis and autophagy in cancer.

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1 Introduction

1.1 Overview

Survivin is one of the most tumour-specific proteins, with its key roles in mitosis and apoptosis making it an important part of the cancer machinery. With it also conferring chemotherapy and radiotherapy resistance, survivin is increasingly being studied as a potential oncotherapeutic target. Survivin is involved in many signalling pathways and has been suggested to cooperate with several different proteins to promote tumourigenesis. As a result, there remains much to be discovered about survivin's roles in cancer and how it can potentially be inhibited pharmacologically.

The studies presented here were an examination of survivin's relationships with two cooperating partners: c-Src and TANK-Binding Kinase 1 (TBK1). This followed on from research suggesting that the N-terminus of survivin can regulate c-Src and is phosphorylated by TBK1 (Dunajová *et al.*, 2016; Chellappan Lab, Moffitt Cancer Centre, unpublished; see Appendix A). The impacts of these interactions on cell division, cell death and cell adhesion were considered.

1.2 Cellular Processes

1.2.1 Cell Division

1.2.1.1 Overview

Cell division is a highly conserved and tightly regulated process that enables a cell to accurately transfer its genome to two daughter cells. In eukaryotes, this comprises four discrete phases – a Synthesis or S-phase in which the cell's DNA is replicated, a Mitosis or M-phase where the chromosomes segregate and the

cell divides, and two Gap phases, G_1 and G_2, which allow the cell to organise its genetic material and adequately prepare itself for the S- and M-phases respectively (Figure 1) (Barnum and O'Connell, 2014).

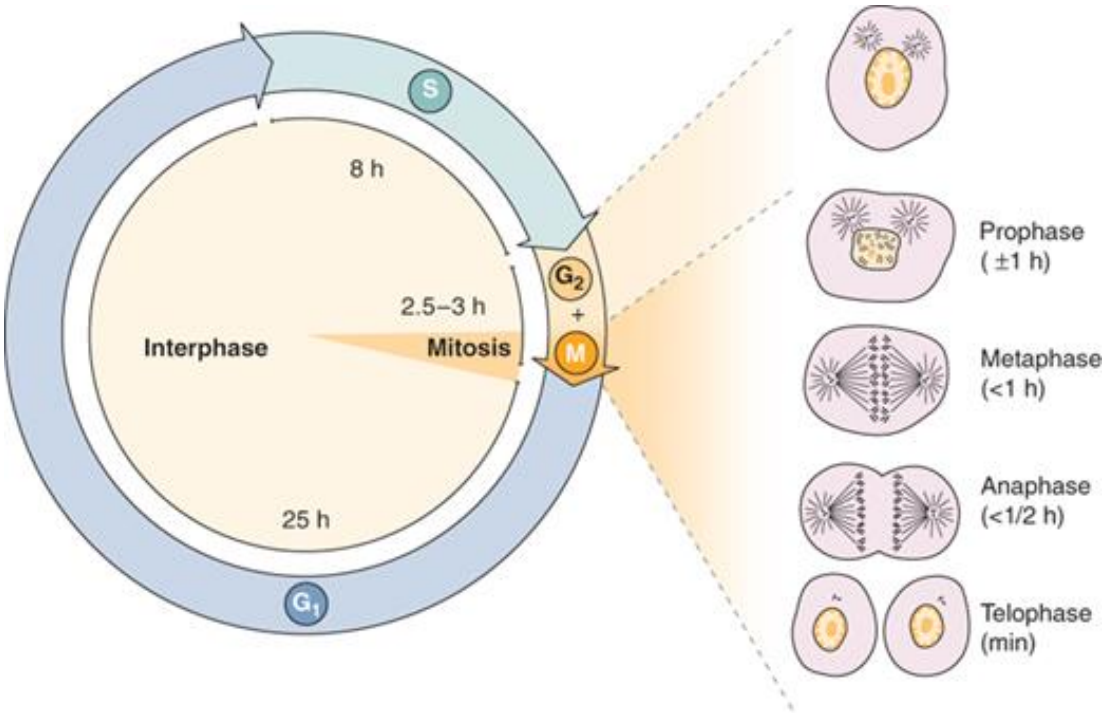


Figure 1 - Overview of the cell cycle. Taken from ABSITE Slayer, 1st Edition (McGraw-Hill Education)

The process of cell replication is initiated in interphase; the cell can either commit to a new cell cycle by entering G_1 or enter a quiescent state known as G_0. Successful passage through the cell cycle is dependent on various checkpoint proteins that regulate cyclin-dependent kinase (CDK) activity to control cell cycle progression and as such, early G_1 is characterised by the generation of D-type cyclins, which are one the terminal products of the RAS/RAF/MAPK pathway (Malumbres and Pellicer, 1998). This leads to the formation of CDK4/6–cyclin-D complexes and the partial phosphorylation and inactivation of the Retinoblastoma (Rb) protein, a potent cell cycle inhibitor, which then triggers the activation of various transcription factors necessary for the transition from G_1 to S-phase (Malumbres and Barbacid, 2001). For all this to happen, cells must have passed DNA replication checkpoints. The kinases ATM (Ataxia telangiectasia

mutated) and ATR (Ataxia Telangiectasia and Rad3 related) are activated by DNA damage and, depending on the type of DNA damage, can initiate pathways leading to the activation of the checkpoint proteins Chk1 and Chk2 and subsequently arrest the cell in G_1 (Maréchal and Zou, 2013). All of these processes allow the cell to integrate various mitogenic signals, gauge that there are sufficient nutrients for replication to begin, and ensure the viability of the soon-to-be replicated DNA.

In S-phase, DNA is unwound by the helicase enzyme, with free nucleotides then binding to the single stranded DNA through the action of DNA polymerase. There is a further chance for detection of DNA damage by the ATR kinase at this point (Branzei and Foiani, 2005).

Following this, the cell enters G_2 phase; this is where the necessary quantities of organelles and proteins for the two daughter cells can be produced. At this point, there is an additional opportunity for ATM and ATR kinase to assess the viability of the DNA (Bertoli, Skotheim and Bruin, 2013) before threshold levels of Maturation Promoting Factor (MPF) are reached, inducing mitosis (Kishimoto, 2015).

1.2.1.2 Mitosis

M-phase consists of six distinct stages: prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis.

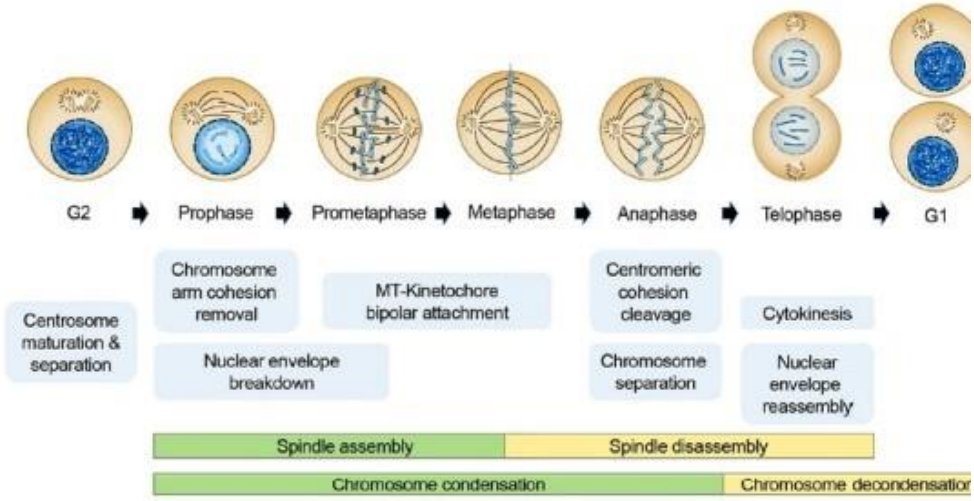


Figure 2 - The stages of mitosis. Adapted from Kim et al (2016).

During G_2, the cell has arranged its replicated DNA into sister chromatids and as it enters prophase, the nuclear envelope disintegrates and the chromatids condense to form two chromosomal arms, linked by a centromere (Figure 2). At prometaphase, kinetochores form around the centromere and attach to duplicated centrosomes, which have separated to opposing poles of the cell, through microtubules (Cheeseman and Desai, 2008). Metaphase then follows, where the tension in the microtubules causes the chromosomes to align across the metaphase plate in the centre of the cell; there is a spindle assembly checkpoint at this point to ensure proper alignment of the chromosomes before progression to anaphase is initiated when the cell stops inhibiting anaphase promoting complex/cyclosome (APC/C) (Gorbsky *et al.*, 1999). Anaphase involves the separation of the sister chromatids through the shortening of the spindle microtubules, which results in the chromosomes being pulled to opposite poles of the cell. Telophase can then commence, with the cytoplasm spit by the

formation of an actin/myosin ring around the cell equator – a process termed cytokinesis (Straight *et al.*, 2003).

1.2.1.3 Chromosomal Passenger Complex (CPC)

The localisation of chromosomes during mitosis is a vitally important part of cell division, and so a complex of Chromosomal Passenger Proteins (CPP) exists to co-ordinate the targeting of chromosomes to the correct areas at different stages of mitosis and cytokinesis (van der Waal *et al.*, 2012). The constitutive proteins are Aurora B Kinase, borealin, inner centromere protein (INCENP) and survivin (Figure 3).

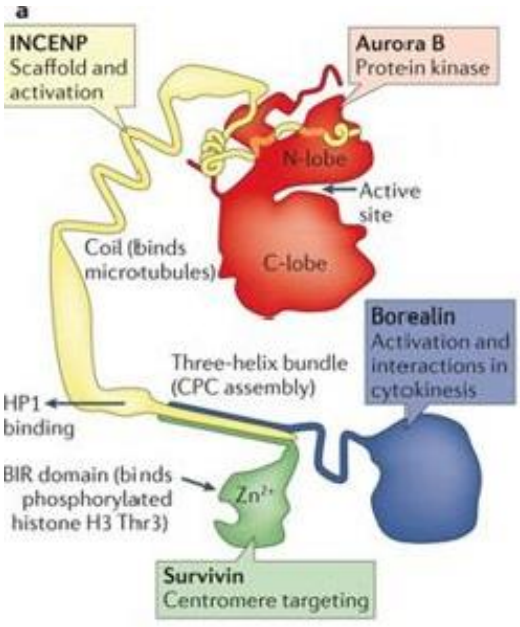


Figure 3 - CPC proteins and their functions. Extracted from Carmena *et al* (2012).

Functionally, the CPC can be split into two modules, with survivin, borealin and the amino terminus of INCENP forming the localisation module which is connected to a kinase module of Aurora B kinase and the central region of INCENP (Carmena *et al.*, 2012). The mitotic localisation of the complex is dependent on the binding of survivin to INCENP, an interaction promoted by borealin, and strikingly, this works even in the absence of the centromere-targeting domain of INCENP (Vader *et al.*, 2006). At the onset of mitosis,

activation of the CPC is initiated by phosphorylation of two histone tails (Yamagishi *et al.*, 2010), one of which can interact with survivin (Jeyaprakash *et al.*, 2011) and the other which recruits Shugoshin-like proteins that interact with borealin (Tsukahara, Tanno and Watanabe, 2010). The subsequent movement of the CPC throughout mitosis allows activation of the relevant proteins at different stages of the cell cycle (Carmena *et al.*, 2012). From prophase to metaphase it is centromeric, where it can regulate chromosome alignment at the metaphase plate through correction of aberrant kinetochore-microtubule attachments (Ruchaud, Carmena and Earnshaw, 2007). The CPC then relocalises to the midbody during the metaphase-anaphase transition where it aids in the formation of the cleavage furrow and separation of the sister chromosomes through microtubule shortening, with the reformation of the nuclear envelope and abscission of the daughter cells following on (Kitagawa and Lee, 2015).

1.2.2 Cell Death

1.2.2.1 Overview

The process of cell death is an equilibrium, with an organism's need to remove old, infected or mutated cells (Fuchs and Steller, 2015) in balance with the need for viable numbers of surviving cells. The molecular pathways that induce cell death are therefore tightly regulated, activated by specific stimuli, and they lead to three main types of cell death: apoptosis, necroptosis and autophagy. These processes can be exploited in disease states. Excessive apoptosis is a common feature of many neurodegenerative diseases, including Alzheimer's disease where mass neuronal death due to apoptosis is a typical phenotype (Shimohama, 2000). Equally, a lack of cell death can be damaging and is a "hallmark of cancer" (Hanahan and Weinberg, 2011) due to the genetic instability caused by the survival of mutated cells.

1.2.2.2 Apoptosis

Apoptosis is generally accepted as the most common and important form of programmed cell death (Elmore, 2007). It is prominent in development; the removal of unwanted cells is vital in sculpting organs, controlling cell numbers and producing differentiated cells (Jacobson, Weil and Raff, 1997). The eventual death of cells subjected to apoptosis is typically induced by morphological changes, such as cell shrinkage and membrane blebbing (Doonan and Cotter, 2008) or by DNA damage in the form of chromatin condensation or nuclear fragmentation (Liu *et al.*, 1998). There are two key pathways of apoptosis, the extrinsic and intrinsic pathways, which coordinate extracellular and intracellular cell death signals respectively before converging at the executioner caspases, 3 and 7, to induce apoptosis (Figure 4). Having previously been seen as distinct pathways, it is now generally accepted that there is some overlap between them (Roy and Nicholson, 2000).

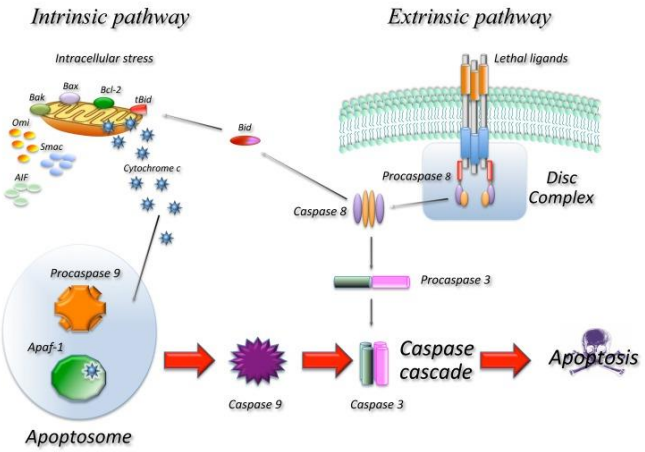


Figure 4 - Schematic of the extrinsic and intrinsic pathways of apoptosis. The extrinsic pathway coordinates the response to cell death stimuli at the plasma membrane whereas the intrinsic pathway responds to intracellular stimuli at the mitochondria. The pathways converge at caspase 3. Extracted from Favaloro et al (2012)

Caspases are a vital part of the apoptotic machinery and can generally be split into two groups: initiator and executioner caspases. Initiator caspases (such as caspases 8,9 and 10) act to trigger apoptotic pathways and executioner caspases (such as caspases 3,6 and 7) are the effectors of apoptosis. The zymogenic nature of caspases allows them to be effective co-ordinators of apoptosis, remaining inert until activation by pro-death stimuli. The two classes of caspases are activated by distinct mechanisms, with initiator caspases requiring dimerisation and executioner caspases requiring cleavage to become active (Boatright and Salvesen, 2003) and this is an important regulatory mechanism to prevent the inappropriate triggering of apoptosis, as well as ensuring a potent response once apoptosis is in motion (Thornberry and Lazebnik, 1998). Regulation of apoptosis can also be effected by the inhibition of caspases by proteins such as survivin and its fellow Inhibitor of Apoptosis family members (see later).

The extrinsic pathway of apoptosis is typically induced through formation of the death-inducing signalling complex (DISC) in response to extracellular pro-death stimuli, a process involving the binding of a plasma membrane death receptor of the TNF (Tumour Necrosis Factor) family, such as Fas, to its extracellular ligand, Fas-L, so that it can recruit death domain-containing protein (FADD) and procaspase 8 (Ouyang *et al.*, 2012). Dimerised caspase 8 can then activate executioner caspases through cleavage, as well as cleave other targets such as BID and Poly(ADP-ribose) Polymerase (PARP). Cleavage of BID is able to induce cell death through increasing outer mitochondrial membrane permeabilisation (MOMP) (H. Li *et al.*, 1998) and cleavage of PARP through preventing DNA repair (Boulares *et al.*, 1999).

The intrinsic pathway of apoptosis is consequentially the same, resulting in the activation of executioner caspases, but the mechanism of action and the cell death signals involved differ. It is an intracellular process centred on

mitochondria, which integrate various pro-death stimuli and trigger the pathway in response. Common stimuli include DNA damage, growth factor withdrawal and oxidative stress (Elmore, 2007; Ott *et al.*, 2007). These all induce changes in mitochondria which lead to apoptosis through MOMP in two main ways. A permeability transition (PT) pore can open on the inner mitochondrial membrane, with the loss of membrane potential leading to the release of pro-apoptotic proteins (cytochrome c, Smac/DIABLO and apoptosis-inducing factor) into the cytosol (Green and Kroemer, 2004). Here, a structure known as the apoptosome can form, consisting of cytochrome c, Apaf-1 (Apoptotic protease activating factor 1) and procaspase 9 (Pan, O'Rourke and Dixit, 1998). Recruitment to this complex enables the autoactivation of procaspase 9, which is then able to cleave executioner caspases (Zou *et al.*, 1999).

A second mechanism of inducing MOMP involves a family of proteins known as Bcl-2. This family includes both pro- and anti-apoptotic proteins, the concentrations of which at the mitochondria determine a cell's fate (Kuwana and Newmeyer, 2003). The pro-apoptotic members of this family BAX and BAK are able to effect MOMP through the opening of outer mitochondrial membrane pores; other members are important in activating BAX and BAK and disrupting anti-apoptotic proteins (Gross *et al.*, 1998; Wei *et al.*, 2000; Letai *et al.*, 2002). It is at this point that there is crossover between the intrinsic and extrinsic pathways as cleavage of BID in the extrinsic pathway inhibits the inhibitors of BAX and BAK, allowing for them to permeabilise the outer mitochondrial membrane (Wei *et al.*, 2001).

1.2.2.3 Autophagy

Autophagy is an intracellular degradation process that uses lysosomes to recycle unwanted cytoplasmic elements (Mizushima, 2007). It can be selective or non-selective, sequestering either the relevant organelles or the bulk of the cytoplasm, and this is generally dependent on whether autophagy is stimulated

by nutrient deprivation or pathological conditions respectively (Xie and Klionsky, 2007). As a catabolic process, autophagy functions to both remove potentially damaging cell matter and produce energy, and its classification as a cell death process has been questioned due to its roles in cell survival (Debnath, Baehrecke and Kroemer, 2005). Evidence for autophagy as a genuine programmed cell death process comes from the highly elevated levels of cell death seen when accompanied by autophagy structures such as autophagosomes, but a direct causal link between autophagy and cell death is yet to be discovered (Yonekawa and Thorburn, 2013).

The mechanism of autophagy first involves formation of a phagophore, which sequesters the organelles and cellular material targeted for degradation (Figure 5). As the phagophore elongates and forms a double membrane, it becomes a vesicle known as an autophagosome. Autophagosomes can fuse with lysosomes to form structures called autolysosomes, and the exposure of the vesicle contents to lysosomal hydolases degrades them. Then, the inner autolysosome membrane can be degraded with the recycled macromolecules released into the cytoplasm (Mizushima, 2007; Xie and Klionsky, 2007).

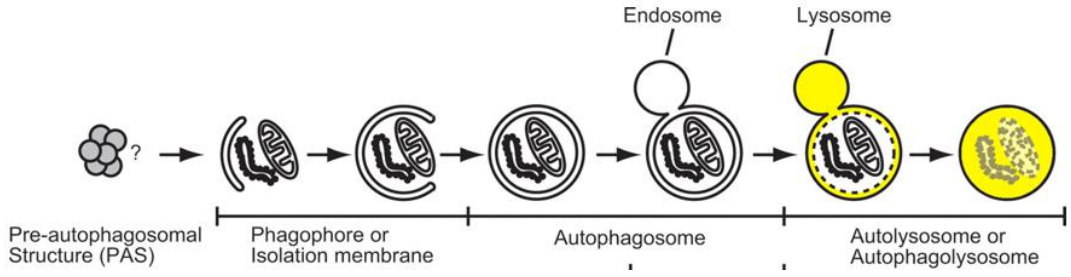


Figure 5 - Overview of the autophagy process. Adapted from Mizushima (2007).

Regulation of autophagy is exceedingly complex, but generally involves various signalling pathways that co-ordinate the expression of the relevant Autophagy-related (ATG) genes to bring about the formation and function of the autophagic machinery (Virgin and Levine, 2009).

The functions of autophagy are broadly to produce energy under nutrient starvation and to remove cellular threats such as pathogens. Autophagy is particularly stimulated by amino acid deficits, and this can be seen in the decreased intracellular and extracellular amino acid levels of autophagy-deficient yeast cells and mice (Kuma *et al.*, 2004; Onodera and Ohsumi, 2005).

The relevance of autophagy to cancer comes through its ability to aid both tumour suppression (through the removal of damaged proteins and organelles) and tumourigenesis (by helping rapidly proliferating cells to maintain energy production) (Yang *et al.*, 2011). Survivin and TBK1 have both been implicated in the function of autophagy, with TBK1 in particular seen as a key link between autophagy and the innate response to various disease states (Wang *et al.*, 2011; Pilli *et al.*, 2012). Autophagy has shown the ability to degrade certain bacteria, viruses and parasites, and has roles in Toll-receptor signalling and MHC Class II antigen presentation (Levine and Deretic, 2007). Defects in autophagy have been linked to several types of disease. Neurodegenerative diseases are commonly related to deficiencies in the clearance of misfolded and aggregated proteins by autophagy (Martinez-Vicente and Cuervo, 2007). Liver disease, muscle disease and cardiac disease have also been linked to the process (Levine and Kroemer, 2008). Through the ATG gene beclin 1, autophagy has been implicated as a tumour-suppression process and consequently, oncogenic loss-of-function mutations in the autophagy pathway have been shown to promote tumour progression (Mathew, Karantza-Wadsworth and White, 2007).

There is a specific type of autophagy for selective degradation of mitochondria, termed mitophagy (Youle and Narendra, 2010). This allows for the removal of dysfunctional mitochondria and prevents the generation of damaging reactive oxygen species in response to mitochondrial stress (Chourasia, Boland and Macleod, 2015). Mitophagy has been proposed to help tumours overcome metabolic stress by altering mitochondrial metabolism (Pavlides *et al.*, 2011).

1.3 Survivin

1.3.1 Overview

Inhibitor of apoptosis proteins (IAPs), first discovered through a baculovirus gene that was able to inhibit apoptosis when transfected into *Spodoptera frugiperda* cells (Crook, Clem and Miller, 1993), are a family of anti-apoptotic proteins that act on the intrinsic pathway of apoptosis both before and after convergence with the extrinsic pathway to prevent cell death (Schimmer, 2004). In humans, eight IAPs have been discovered with the common link of a zinc finger fold of around 70 amino acids, termed a Baculovirus IAP Repeat (BIR) domain (Srinivasula and Ashwell, 2008). The smallest member of this family at 142 amino acids and 16.5 kDa is survivin. Since its discovery in 1997 (Ambrosini, Adida and Altieri, 1997), several more functions of survivin have been elucidated, resulting in a shift in the scientific consensus from a simple anti-apoptotic protein to one that is pivotal in various processes of both cell proliferation and cell death, as well as a central part of many molecular pathways, such as its pivotal role in the CPC (van der Waal *et al.*, 2012). Further to this, it has high tumour specificity and is associated with poor prognosis in many cancers, in part due its ability to confer resistance to chemotherapy and radiotherapy, making it an attractive oncotherapeutic target (Velculescu *et al.*, 1999; Asanuma *et al.*, 2005; Tirrò *et al.*, 2006).

1.3.2 Structure

Overview

Whereas the majority of IAPs have multiple BIR domains, survivin has only a singular BIR domain and lacks the additional carboxy-terminal domains of some of the other members, such as Caspase Activation Recruitment (CARD) or Really

Interesting New Gene (RING) domains (Figure 6).

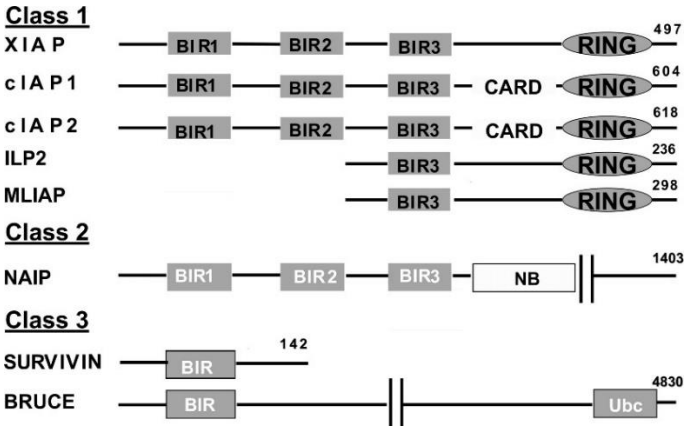


Figure 6 - Structures of IAP family proteins. Taken from Schimmer (2004).

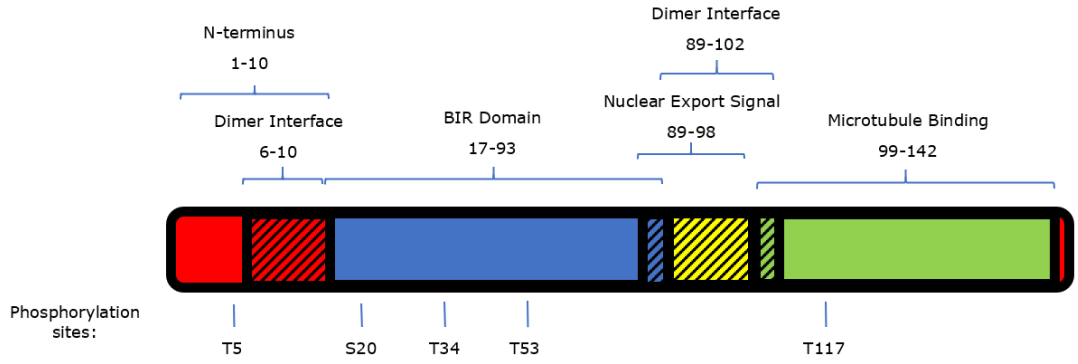


Figure 7 - Basic structure of survivin and its phosphorylation sites

The N-terminus of survivin (amino acids (aa) 1-10) functions as a mitochondrial targeting sequence and is followed by a BIR domain (aa 17-93), nuclear export sequence (NES; aa 89-98) and an alpha-helical C-terminus (aa 99-142) (Figure 7) (Dunajová *et al.*, 2016; Verdecia *et al.*, 2000; Colnaghi *et al.*, 2006; Cheung *et al.*, 2013). Structural integrity of the BIR domain is maintained by a Zinc finger, formed of cysteines 57, 60 and 84, and histidine 77 (Nogueira-Ferreira *et al.*, 2013).

Dimerisation

Survivin crystallises into a homodimer, yet many of its functions require it to be monomeric. These include its role in the CPC and some of its anti-apoptotic activity (Bourhis, Hymowitz and Cochran, 2007; Pavlyukov *et al.*, 2011). Dimerisation has been shown to interfere with the shuttling of survivin out of the nucleus, with the interaction between its NES and the export receptor CRM1 antagonised, suggesting that it is a key determinant of survivin's subcellular localisation (Dieuwke *et al.*, 2007). Regulation of this comes via acetylation on lysine 129 (K129), a reaction dependent on CREB-binding protein, which predictably promotes nuclear accumulation of survivin (Wang *et al.*, 2010). Both constitutive acetyl mimetics and non-acetylatable mutants of survivin cause mitotic defects, indicating that cyclical acetylation and deacetylation at K129 is necessary for functional mitosis (Aljaberi, Webster and Wheatley, 2015). Taken together, it is apparent that the monomer-dimer balance of survivin has a substantial effect on its localisation and therefore its function.

N-terminus

In addition to the survivin that is shuttled between the nucleus and cytoplasm, there is a distinct pool of survivin in the mitochondria (Dohi *et al.*, 2004). This is facilitated by its amphiphilic N-terminus, which is able to function as a mitochondrial targeting sequence even when truncated from the rest of the protein (Dunajová *et al.*, 2016). Further N-terminal specific functions of survivin are its ability to bind and regulate the activity of the non-receptor tyrosine kinase c-Src and to reduce sensitivity to X-irradiation (Wheatley, 2015; Dunajová *et al.*, 2016). The interaction between survivin and c-Src is theorised to be due to the recruitment of to survivin's proline-rich N-terminus to the SH3 domain of c-Src and through this, survivin has been shown to regulate c-Src (see section 1.5.5).

BIR Domain

Survivin is somewhat atypical as an IAP in that it has only a single BIR domain. Despite being a domain specific to anti-apoptotic proteins, the BIR domain is vital for survivin to both inhibit cell death and to function in the CPC (Ambrosini, Adida and Altieri, 1997; Carmena *et al.*, 2012). Two known cofactors are mitotically-phosphorylated histone 3, which helps target the CPC and heat shock protein 90, which may be involved in transporting survivin into the mitochondria (Fortugno *et al.*, 2003; Niedzialkowska *et al.*, 2012).

1.3.3 The survivin gene

The survivin gene was first identified through the homology of its coding region to that of the effector cell protease receptor, EPR-1 (Ambrosini, Adida and Altieri, 1997). It is 11,451 bases long with four exons and five introns, a cytogenetic band at 17q25.3, and stark oncofetal expression - neoplastic and embryonic tissues show significantly increased expression of "wild type" survivin compared to normal adult tissue (Adida *et al.*, 1998; Velculescu *et al.*, 1999). The human gene allows for splicing into several isoforms, five of which have known functions: survivin-2α, survivin 3α, survivin-2β, survivin-3β and survivin-ΔEx3 (Sah and Seniya, 2015). Each has a distinct cellular localisation and structure (Mahotka *et al.*, 1999).

Survivin is expressed in mitotic cells and at various stages of embryonic development but not in differentiated cells, with the exception of activated T lymphocytes and erythroblasts (Adida *et al.*, 1998; F. Li *et al.*, 1998; Leung *et al.*, 2007; Keerthivasan *et al.*, 2012). Correct survivin expression in mitosis is ensured by three cell cycle-dependent elements and a cell cycle homology region in its promoter. Tumour suppressors such as p53 repress the survivin promoter, whereas it is activated in certain signalling pathways, including NF-κB and MAPK (Boidot, Végran and Lizard-Nacol, 2014).

1.3.4 Cellular Functions

1.3.4.1 Subcellular Localisation

Survivin has roles in several subcellular compartments and its travel between these is often dependent on its dimerisation status, as previously mentioned. In normal cells it is predominantly nuclear, with expression peaking in G2 and mitosis (Wheatley *et al.*, 2001).Cytoplasmic expression of survivin has also been noted in cancer patients, where its anti-apoptotic functions can be exploited (Li *et al.*, 2005), although it has been suggested that this is dependent on survivin first being enriched in the mitochondria before being discharged back in to the cytoplasm (Dohi, Xia and Altieri, 2007). There is also a mitochondrial pool of survivin that appears to be uniquely expressed by cancer cells, where it can influence mitochondrial dynamics, as well as acting as a store before release into the cytoplasm (Dohi *et al.*, 2004; Rivadeneira *et al.*, 2015).

1.3.4.2 Mitosis

In mitosis, survivin is part of the CPC and helps guide it to the centromeres during prophase and metaphase, where it can ensure proper chromosome alignment through the correction of faulty kinetochore-microtubule attachments (Vader *et al.*, 2006). Together with borealin, it is key in recognising and binding mitotically-phosphorylated histone 3 and it synchronises mitosis with cytokinesis through confirming that there is sufficient chromosome tension to proceed (Lens *et al.*, 2003; Niedzialkowska *et al.*, 2012).

Certain cells also require survivin to regulate the G1/S transition, including T lymphocytes, haematopoietic progenitor cells, hepatoma cells, and breast cancer cells (Fukuda and Pelus, 2006).

1.3.4.3 Apoptosis

The oncological potential of survivin is underpinned by its critical roles in the cellular processes of both life, through mitosis, and of death, through apoptosis. Survivin's impact here can be clearly seen in the induction of apoptosis in cells treated with an antisense oligonucleotide for survivin (Olie *et al.*, 2000).

There has been much speculation and conflicting reports on exactly where in the intrinsic pathway of apoptosis survivin acts. It has been suggested that as the BIR domains of X-linked inhibitor of apoptosis protein (XIAP) are able to directly inhibit caspases 3,7 and 9, with BIR2 causing steric hindrance of caspases 3 and 7 (Sun *et al.*, 1999; Riedl *et al.*, 2001) and BIR3 preventing caspase 9 from dimerising (Shiozaki *et al.*, 2003), that survivin may also directly interact with caspases to inhibit apoptosis. In one study, survivin was shown to have an inhibitory effect on caspases 3 and 7, although the specificity of the interaction is questionable and the experiments were not performed at normal physiological conditions (Shin *et al.*, 2001), and a further study also claimed to show a direct interaction between survivin and caspases 3 and 7, but the negative control also bound to caspase 7, there were no positive controls, and there was no mention of the temperature at which the binding occurred (Tamm *et al.*, 1998). Subsequent studies have attempted to validate the finding of a direct inhibitory effect of survivin on caspases but have been unable (Banks *et al.*, 2000; Verdecia *et al.*, 2000; Song, Yao and Wu, 2003).

Some of the anti-apoptotic effects of survivin appear to be linked to its subcellular localisation. It was found that only survivin molecules that had been released from the mitochondria into the cytosol could form a complex with XIAP to inhibit apoptosis (Dohi, Xia and Altieri, 2007). Survivin that was exclusively cytosolic could not associate with XIAP, and this was linked to phosphorylation at Serine 20. The mechanism of apoptosis inhibition by a survivin-XIAP complex is

currently undefined and was found to be unrelated to interactions with caspase 9 (Zumbrägel *et al.*, 2017).

Survivin can also inhibit apoptosis through interactions with other proteins. The effects of survivin dimerisation are particularly interesting here, as monomeric survivin interacts with Smac/DIABLO and XIAP both in vitro and in vivo, conferring even greater resistance to apoptosis than the wild type (Pavlyukov *et al.*, 2011). However, only the survivin dimer was able to enhance tubulin stability, suggesting that the monomer-dimer balance of survivin is key in regulating apoptosis.

Hepatitis B X-interacting protein (HBXIP) appears to be an important cofactor for survivin in the inhibition of apoptosis. Individually, they are unable to suppress caspase activation but when added in combination, they prevent cytochrome c release through blocking the recruitment of procaspase 9 to the apoptosome (Marusawa *et al.*, 2003).

A role for survivin in the extrinsic pathway has also been suggested, with the finding that it inhibits caspase 8 activation, but it does not bind the caspase directly (Tamm *et al.*, 1998). Overall, survivin appears to have influence on the inhibition of apoptosis at various stages and through acting with several cofactors.

1.3.4.4 Mitochondria

Mitochondrial survivin is unique to cancer cells and was first discovered due to its anti-apoptotic effects but since then, evidence of a much broader role in influencing mitochondrial dynamics has emerged. Survivin has been implicated in a glycolytic switch known as the "Warburg effect", whereby cancer cells undergo a shift from oxidative phosphorylation to glycolysis to produce ATP, through inhibiting complex I activity (Vander Heiden, Cantley and Thompson, 2009; Hagenbuchner *et al.*, 2012). This was said to be induced in part by

mitochondrial fragmentation caused by survivin, however the group used a tetracycline vector to produce survivin-expressing cell lines and tetracyclines can cause a similar mitochondrial fragmentation phenotype (Moullan *et al.*, 2015). There is also conflicting evidence that survivin promotes oxidative phosphorylation (Rivadeneira *et al.*, 2015). In this case, the mechanism of action was enhancement of complex II and this, together with the fact that a different cell line was used, may explain the discrepancy. Unpublished data from the Wheatley group corroborates the Hagenbuchner *et al.*, (2012) findings that survivin promotes glycolysis rather than oxidative phosphorylation.

1.3.4.5 Autophagy

Mitochondrial survivin has also been suggested to play a role in the inhibition of autophagy. A complex involving PTEN-induced-kinase-1 (PINK1), beclin 1, parkin and survivin has been proposed to regulate mitophagy through keeping the autophagy-inducing beclin 1 bound until an autophagic threshold has been reached (Hagenbuchner, 2016). Further evidence for survivin as an autophagy inhibitor is provided by studies showing that downregulation of survivin, through the pharmacological inhibitor YM155 or arsenic trioxide, induces autophagy (Chiu, Ho and Wang, 2011; Wang *et al.*, 2011).

1.3.4.6 Post-translational modifications (PTMs)

Survivin function can be regulated through phosphorylation and there are currently six known sites where this occurs: Threonine (T); T5; T34; T48; T53; T117; Serine (S) 20 (Wheatley *et al.*, 2004; Dohi, Xia and Altieri, 2007; Osborne and Wheatley, 2009; Barrett, Colnaghi and Wheatley, 2011; Cheung *et al.*, 2013; Wheatley lab, unpublished). Phosphorylation of survivin appears to be an important regulator of which pathways it can participate in (Nogueira-Ferreira *et al.*, 2013). Survivin is phosphorylated at T34 at the onset of mitosis and interestingly, a constitutively active mimetic of this was found to be inhibitory to

mitosis, as well as apoptosis (O'Connor *et al.*, 2000). Phosphorylation at T48 and S20 are also important PTMs for mitosis.

As previously mentioned, acetylation at K129 is a key determinant of survivin subcellular localisation, causing survivin to dimerise and therefore prevent its nuclear export (Wang *et al.*, 2010).

K63-linked ubiquitination of survivin has distinct effects on its stability and its ability to align chromosomes. Ubiquitinated survivin has a half-life of 30-120 minutes before degradation by the 26S proteasome (Zhao *et al.*, 2000). Regulation of this appears to be cell cycle-dependent, with survivin needing to be ubiquitinated to associate with centromeres and then de-ubiquitinated, mediated by hFAM, to disassociate (Vong *et al.*, 2005).

1.3.5 Cancer-specific Functions

1.3.5.1 Regulation and Expression

The tumour-specific deregulation of survivin in cancer is undoubtedly vital to tumour progression. The increase in survivin expression has been linked to changes in the survivin promoter, which shows far greater activity in cancer cell lines than normal cell lines (Li and Altieri, 1999; Pisarcik *et al.*, 2002). In ovarian cancer, the increased incidence of demethylation of survivin exon 1 compared to in normal ovaries has been linked to deregulation of survivin expression (Hattori *et al.*, 2001).

The wild-type form of the tumour-suppressor p53 strongly suppresses survivin expression by binding to its promoter, but loss-of-function mutations of p53 have been shown to occur in almost every type of cancer and this has been implicated in the cancer-specific deregulation of survivin expression (Hoffman *et al.*, 2002; Rivlin *et al.*, 2011).

1.3.5.2 Chromosome Instability

In normal cells, apoptosis is a vital cog in the cellular regulation machinery. Through the various checkpoints of the mitotic cycle, defects in chromosome segregation can be detected and the relevant cells destroyed to prevent aneuploidy and provide some level of genetic stability (Reed and Bischoff, 2000). This regulation is an essential host defence mechanism against pathological cell proliferation. In this context, the marked over-expression of survivin in the vast majority of cancers can be seen as an attempt by tumour cells to induce genome instability, which has been described as the underlying factor behind many characteristics of neoplastic disease (Hanahan and Weinberg, 2011). Survivin overexpression has been shown to induce chromosome instability at mitosis through aberrations in kinetochore-microtubule attachments, resulting in chromatin being trapped at the cleavage furrow and exposed to damage and then DNA-damage response processes (Conde *et al.*, 2017). The subsequent aneuploidy is correlated with poor prognosis and chemoresistance (Rajagopalan and Lengauer, 2004).

1.3.5.3 Retrospective Studies and Oncotherapeutic Potential

The correlation between survivin and disease outcomes has been examined through retrospective studies of various types of cancer. These include bladder, breast, central nervous system, hepatocellular, lung and pancreatic tumours (Fukuda and Pelus, 2006). In general, elevated survivin levels are associated with shorter disease-free interval and survival. However, survivin's function as a prognostic marker has been poor for some breast, lung and gastric cancers and osteosarcoma (Fukuda and Pelus, 2006).

Survivin's high tumour specificity, correlation with poor disease outcomes and contribution to chemo- and radiotherapy resistance has made it an attractive target for pharmacological inhibition. Attempts to inhibit survivin expression

have led to the development of antisense oligonucleotides such as LY2181308 which, although showing promising results in vitro, have shown little efficacy in vivo, particularly for solid tumours (Rödel *et al.*, 2008; Tanioka *et al.*, 2011).

High-Throughput Screening identified YM155 as a potential small-molecule inhibitor of survivin (Nakahara *et al.*, 2007). It was thought to act by binding directly to survivin's promoter but in fact acts to inhibit survivin upstream transcription factor Sp1 (Cheng *et al.*, 2012). Promising results led to Phase II trials, but the need for continuous infusion has meant that it is considered unviable for now and there are no present ongoing studies into it (Peery, Liu and Zhang, 2017).

Inhibiting survivin expression is seen as difficult because of survivin's lack of enzymatic activity. As such, the focus has now shifted to disrupting survivin by targeting its homodimerisation. One molecule, LQZ-F7, has been discovered to block survivin homodimerisation and consequently induce survivin degradation and inhibit cancer cell survival. Studies in xenograft tumours have been promising and this appears to be the future of survivin-related therapies (Peery, Liu and Zhang, 2017).

1.3.6 Phosphorylation at T5

Preliminary data has suggested that survivin can be phosphorylated at T5 by TBK1 (Chellappan Lab, Moffitt Cancer Centre, unpublished; see Appendix A). Unpublished research in the Wheatley lab has found that phosphorylation at this site can prevent the mitochondrial targeting of survivin and cause aberrant localisation in mitosis. As T5 is adjacent to survivin's dimer interface, promotion of homodimerisation has also been seen.

1.4 TANK-Binding Kinase 1

1.4.1 Overview

TBK1 (TANK {TRAF (TNF (tumour necrosis factor) receptor-associated factor)-associated NF-κB activator}-binding kinase 1) is an ubiquitously expressed (Tojima *et al.*, 2000) serine/threonine kinase and a non-canonical member of the IκB kinase (IKK) family – a group of proteins which inhibit the nuclear factor-kappa B (NF-κB) pathway (Pomerantz and Baltimore, 1999). Its primarily studied function has been the regulation of type I interferon (IFN) production (Fitzgerald *et al.*, 2003) but TBK1's activity in several signalling pathways has implicated it in a number of other cellular processes such as autophagy (Thurston *et al.*, 2009), apoptosis (Lafont *et al.*, 2018) and insulin signalling (Uhm *et al.*, 2017); these functions can be utilised to induce oncogenic transformation and promote cell proliferation under pathogenic conditions (Ou *et al.*, 2011). Mutated forms of the protein have been linked to various neuroinflammatory conditions, including childhood herpes simplex encephalitis (HSE) (Ahmad *et al.*, 2018), amyotrophic lateral sclerosis (ALS) (Chase, 2015) and frontotemporal dementia (FTD) (Gijselinck *et al.*, 2015).

1.4.2 Structure

TBK1 comprises 729 amino acids and four functional domains. From N- to C-terminus these are: kinase domain (KD); ubiquitin-like domain (ULD); scaffold/dimerization domain (SDD); C-terminal domain (CTD) (Figure 8).



Figure 8 - Domains of TBK1. KD: kinase domain; ULD: ubiquitin-like domain; SDD: scaffold/dimerisation domain; CTD: C-terminal domain. Adapted from Tu et al. (2013).

It is dimeric, with dimerisation assumed to be mediated in the same way as fellow IKK family member IKKβ - through leucine zipper and helix-loop-helix motifs in the SDD (Xu *et al.*, 2011). Studies on IKKβ have also shown that the ULD is required for kinase activity (May *et al.*, 2004) and interaction between the ULD and KD in TBK1 suggests that it may be performing a similar function (Tu *et al.*, 2013). This is supported by the finding that deletion of the ULD impairs kinase activation of TBK1 (Ikeda *et al.*, 2007).

TBK1 requires transautophosphorylation at S172 to become active (Clark *et al.*, 2009) and this induces conformational changes in the KD that aid in the autoactivation of other nearby TBK1 molecules (Ma *et al.*, 2012). At low concentrations, TBK1 appears to be maintained in an inactive state through its KD structure which places the kinase active sites on opposite faces of the dimer, making it difficult for the dimer to phosphorylate itself (Ma *et al.*, 2012). It is hypothesised that high TBK1 concentration caused by recruitment of TBK1 dimers to signalling complexes enables the activation-loop swapping that results in transautoactivation (Ma *et al.*, 2012).

K63-linked polyubiquitination at K30 and K401 is a further regulatory mechanism of TBK1 activation. Mutating these residues into inert forms causes disruption downstream activity in the IRF3 signalling pathway and of S172 phosphorylation, with ubiquitination at these sites seemingly a prerequisite for TBK1 activity (Tu *et al.*, 2013).

1.4.3 Cellular Functions

Immune response

Coordination of the innate immune response to viral infection requires the activation of several signalling pathways, and TBK1 is integral in two of these: NF-κB and interferon regulatory factor 3 (IRF3). TBK1 is recruited to the IRF3 pathway by a number of adaptor proteins, including TRAF (Chariot *et al.*, 2002)

and MITA/STING (stimulator of interferon gene) (Zhong *et al.*, 2008). Once activated, it can phosphorylate the C-terminus of IRF3 to induce its dimerisation and to localise it to the nucleus (Clément *et al.*, 2008).

NF-kB pathway

The role of TBK1 in NF- κB activation is less defined. It was found to be non-essential to activation of the pathway in response to lipopolysaccharide, a bacteria-associated motif (Perry *et al.*, 2004). However, it has also been shown to activate NF- κB reporter genes (Tojima *et al.*, 2000) through an interaction with TANK (Pomerantz and Baltimore, 1999).

Apoptosis

Knockdown of TBK1 in mice is embryonically lethal, resulting in widespread liver degeneration and apoptosis (Bonnard *et al.*, 2000), suggesting TBK1 is an active suppressor of cell death. Indeed, it has been shown to regulate Tumour Necrosis Factor (TNF) induced cell death through inhibitory phosphorylation of receptor-interacting protein kinase 1 (RIPK1), preventing its association with caspase 8 and limiting the subsequent caspase cascade (Xu *et al.*, 2018). This was independent of activation of the NF- κB pathway, which further acts to regulate RIPK1 through cIAP1/2-induced K63-linked polyubiquitination (Bertrand and Vandenabeele, 2010) in a manner independent of TBK1 (Xu *et al.*, 2018).

Conversely, C-terminal-phosphorylated IRF3 is strongly pro-apoptotic (Heylbroeck *et al.*, 2000), a post-translational modification performed by TBK1. Functional redundancies between TBK1 and IKKε in IRF3 activation may explain the apoptotic phenotype of $TBK1^{-/-}$ mice. It appears that TBK1 can act to both promote and restrain cell death depending on the stimulus, with TNF inducing an anti-apoptotic response and viral infection inducing a pro-apoptotic response.

Autophagy

A role for TBK1 in autophagy has been proposed, with the finding that it directly phosphorylates the autophagic adaptor optineurin (OPTN) to enhance its interaction with ubiquitin chains and proteins essential for autophagy (Weidberg and Elazar, 2011). TBK1 is promoted by the PINK1-PARKIN pathway, which facilitates mitophagy through the polyubiquitination of damaged mitochondria, and it phosphorylates four receptors at several autophagy-relevant mitochondrial locations (Heo *et al.*, 2015; Richter *et al.*, 2016). Further, TBK1 was found to be essential for the maturation of autophagosomes in autolysosomes and as such is a key regulator of autophagy (Pilli *et al.*, 2012). The removal of mycobacteria and damaged mitochondria by autophagy appears to be dependent on TBK1 activity.

Polyubiquitination is also used in autophagy to target proteins for degradation, with the autophagy receptor p62 a key part of this process (Shaid *et al.*, 2012). TBK1 has a large influence in this as it phosphorylates p62 at S403, which greatly increases the affinity of p62 to ubiquitin chains (Moscat and Diaz-Meco, 2009). This can be clearly seen in the impairment of autophagy when TBK1 is deleted in mice, with p62- and ubiquitin-positive aggregates accumulating in Purkinje cells (Duan *et al.*, 2019).

1.4.4 Cancer-Specific Functions

The activation and function of TBK1 in cancer are related to the Ras signalling pathway, which is associated with deregulated tumour cell growth, invasion and angiogenesis (Shields *et al.*, 2000). The three RAS genes code for the proteins HRas, KRas and NRas – small GTPases which normally are activated when bound to GTP and de-activated when bound to GDP (Bourne, Sanders and McCormick, 1991). Mutations in these occur frequently in cancer, present in around 20% of tumours, and this leads to constitutive activation in the absence of GTP (Barbacid, 1987; Bos, 1989). TBK1 has been found to be essential in preventing apoptosis in KRas-driven cancers (Barbie *et al.*, 2009).

Ras proteins can also propagate cancer through the phosphatidylinositol-3 kinase (PI3K)/Akt pathway, associated with tumour cell growth, survival and angiogenesis (Vara *et al.*, 2004; Karar and Maity, 2011). Here, TBK1 is directly implicated in the activation of Akt and enhancement of its functions at the exocyst (Ou *et al.*, 2011). Knockdown of TBK1 severely impairs Akt phosphorylation and inhibits bladder tumour growth as a result (Chen *et al.*, 2017).

Closely related to the Ras proteins are the Ral GTPases RalA and RalB, and TBK1 forms an important part of their oncogenic functions. At the exocyst, RalB forms an anti-apoptotic complex with Sec5 which recruits TBK1, with this localisation shown to be important for Akt activation. This leads to the phosphorylation of TBK1 and RalB-induced oncogenic transformation was found to be strongly enhanced by TBK1 kinase activity (Chien *et al.*, 2006). Taken together, this indicates that there are multiple roles for the Ras pathway in upregulating TBK1 in cancer.

1.5 c-Src

1.5.1 The Src family

The roots of the Src family of proteins lie in the early 20th century, when it was first discovered that certain viruses could induce tumours in susceptible hosts (Rous, 1910). Through analysis of the Rous Sarcoma Virus, the gene responsible for its ability to malignantly transform cells was identified and termed viral-Src (v-Src) (Martin, 2001). Since then, the cellular counterpart of v-Src (c-Src) and eight other Src family kinases (SFK) have been found in mammals (Fyn, Yes, Blk, Fgr, Hck, Lck, Lyn and Frk) as well as one only in chickens (Yrk) (Thomas and Brugge, 1997). They are non-receptor tyrosine kinases and can be further characterised according to their expression – Src, Yes and Fyn are ubiquitously

expressed; Blk, Fgr, Hck, Lck and Lyn are mostly expressed by haematopoietic cells; Frk is primarily found in epithelial-derived cells (Thomas and Brugge, 1997).

1.5.2 Structure

All SFKs have the same basic structure (Figure 9), with a poorly conserved unique region of 40-70 residues conferring the differences between the proteins. The short SH4 domain is required for myristylation (Cross *et al.*, 1984), which facilitates release from ribosomes and binding to membranes thereafter (Resh, 1994). The SH3 domain is able to bind proline-rich sequences and the SH2 domain binds to tyrosine phosphorylation sites, and these are followed by a protein kinase domain which can be autophosphorylated at tyrosine 416 to increase activity. SFKs can be regulated by C-terminal Src Kinase (CSK), which phosphorylates the C-terminus at tyrosine 530 (Y530; Y527 in avian c-Src) to decrease activity (Brown and Cooper, 1996). Initially, c-Src is cytoplasmic and is held in an inactive state through the binding of CSK-phosphorylated Y530 to the SH2 domain and of a polyproline type II (PPII) helix in the SH2 domain to the SH3 domain (Cowan-Jacob *et al.*, 2005).

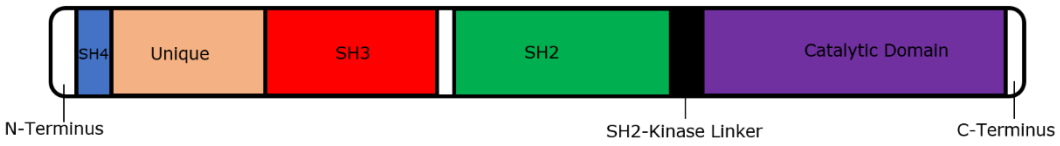


Figure 9 - Basic structure of Src family kinases

1.5.3 Cellular Functions

In normal tissues, c-Src is multifunctional and is implicated in many fundamental processes. It appears to be important in embryonic development, with roles in neural network formation seen in chicken (Sorge, Levy and Maness, 1984) and mice (Howell, Gertler and Cooper, 1997). In adult tissues, c-Src is primarily

found localised to intracellular membranes, having been released from the ribosome through myristylation (Resh, 1994). Its binding to the lipid bilayer is seemingly dependent on this myristylation of its N-terminus to facilitate hydrophobic interactions with the interior of the membrane (Sigal *et al.*, 1994). c-Src is tightly regulated through phosphorylation at Y530, and the importance of this is seen in the cellular transformation that occurs when it is constitutively active (Piwnica-Worms *et al.*, 1987; Reynolds *et al.*, 1987). This gives an insight into c-Src's many cellular functions, a product of its involvement in several different receptor pathways. It interacts with various proteins, including cell surface receptors (Biscardi *et al.*, 2000; Owens *et al.*, 2000; Moro *et al.*, 2002), steroid hormone receptors (Migliaccio *et al.*, 2000), STATs (Silva, Boerner and Parsons, 2003) and focal adhesion kinase (FAK) (Kaplan *et al.*, 1994).

The G1 and G2/M phases of the cell cycle see increased c-Src activity (David-Pfeuty, Bagrodia and Shalloway, 1993; Roche, Fumagalli and Courtneidge, 1995), due to a partial dephosphorylation of Y530, mediated by Cdc2 (Shenoy *et al.*, 1992; Kaech *et al.*, 1993). C-Src has also been linked to various growth factor signalling pathways through binding to receptor tyrosine kinases such as Epidermal Growth Factor Receptor (EGFR) and Platelet Derived Growth Factor Receptor (PDGFR) (Ralston and Bishop, 1985; Maa *et al.*, 1995; Abram and Courtneidge, 2000).

At cell junctions, c-Src has been associated with adhesion, invasion and motility, and therefore has vast influence on cell migration. Elevated levels of activated c-Src induce structural changes in cell adhesion from cadherin-mediated adhesion to focal adhesions based around integrins (Avizienyte and Frame, 2005). This is achieved through formation of a signalling complex with FAK and by phosphorylation of β-catenin, which destabilises E-cadherin to inhibit cadherin-mediated junctions (Roura *et al.*, 1999; Mitra, Hanson and Schlaepfer, 2005). E-cadherin suppresses cell motility, so its destabilisation is an important function of

the FAK-Src signalling complex; motility is inhibited in cells that form focal adhesions in the absence of the complex (Gilmore and Romer, 1996; Chen *et al.*, 1997). Similarly, as E-cadherin is an inhibitor of cell invasion, loss of E-cadherin induced by c-Src facilitates cell migration through the extracellular matrix (Frixen *et al.*, 1991). It has also been suggested that c-Src can further promote this through regulation of metalloproteinases (Noritake *et al.*, 1999).

1.5.4 Cancer-specific Functions

The ability to potentiate cell growth and to increase cellular invasion and motility make c-Src an ideal oncoprotein and it follows that elevated c-Src is a common feature of a vast range of cancers (Irby and Yeatman, 2000). In colon cancer, it correlates strongly with disease progression, with the highest levels seen in patients with liver metastases (Talamonti *et al.*, 1993).

There are a number of ways for cancer cells to upregulate c-Src activity. Known activators of c-Src such as EGFR and PDGFR have been implicated, with oncogenic transformation and hyperproliferation of nontumorigenic human mammary epithelial cells requiring both c-Src and EGFR (DeMali *et al.*, 1999; Tice *et al.*, 1999; Dimri *et al.*, 2007). Alternatively, the upregulation of c-Src has been linked to post-translational modifications in some cancers. In hepatocellular carcinomas, negative regulation of c-Src through phosphorylation at Y530 is reduced via reduced CSK levels and phosphatases that dephosphorylate c-Src at the same site have been reported in breast cancer cell lines (Egan *et al.*, 1999; Masaki *et al.*, 1999). A further method of elevating c-Src activity is through mutations in the SRC gene, with point mutations resulting in the truncation of c-Src just terminal to Y530 being found in subsets of colon and endometrial cancers (Irby *et al.*, 1999; Sugimura *et al.*, 2000).

It is hard to define a precise mechanism for the neoplastic deregulation of c-Src as it appears to differ between types of cancer but the end result of elevated c-Src activity is undoubtedly vital for tumour progression.

1.5.5 Interaction with survivin

Src family proteins commonly recruit substrates through their hydrophobic SH3 domains, which associate with hydrophobic amino acid motifs (Weng *et al.*, 1994). The SH3 domain can bind in two orientations: Class 1 binds the N-terminal region of the domain to the C-terminal region of the substrate and Class 2 binds the C-terminal region of the domain to the N-terminal region of the substrate (Feng *et al.*, 1994). The N-terminus of survivin is proline-rich and forms a hydrophobic PPII helix, which would suggest that any binding with c-Src would be in a Class 2 orientation. Evidence of this interaction is presented by Dunajová *et al.* (2016) with the finding that a construct of the first 10 amino acids of survivin was able to co-immunoprecipitate and negatively regulate c-Src but a construct of the latter 132 amino acids was not. The group also found that this regulation led to a decrease in cell adhesion. This would seem to suggest that survivin and c-Src can act in concert to provide a beneficial environment for cell proliferation and metastasis.

An indirect association between survivin and c-Src can be seen at adherens junctions. The efficacy of the adhesion receptor E-cadherin is regulated by the phosphorylation of its binding partner, β-catenin, by c-Src (Roura *et al.*, 1999), leading to the destabilisation of E-cadherin and subsequently the disintegration and loss of polarity of cell-cell junctions (Palovuori, Sormunen and Eskelinen, 2003). This is correlated with an increase in survivin expression (Capra and Eskelinen, 2017). Disintegration of cell-cell junctions is a cornerstone of epithelial-mesenchymal transition (EMT) (Ikushima and Miyazono, 2010), which is crucial in the development of metastatic potential in tumours (Thiery *et al.*, 2009), so c-Src-induced upregulation of survivin in this manner may explain why

elevated expression of both proteins is correlated with malignancy and poor prognosis in many cancers (Diaz *et al.*, 2006; Khoury *et al.*, 2009).

1.6 Knowledge Gap and Aims

Whilst it appears clear that there is an interaction between survivin and c-Src that facilitates tumour growth and progression, the nature of this interaction is yet to be elucidated. Previous research has suggested that the N-terminus of survivin is crucial to the regulation of c-Src (Dunajová *et al.*, 2016), yet the specifics of this interaction have not been studied. It has been theorised that the interaction could be influenced by phosphorylation of survivin's N-terminus, with preliminary research suggesting that TBK1 could be involved (Chellappan Lab, Moffitt Cancer Centre, unpublished; see Appendix A). There is still much to be discovered about the influence of TBK1 on survivin, although it has been shown that siRNA inhibition of TBK1 decreases survivin expression (Wang *et al.*, 2017).

There were two aims to this thesis. Firstly, to examine whether the interaction between survivin and c-Src is dependent on phosphorylation of survivin and T5 and secondly, to study whether inhibition of the suggested T5 kinase (TBK1) had an effect on the expression of survivin in HeLa cells.

2 Materials and Methods

All materials sourced from Sigma unless otherwise stated.

2.1 Cloning

2.1.1 Production of mutant survivin plasmids via Site-Directed Mutagenesis (SDM)

Polymerase Chain Reaction (PCR) and Ligation

The New England Biolabs' Q5 Site-Directed Mutagenesis kit was used on constructs of survivin in the vector pGEX 4T1 to produce substitutions at the fifth amino acid. The reagents described in Table 1 were used for each reaction, with the primers described in 2.2.1. Thermal cycling was performed as in Table 2, with a melting temperature (T_m) of 72°C for the T5A mutant. Kinase, Ligase and DpnI (KLD) treatment was then performed using: 2X KLD Reaction Buffer; 10X KLD Enzyme Mix; 1µl PCR product; distilled water.

Table 1 - PCR Constituents for SDM

Constituent	**Volume (µl)**	**Final Concentration**
2X Q5 Hot Start High-Fidelity Master Mix (New England Biolabs (NEB))	12.5	1X
Forward Primer	1.25	10 mM
Reverse Primer	1.25	10 mM
Vector	1	1 ng/µl

Sterile Distilled Water (SDW)	9	Not applicable
Final Volume	25	

Table 2 - Thermal Cycling Programme for SDM PCR reaction

Step	**1**	**2**	**3**	**4**	**5**	**6**
Temperature (°C)	98	98	72	72	Return to Step 2	72
Time (s)	30	10	30	190	X30	120

Transformation of SDM products into DH5α cells

5 µl of SDM product was added to 100 µl of competent DH5α cells and the mixture was left on ice for 30 minutes, heat shocked at 42°C for 1 minute, then left on ice for a further 2 minutes. 900 µl Luria-Bertani (LB) broth (10 g/l tryptone; 10 g/l NaCl; 5 g/l yeast extract) was added and the solution was incubated in an orbital shaker for 90 minutes at 37°C and 200 rpm. This was followed by pelleting in a centrifuge at 16200 xg and resuspension in 1/3 of the supernatant, with the resulting culture then spread onto LB+ampicillin agar plates (10 g/l tryptone; 5 g/l yeast extract; 5 g/l NaCl; 30 g/l agar; 0.1 g/l ampicillin). These plates were incubated overnight at 37°C.

2.1.2 Production of mutant survivin plasmids via PCR cloning

PCR

Sub-cloning was used to insert pre-made survivin constructs with mutations at the fifth amino acid into a pGEX vector. The reagents were assembled as in

Table 3, with the primers detailed in 2.10.1, then incubated in a thermal cycler under the conditions set out in Table 4.

Table 3 - PCR Constituents for PCR Cloning

Constituent	**Volume (µl)**	**Final Concentration**
5X Phusion Buffer (Finnzymes)	10	1X
dNTPs	1	1 mM
Forward Primer	2.5	1 mM
Reverse Primer	2.5	1 mM
Vector	1	1 ng/µl
Sterile Distilled Water (SDW)	32.5	Not applicable
Phusion High-Fidelity DNA Polymerase (Finnzymes)	0.5	0.01 U/µl
Final Volume	50	

Table 4 -Thermal Cycling Conditions for PCR reaction

Step	**1**	**2**	**3**	**4**	**5**	**6**
Temperature (°C)	98	98	68	72	Return to Step 2	72
Time (s)	180	30	45	60	X39	300

Agarose Gel Electrophoresis

Agarose was dissolved in tris-acetate-EDTA (TAE) buffer (40 mM Trizma; 5.7% acetic acid; 1 mM EDTA) at 1% concentration by microwaving. Once cooled, SYBR® safe (Invitrogen) was added with a final concentration of 0.01%. The resulting gel was loaded with the PCR products, then run at 100V in TAE buffer for 40 minutes.

Gel Extraction

DNA fragments were excised from agarose gels with a scalpel. Buffer QG (QIAGEN) was added at a ratio of 3 µl buffer to 1 µg DNA and the resulting solution was incubated at 50°C for 10 minutes to dissolve the gel. The sample was then centrifuged at 12500 xg for one minute in a QIAquick spin column (QIAGEN). 750 µl Buffer PE (QIAGEN) was then added to the column and the centrifugation was repeated. The DNA was eluted by adding 30 µl SDW to the column, leaving it to stand for a minute, then repeating the centrifugation. Concentrations were determined by Nanodrop 2000 (Thermo Scientific).

Restriction Digest

Samples were set up as in the table below and were run in a thermal cycler at 37°C for 1 hour then 65°C for 20 minutes.

Table 5 - Restriction digest components

	Insert double digest	**Vector double digest**	**Vector single digest (EcoR1)**	**Vector single digest (Xho1)**	**Vector negative control**
EcoR1 Buffer (NEB)	1X	1X	1X	1X	1X

Insert	0.5 ng/µl	-	-	-	-
Vector	-	1 ng/µl	1 ng/µl	1 ng/µl	1 ng/µl
EcoR1 (NEB)	20 U	20 U	20 U	-	-
Xho1 (NEB)	20 U	20 U	-	20 U	-
CIP (NEB)	-	10 U	10 U	10 U	-

The products were then separated by agarose gel electrophoresis and the relevant fragments were extracted.

Ligation

Ligation reactions were performed as in the table below and incubated at 16°C overnight. Insert amount was calculated using the following equation:

$$Insert\ mass\ (ng) = 3 \times \frac{Insert\ length\ (bp)}{Vector\ length\ (bp)} \times Vector\ mass\ (ng)$$

Table 6 - Ligation reaction components

Constituent	**Volume (µl)**	**Final Concentration**
10X T4 Ligase Buffer (NEB)	2	1X
Vector	-	2.5 ng/µl
Insert	-	-
T4 Ligase (NEB)	1	400 U

Ligation products were then transformed into DH5α cells following the same protocol as for the SDM products.

2.1.3 Selection of colonies

Two colonies from each plate were picked using a sterile 200 µl pipette tip and inoculated into separate LB-Ampicillin solutions of 4 ml LB broth with 100 µg/ml ampicillin, then incubated in an orbital shaker at 37°C overnight.

2.1.4 Miniprep purification

Overnight cultures were centrifuged at 9600 xg for 1 minute. Pellets were resuspended in 250 µl of chilled Buffer P1 (QIAGEN) and then lysed by adding 250 µl Buffer P2 (QIAGEN), overtaxing 6 times and then incubating for 5 minutes at room temperature. Lysis was neutralised by adding 350 µl Buffer N3 (QIAGEN) and overtaxing a further 6 times. The lysate was then centrifuged for 8 minutes at 12500 xg. The resulting supernatant was applied to a QIAprep 2.0 spin column (QIAGEN) and centrifuged for 1 minute at 12500 xg. A spin column was washed by adding 750 µl Buffer PE (QIAGEN) and repeating the centrifugation, followed by a further centrifugation on the empty column. DNA was eluted by applying 30 µl SDW to the centre of the spin column filter, leaving it to stand for 1 minute, then centrifuging at 12500 xg for 1 minute once more.

2.2 Protein Expression

Transformation of plasmids into BL21 cells

100 ng of plasmid was added to 100 µl of competent BL21 cells and left on ice for 30 minutes following gentle agitation. The mixture was then heat shocked at 42°C for 10 seconds then left on ice for 5 minutes, before adding 950 µl Super Optimal broth with Catabolite repression (SOC) media. This was placed in a shaking incubator at 37°C and 200 rpm for 60 minutes. 10- and 100-fold serial dilutions were made of the resulting solution and 100 µl of each was spread on a LB+ampicillin agar plate and incubated at 37°C overnight. Colonies were selected as before and grown in 4 ml LB + 100 µg/ml ampicillin cultures.

Culture conditions and IPTG induction

Overnight cultures were used to inoculate LB-Ampicillin solutions ranging from 75 ml to 300 ml at a 1:100 dilution. Cultures were grown at 37°C and 200 rpm and samples were periodically taken for OD_{600} analysis by spectrophotometer (Bio Rad Smart Spec™ 3000). Once the cultures had reached an OD_{600} of 0.6-0.8 they were induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated on a rotator for either 5 hours at 30 °C or overnight at room temperature.

Bacterial lysis and harvesting of proteins

The cultures were centrifuged at 3220 xg for 15 minutes at 4°C, then the pellets were resuspended in 10-20 ml Tris-buffered saline (TBS). The centrifugation was repeated, then the pellets were resuspended in 4 ml B-PER Bacterial Protein Extraction Reagent (Thermo Scientific) per 1 g of pellet, supplemented with protease inhibitors (100µm PMSF (Phenylmethylsulphonyl fluoride), 1µg/mL CLAP (Chemostatin, Leupeptin, Antipain, Pepstatin A), 20mM NaV, 2mM $MgCl_2$, 25 units Benzonase and 2mM β-glycerophosphate). The lysate was incubated at room temperature for 15 minutes before centrifugation at 15000 xg for 5 minutes at 4°C. The resulting supernatant, containing the soluble proteins, was extracted and the pellets discarded.

Protein purification via Glutathione Sepharose 4B beads

A 50% slurry of 20-40 µl Glutathione Sepharose 4B beads (Sigma) was prepared as per the manufacturer's instructions. The beads were resuspended in the cell lysate, then incubated with end-over-end rotation at either room temperature for 1 hour or at 4°C overnight. Following incubation, the beads were pelleted at 500 xg for 4 minutes at 4°C and the supernatant discarded. The beads were resuspended in 500 µl TBS, then pelleted again. This was repeated twice more, for a total of three washes.

To elute the purified proteins, 0.5 ml of elution buffer (50 mM Tris-HCl; 20 mM reduced glutathione; pH 8.0) was added to the beads for each 1 ml of the original slurry. This was incubated with end-over-end rotation at room temperature for 10-15 minutes, before the elute was extracted. This was repeated twice more, for a total of three elutions.

Bradford Assay

Proteins were diluted in 500 µl Sterile Distilled Water (SDW) and 500 µl Coomassie brilliant blue G-250 based dye (Bio-Rad protein assay) along with a series of Bovine Serum Albumin (BSA) standards (0 mg/ml, 1 mg/ml, 2.5 mg/ml, 5 mg/ml, 10 mg/ml, 15 mg/ml and 25 mg/ml) in the same diluents. Samples were vortexed and after 10 minutes the intensity was measured in a spectrophotometer (Bio Rad Smart Spec™ 3000) at wavelength 595 nm.

In-vitro translation

In-vitro translation of proteins was performed using TNT Coupled Reticulocyte Lysate Systems (Promega). The components were assembled as per Table 7 and incubated at 30 °C for 90 minutes.

Table 7 - In-vitro translation components

Component	Volume
TNT Rabbit Reticulocyte Lysate	25 µl
TNT Reaction Buffer	2 µl
TNT RNA Polymerase (T7)	1 µl
Amino Acid Mixture, Minus Leucine (1mM)	0.5 µl
Amino Acid Mixture, Minus Methionine (1mM)	0.5 µl
RNasin Ribonuclease Inhibitor (40u/µl)	1 µl

DNA template	1 µg
Nuclease-Free Water	To a final volume of 50 µl

2.3 Cell culture

Passaging

HeLa (cervical carcinoma) cell lines were maintained by standard practices. They were grown in 25 cm^2 flasks (T-25; Thermo Fisher) then passaged once they had reached ~90% confluency. This involved harvesting with either trypsin-EDTA solution (T4174) diluted in Dulbecco's phosphate buffered saline (DPBS; D8537) or a cell scraper (Fisher Scientific). Non-transfected cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; D6429), supplemented with 10% (v/v) foetal bovine serum (F7524), 100 µg/ml penicillin and 100 µg/ml streptomycin (Invitrogen), referred to as A_2 media from here on. 1 mM G418 (ACROS organics) was additionally included in media for transfected cell lines.

Whole cell extracts

Cells were harvested in the same way as for passaging, then centrifuged at 500 xg for 4 minutes, washed with DPBS, and centrifuged again. Pellets were resuspended in NP40 buffer (10mM Tris-HCl pH 7.5; 150mM NaCl; 0.5 mM EDTA; 0.5 % (v/v) NP40), supplemented with 100µm PMSF, 1µg/mL CLAP, 20mM NaV, 2mM $MgCl_2$, 25 units Benzonase and 2mM β-glycerophosphate. They were then left to lyse on ice for 30 minutes.

Transfections

3.0 x 10^4 HeLa cells in antibiotic-free media were seeded into each well of a µ-Slide 8 Well imaging chamber (Ibidi) and incubated for 24 hours at 37°C. Torpedo transfection reagent (Ibidi) and the DNA for transfection were prepared at a 3:1 ratio of Torpedo (µl) to DNA (µg) and diluted individually in PBS before

being mixed and incubated at room temperature for 20 minutes. The transfection mix was then added to the cells in the imaging chamber and incubated at 37°C for a further 24 hours before imaging.

2.3.1 Drug Treatments

Eg5 Inhibition

HeLa cells were treated with an Eg5 inhibitor to induce mitotic arrest and therefore increase survivin content. Cells in T-25 flasks were grown in A_2 media with 2 μM Eg5 inhibitor for 4 hours at 37°C. The flasks were then struck three times to shake off mitotic cells, with the media harvested to make whole cell extracts from.

TBK1 and Proteasome Inhibition

1.5 x 10^6 cells from various transfected and non-transfected HeLa lines were seeded down into 10 cm^2 plates (Thermo Fisher) and grown at 37°C for 24 hours. The TBK1 inhibitor BX795 and the proteasome inhibitor MG-132 were then added to the relevant plates, for a final concentration of 1 μM and 50 μM respectively. The cells were incubated for a further 2 hours at 37°C.

2.4 SDS-PAGE

Gel production

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was used to separate proteins. Polyacrylamide gels were made with a 6% stacking layer and then a 12% or 15% resolving layer depending on the size of the analysed proteins. The compositions are detailed in the tables below.

Constituent	**Final Concentration**
Trizma pH 8.8	390 mM

Acrylamide:bisacrylamide	12% or 15%
SDS	3.5 mM
TEMED	3.4 mM
Ammonium persulphate	0.4 mM

Table 8 - SDS-PAGE resolving layer components

Constituent	**Final Concentration**
Trizma pH 6.8	130 mM
Acrylamide:bisacrylamide	6%
SDS	3.5 mM
TEMED	6.8 mM
Ammonium persulphate	0.4 mM

Table 9 - SDS-PAGE stacking layer components

The gels were then either stained with Coomassie Brilliant Blue R-250 (Thermo Fisher) for visualisation of proteins or used for immunoblotting.

Protein Expression Assay

Whole cell extracts were boiled in 5x SDS sample buffer (5% SDS (w/v, Fisher), 250mM Tris-HCl, 50% Glycerol (v/v Fisher), 0.05% Bromophenol blue (v/v), 250 mM β-mercaptoethanol (v/v)) for a final concentration of 1x at 95°C for 8 minutes. They were then loaded into 12% or 15% SDS-PAGE gels and ran at 120V for 20 minutes then 200V for 40 minutes in 1x SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS).

GST Pull Down Assay

20-40 µg of "bait" protein was added to a 50% slurry of 20-40 µl Glutathione Sepharose 4B beads (Sigma), prepared as per the manufacturer's instructions, and incubated with end-over-end rotation at either room temperature for 1 hour or at 4°C overnight. The beads were washed three times, as in 2.2, before whole cell extracts of non-transfected HeLa cells were added and incubated at 37°C and 200 rpm for 30 minutes. The samples were then boiled in SDS sample buffer and ran on SDS-PAGE gels as in 2.4.

2.5 Immunoblotting

A wet transfer system using 1x transfer buffer (24 mM Tris, 195 mM Glycine, 0.05% SDS, 10% Methanol) was used to transfer SDS-PAGE gels onto a 0.22 µM nitrocellulose membrane (BIOTRACE, PALL life sciences), which was run at 350 mA for 90 minutes. The membrane was then blocked with either 5% non-fat milk (Marvel, in TBS + 0.1% Tween 20 (TBST) or 5% BSA (in TBST) for 20-60 minutes with gentle agitation at room temperature. Primary antibodies (see 2.6) were made in the appropriate buffer then incubated on the membranes at 4°C overnight. This was followed by three 5-minute washes in TBST and incubation with the relevant horseradish peroxidase-conjugated secondary antibody (see 2.6) for one hour at room temperature.

Enhanced chemiluminescent (ECL) reagents (Amersham) were mixed 1:1 then incubated on membranes in the dark for 1 minute before exposure to detection film (Roche).

Expression of proteins on immunoblots was analysed in a semi-quantitative manner by scanning the films and then measuring the pixel intensity of each band, normalised to controls, using the image processing package ImageJ.

2.6 Antibody specification and sourcing

Table 10 - Summary of antibodies used. BSA = Bovine Serum Albumin. PBS = Phospho-buffered saline. TBST = Tris-buffered saline + 0.1% Tween.

Antibody Specificity	Host Species	Dilution WB	Buffer	Source
B512 (Tubulin)	Mouse	1/2000	5% Milk TBST	Sigma T5168
c-Src	Rabbit	1/500	5% Milk TBST	Santa Cruz
GFP	Rabbit	1/500	5% Milk TBST	Sigma G1546
GST	Rabbit	1/1000	5% BSA TBST	Cell Signalling Technology
p-Src (Tyr416)	Rabbit	1/1000	5% BSA TBST	Cell Signalling Technology
Survivin	Rabbit	1/1000	5% Milk TBST	Novus AB469
Voltage dependent anion channel (VDAC)	Rabbit	1/1000	5% Milk TBST	Cell Signalling Technology
Mouse HRP conjugated	Rabbit	1/1000	5% Milk TBST	Dako PO161
Rabbit HRP conjugated	Goat	1/1000	5% Milk TBST	Dako PO448
Anti-Rb Texas Red	Goat	1/1000	1%BSA/PBS/ Azide	Sigma

2.7 Microscopy

Live imaging

Transfected or drug-treated cells grown in µ-Slide 8 Well imaging chambers (Ibidi) had their media changed to CO_2 independent media (DMEM, Invitrogen) supplemented with 2 mM L-glutamine and 10% Foetal Calf Serum, and were then all stained with nucblue (Thermo Fischer Scientific) to visualise DNA and 100 nM MitoTrackerRed CMXRos (Invitrogen) to visualise mitochondria, and grown for 15 minutes at 37°C. An inverted microscope (DMRIB Olympus, Delta Vision Elite) with a 60x lens (NA1.4, oil) was used to take images. Images were either taken at a single plane or with z-stacks of 0.3 µm slices which were then de-convolved.

Images were processed using ImageJ and the levels of protein expression were quantified by measuring pixel intensities of expressing cells in the relevant channels.

Fixed imaging

Cells were grown in either µ-Slide 8 Well imaging chambers (Ibidi), as described in 2.3, or on ethanol washed coverslips in 24 well plates, then fixed with 4% paraformaldehyde/PBS (Sigma) for 5 minutes at room temperature. The wells were washed three times for 5 minutes with PBS then 0.15% Triton in PBS was added for 2 minutes to permeabilise the cells. The wash steps were repeated and then the cells were blocked with 1% BSA/PBS/Azide (PAA, Sigma) for 15 minutes. Following this, primary antibodies were prepared as per 2.6 and added to the cells for 1 hour in the dark at room temperature with gentle agitation. The cells were then washed with PBS again, before the secondary antibodies were added and incubated with the same conditions as the primary antibodies. Finally, 4′,6-diamidino-2-phenylindole (DAPI) was added for 20 minutes to stain for nuclei. Cells in imaging chambers were then stored in 1% BSA/PBS/Azide (PAA,

Sigma) and those on coverslips were mounted with Mowiol. The equipment and parameter used to image and process the slides were the same as for live imaging.

2.8 Online Resources

Several online tools were used to elicit relevant DNA and protein sequences, as well as to gain information on the proteins being studied. These are summarised in Table 9.

Table 11 - Summary of online resources used.

Tool	Use	Website
Translate Tool	DNA to protein sequence translation	https://web.expasy.org/translate/
Prot Param	pI and hydropathicity summary	https://web.expasy.org/protparam/
Reverse Complement Tool	Generation of reverse complements for primers	http://www.bioinformatics.org/sms/rev_comp.html/
uniprot	Protein database	https://www.uniprot.org/
Gene Cards	Protein/gene database	https://www.genecards.org/
Pubmed	Literature search engine	https://ncbi.nlm.nih.gov/pubmed/

2.9 Statistical analyses

Data generated from microscopy were collated, with the mean and standard deviation calculated for each group of results, then assessed for significant differences using a paired samples t-test.

To assess changes in survivin expression after drug treatment, the expression data were first normalised to controls, then log-fold change was calculated by

comparing expression in treated cells to untreated cells. This was then analysed by paired sample t-tests.

Data from co-immunoprecipitations were first assessed for normality using the Shapiro-Wilks normality test, then analysed for significant differences using an unpaired t-test with Welch's correction.

All analyses were performed using Prism 7 (GraphPad) software.

2.10 Materials

2.10.1 Primers

T5A (Site-Directed Mutagenesis)

Forward: 5'-GCATTGCCCCCTGCC-3'

Reverse: 5'-TACCCACGGGGCCGT-3'

T5E (Sub-cloning)

Forward: 5'-TAAGCAGAATTCATGGGTGCCCCGGAGTTGCCC-3'

Reverse: 5'-TGCTTACTCGAGCAGCTGGCTGCCATGGATTGA-3'

3 Results

3.1 PCR cloning of T5 mutants

As described in Materials and Methods, two cloning methods were used to obtain T5 mutants in pGEX vectors, so that they could be expressed by E.Coli. The T5A and T5E mutants were created to provide mimics of T5 in both it's inert and phosphorylated states respectively so that the effects of mutation on the binding of survivin to c-Src could be studied.

SDM was used on a full-length survivin construct in pGEX to induce and amplify the T5A mutation (Figure 10). The T5E mutation was created through sub-cloning; a previously made T5E in a pcDNA3.1 vector was spliced out and then ligated into an empty pGEX vector (Figure 11).

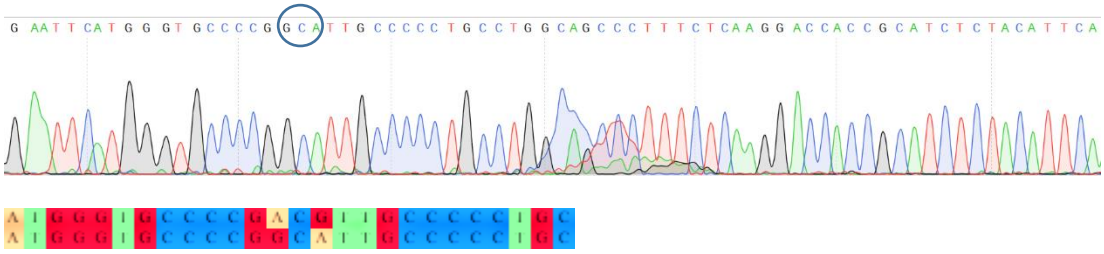


Figure 10 - Chromatogram and visualisation of mutation from Threonine (ACG) to Alanine (GCA)

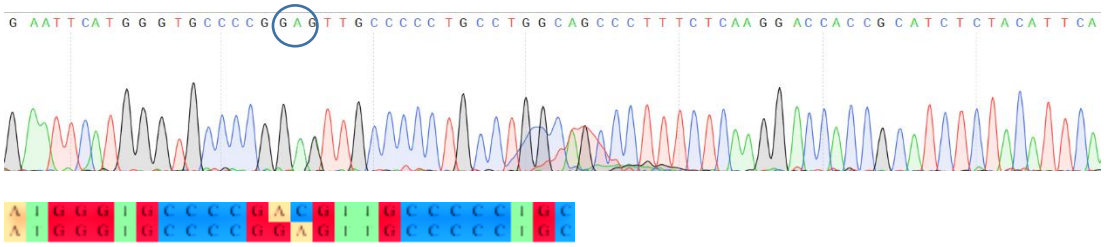


Figure 11 - Chromatogram and visualisation of mutation from Threonine (ACG) to Glutamic Acid (GAG)

Next Generation Sequencing provided chromatogram verification that the mutations had been successfully cloned.

3.2 Protein Expression

Expression of the mutant survivin plasmids was achieved through firstly transforming them into competent BL21 E.coli cells, growing them at 37°C until they reached the exponential growth phase, then inducing expression through IPTG, a molecular mimic of allolactose which triggers transcription of the lac operon. Soluble proteins were isolated by pelleting the cultures twice, such that the supernatants could then be bound to GST beads and washed with TBS for purification (Figure 12). The decision was made to not use Wild-Type survivin as there was precedent for using GST-tagged survivin to show that regulation of c-Src was dependent on survivin's N-terminus (Dunajová *et al.*, 2016) and the GST tag was useful for Western blotting as WT survivin's low molecular weight compared to the other relevant proteins made it difficult to resolve all the necessary proteins on the same gel.

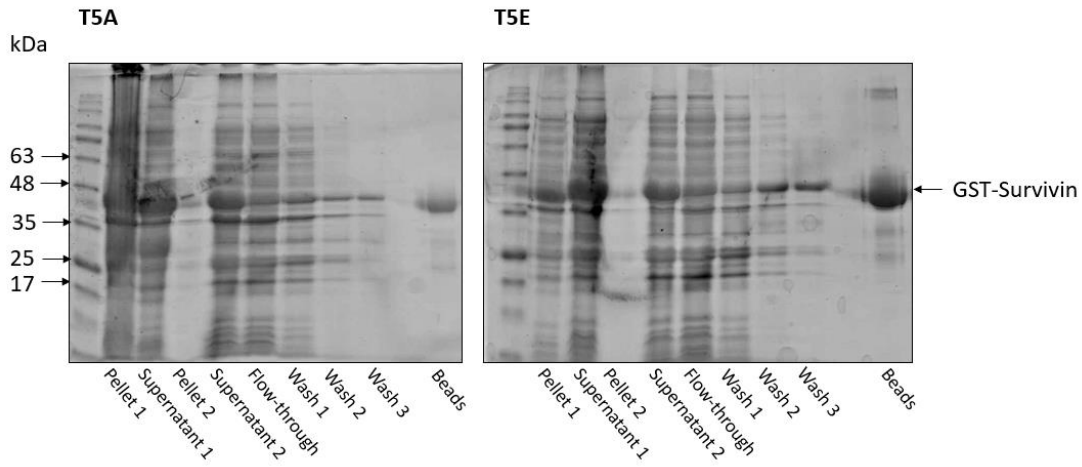


Figure 12 - – SDS-PAGE analysis of the protein expression and purification process. Survivin mutants were transformed into BL21 cells then grown until the optical density at 600 nm (OD600) was 0.6-0.7. Cultures were then treated with 0.5mM IPTG to induce protein expression and pelleted twice to separate soluble and insoluble proteins. The supernatants were bound to GST beads, with the resulting flow-through kept for analysis, and the protein-bound beads were then washed 3 times.

SDS-PAGE analysis confirmed the efficacy of the purification and showed that the proteins were sufficiently soluble.

3.3 Microscopy

3.3.1 Inhibition of TBK1 reduces survivin expression in HeLa cells transfected with full-length survivin, but not those transfected with survivin $_{1-10}$

Inhibition of TBK1 through siRNA has previously been shown to reduce survivin expression in head and neck squamous cell carcinoma (Wang *et al.*, 2017). As preliminary data had suggested TBK1 as the kinase for survivin T5 phosphorylation (Chellappan Lab, Moffitt Cancer Centre, unpublished; see Appendix A), the effects of TBK1 inhibition, this time through the blocking of its activation, was studied in HeLa cells.

To determine the effects of TBK1 on survivin expression, HeLa cells expressing either survivin-GFP or survivin$_{1-10}$-GFP (green) were treated with BX795, an inhibitor of TBK1 activation, and then stained with DAPI (blue) and with antibodies for VDAC (red) and TBK1 (far red) in order to visualise the survivin constructs, nuclei, mitochondria and TBK1 respectively. The cells were fixed and then imaged using fluorescence microscopy (Figure 13) and this was preferred to live imaging as the changes did not need to be seen in real time. This was subsequent to a previous study that used western blotting to show that TBK1 inhibition by siRNA decreases survivin expression (Wang *et al.*, 2017). It was decided that the effects of TBK1 inhibition on transfected rather than endogenous survivin would be studied as this would allow direct comparison with the survivin$_{1-10}$-GFP construct and also for ease of study as survivin is endogenously expressed at quite low levels and therefore changes in expression can be difficult to quantify or visualise.

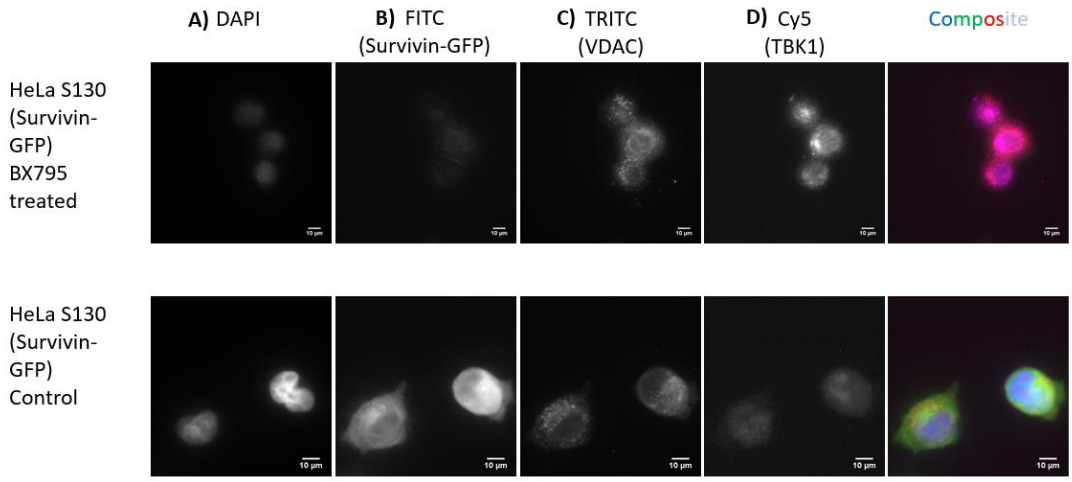


Figure 13 - HeLa cells transfected with survivin-GFP were either treated with BX795 or left untreated, then stained for visualisation with fluorescence microscopy to examine the nuclei (A), construct expression (B), mitochondria (C) and TBK1 expression (D).

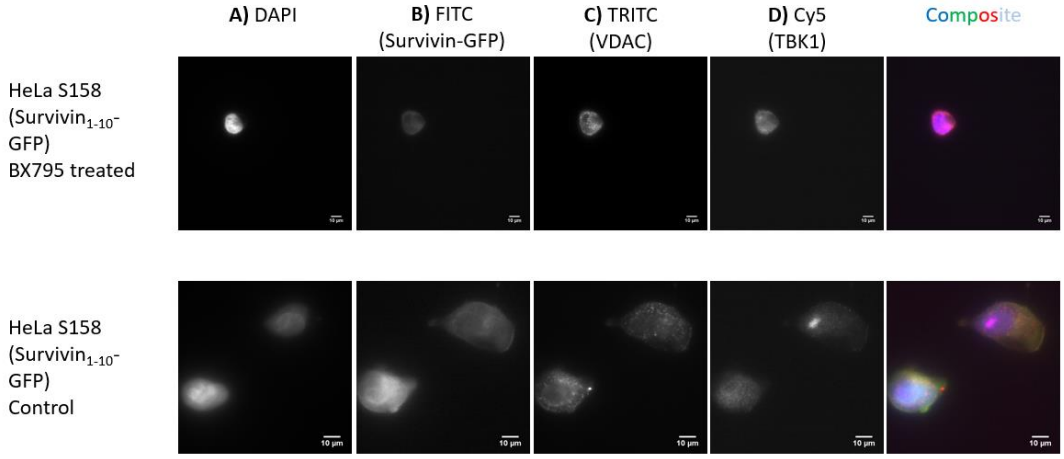


Figure 14 - HeLa cells transfected with survivin1-10-GFP were either treated with BX795 or left untreated, then stained for visualisation with fluorescence microscopy to examine the nuclei (A), construct expression (B), mitochondria (C) and TBK1 expression (D).

The pixel intensities of cells in the FITC channel were quantified (Table 12) and plotted (Figure 15).

Table 12 - FITC channel pixel intensities for HeLa cells transfected with survivin-GFP or survivin$_{1-10}$-GFP, with and without BX795 treatment.

	Mean Pixel Intensity	**Standard Deviation**	**Number of images**
HeLa Survivin-GFP BX795 Treated	109	1.13	6
HeLa Survivin-GFP Untreated	169	27.8	7
HeLa Survivin$_{1-10}$-GFP BX795 Treated	108	1.27	8
HeLa Survivin$_{1-10}$-GFP Untreated	110	2.24	5

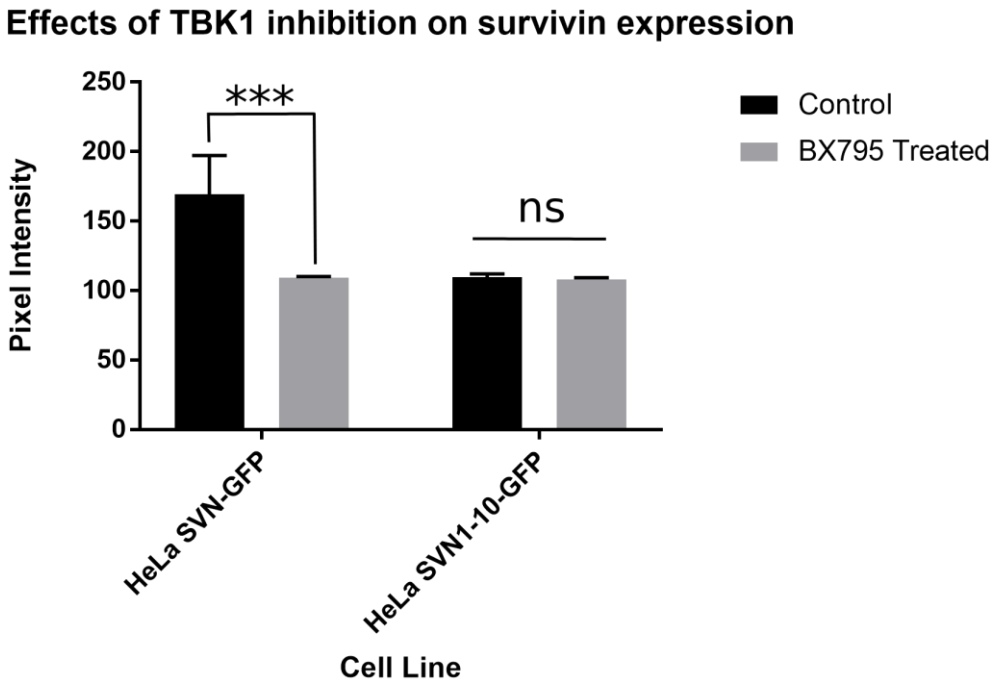


Figure 15 - Survivin expression in response to TBK1 inhibition. HeLa cells expressing survivin-GFP saw a decrease in survivin expression when treated with BX795. The treatment had no effect in HeLa cells expressing survivin1-10-GFP.

HeLa cells transfected with survivin-GFP and treated with BX795 had a mean pixel intensity (MPI) in the FITC channel of 109. For untreated cells, the MPI was 169. This difference was statistically significant (P=0.0003). Conversely, HeLa cells transfected with survivin$_{1\text{-}10}$-GFP showed no change in survivin expression, with treated and untreated cells returning MPIs of 108 and 110 respectively.

These data raise the possibility that the effects of TBK1 on survivin expression are due to an interaction, either direct or indirect, outside of survivin's N-terminus and therefore independent of the phosphorylation of survivin at T5 by TBK1.

3.4 Pull-Down Assays

3.4.1 Inhibition of TBK1 may reduce survivin expression in HeLa cells transfected with GFP or survivin-GFP, but not in non-transfected HeLa cells

In order to further explore the effects of TBK1 on survivin levels, immunoblotting was used to provide more quantifiable results.Three sets of HeLa cell lines (non-transfected; GFP; survivin-GFP) were treated with a TBK1 inhibitor (BX795) and/or a proteasome inhibitor (MG-132), with survivin expression then analysed via Western blot (Figure 16). Previous research has shown that survivin can be degraded by the ubiquitin-proteasome pathway (Zhao *et al.*, 2000), so MG-132 was used to ensure that any changes in expression were not due to ubiquitin-mediated degradation of survivin. Levels of survivin expression were measured in a semi-quantitative manner in order to provide a degree of objectivity in determining the effects of the treatment.

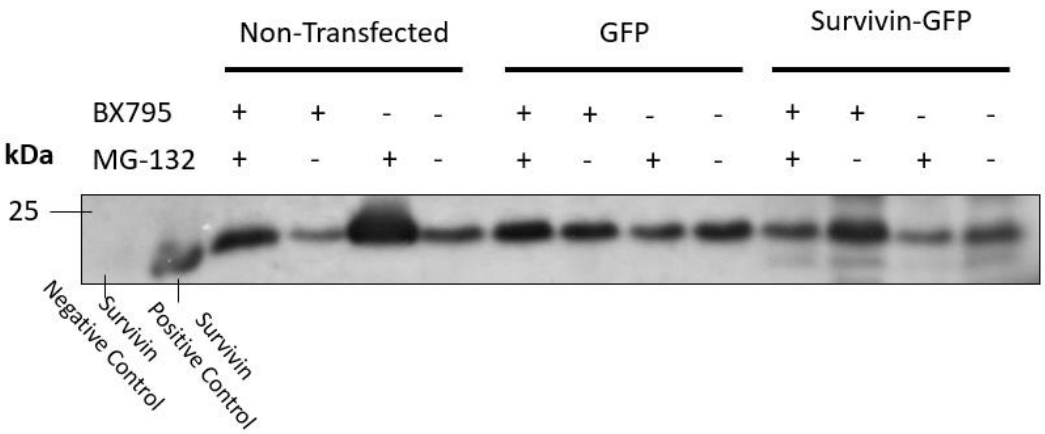


Figure 16 – Western blot of endogenous survivin in three cell lines in response to TBK1 inhibition and proteasome inhibition.

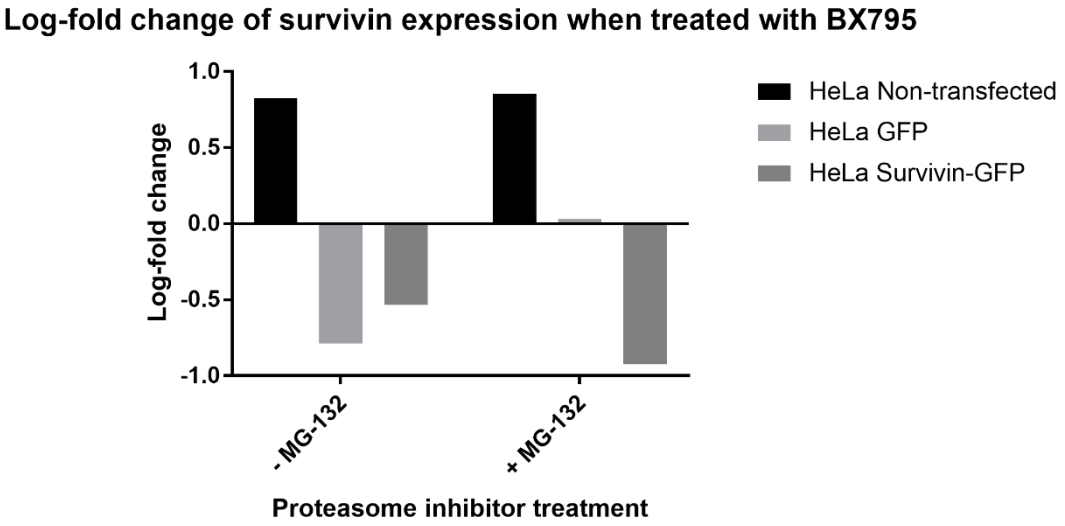


Figure 17 - Log-fold change of survivin expression in cells treated with BX795 compared to untreated cells. Results are shown both with and without proteasome inhibitor treatment.

Log-fold change was used to assess the amount of survivin in BX795 treated cells in comparison to non-treated cells across the three HeLa cell lines and with or without proteasome inhibitor treatment. This was to normalise changes in survivin content between the cell lines so that they could be evaluated against each other. Non-transfected HeLa cells showed an upregulation of survivin when treated with BX795 whereas both transfected cell lines showed a downregulation (Figure 17). This is in accordance with both the microscopy data and the previous study demonstrating that TBK1 inhibition reduces survivin expression in transfected cell lines (Wang *et al.*, 2017). An attempt was made to prevent survivin degradation through the use of MG-132, but the similarity of survivin content in treated and untreated cells suggests it didn't work. The results were not able to be analysed for statistically significant differences due to the lack of repeats.

3.4.2 Mimicking survivin phosphorylation at T5 allow it to pull-down c-Src

The N-terminus of survivin (aa 1-10) alone is sufficient to regulate c-Src (Dunajová *et al.*, 2016), so to this end pull-down assays were performed with an inert construct at T5 (T5A) and one that mimicked T5 phosphorylation (T5E) to examine whether the phospho-status of survivin could be influencing this interaction (Figure 18). Pull-down assays useful in that they can determine whether there is an interaction, however they are an indirect measure and cannot account for other cofactors. In-vitro translation of c-Src was attempted to look for a direct binding with the survivin mutants, as predicted in 1.5.5, but issues with running the products on SDS-PAGE meant the results were inconclusive.

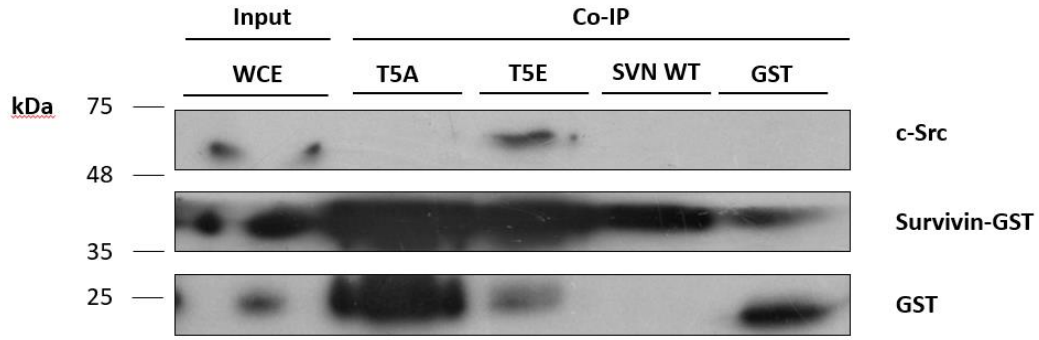


Figure 18 – Pull-down assay of survivin mutants and c-Src from HeLa cell extracts. Immunoblotting showed that the phospho-mimetic mutant (T5E) was able to pull-down c-Src but the wild-type (SVN WT) and the non-phosphorylatable mutant (T5A) were not.

The SDS-PAGE results showed a clear pull-down in the T5E lanes and very little in the T5A lanes for all three replicates (see Appendix B). It seems apparent that mimicking phosphorylation at T5 facilitates survivin's ability to bind c-Src. Interestingly, wild-type survivin (full length, non-phosphorylated) could not precipitate c-Src either, indicating that c-Src can only bind to T5-phosphorylated survivin.

4 Discussion

4.1 Survivin and TBK1

4.1.1 Basis for studying an interaction between survivin and TBK1

At first glance, the prospect of an interaction between survivin, an anti-apoptotic protein with important roles in the cell cycle, and TBK1, predominantly an immune system protein involved in co-ordinating the interferon response, may appear unlikely. However, they are both proteins that participate in several signalling pathways and, under oncogenic conditions, TBK1 is active in two pathways, NF-κB and KRas, which have been shown to influence survivin expression (Tojima *et al.*, 2000; Barbie *et al.*, 2009; Sebti, 2013; Ni *et al.*, 2014). Coupled with the fact that TBK1 inhibition has proven to repress survivin expression (Wang *et al.*, 2017) and the preliminary data suggesting that TBK1 phosphorylates survivin at T5 (Chellappan Lab, Moffitt Cancer Centre, unpublished; see Appendix A), it now seems quite possible that these two proteins are interacting, both directly and indirectly, to produce favourable conditions for tumour growth.

4.1.2 Effects of TBK1 inhibition on survivin expression

The data presented here suggest that treating HeLa cells transfected with GFP or survivin-GFP with a TBK1 inhibitor results in lowered survivin expression, both on microscopy and by western blotting, although only the microscopy data was statistically significant due to an insufficient number of western blot repeats. This is consistent with a previous study showing reduced survivin expression in head and neck squamous cell carcinoma cell lines transfected with TBK1 siRNA (Wang *et al.*, 2017). It should be noted that whilst the microscopy data relate to expression of GFP-tagged survivin, the western blot examines endogenous

survivin and the accordance between both sets of results indicates that the lowered survivin expression on microscopy is a true result rather than a GFP-induced effect.

Whilst those results hold true for HeLa cells transfected with full-length survivin, those transfected with survivin$_{1\text{-}10}$ did not see the same effects. This suggests that the mechanism of TBK1 regulation of survivin expression is not linked to phosphorylation of survivin at T5. It is not possible to ascertain from this data whether the interactions shown are direct or indirect, so it may be that TBK1 is influencing survivin through a common signalling pathway or through the recruitment of cofactors.

The caveat to all this is that our data suggests that this result is not replicable in non-transfected HeLa cells. Considering the only previous study was done on siRNA-transfected cell lines, this raises serious questions as to whether the findings are due to the effects of transfection on the survivin promoter and whether the same results would be seen in vivo. Inhibition of TBK1 appears to increase survivin expression in non-transfected HeLa cells, the opposite result to transfected cells, which could indicate that TBK1 is regulating survivin through an interaction with its promoter and that integration of transfected DNA into the genome is interfering with this.

4.1.3 Potential reasons for an interaction

Apoptosis

The impact of TBK1 on survivin expression appears to be unlinked to its potential phosphorylation of survivin's N-terminus, as shown by the survivin$_{1\text{-}10}$-GFP microscopy data. Therefore, the key to understanding the relationship between the two proteins may lie in the areas where they perform similar cellular functions, particularly in cancer cells. Both have been implicated in the inhibition of apoptosis, specifically in the extrinsic pathway. Survivin has been found to

reduce caspase 8 activation in response to Bax- or Fas-induced apoptosis but it does not bind the caspase directly, suggesting that there are potential cofactors involved (Tamm *et al.*, 1998). TBK1 also functions to prevent caspase 8 activation, through a more defined mechanism. It can regulate the autophosphorylation of RIPK1, which prevents it from forming a complex with, and subsequently activating, caspase 8 (Heger and Dixit, 2018). Interestingly, it appears that TBK1 requires at least one as-yet-unidentified adaptor protein to prevent TNF-induced apoptosis in this manner (Lafont *et al.*, 2018). From this, it appears possible that survivin and TBK1 are acting in concert to inhibit the extrinsic pathway of apoptosis.

Autophagy

The theories presented so far have rested on the assumption that TBK1 upregulates survivin, as suggested by the microscopy data and the results from transfected cell lines. However, it is also possible that the data from non-transfected cells are correct and TBK1 is involved in reducing survivin expression. This would fit in the context of autophagy, where the two proteins have opposing effects. The pro-autophagy PINK1/Parkin pathway links TBK1 and survivin in an interesting way: it is correlated with increased TBK1 expression and reduced survivin expression (Lee *et al.*, 2012; Richter *et al.*, 2016). Oncogenic activation of the Ras signalling pathway, which upregulates TBK1 activity, is also pro-autophagic and inhibits beclin 1 expression, and knockdown of beclin 1 has been shown to downregulate survivin (Niu *et al.*, 2010; Elgendy *et al.*, 2011). If TBK1 is acting to inhibit survivin, then it is possible that the PINK1/Parkin pathway is exploiting this to promote autophagy or that the Ras pathway is the mechanism for TBK1-induced reduction in survivin expression.

A further potential mechanism for a direct inhibition of survivin by TBK1 can be seen in *Drosophila*, where a TBK1 homolog (DmIKKε) can degrade both the *Drosophila* IAP and human XIAP through phosphorylation (Kuranaga *et al.*,

2006). The phosphorylation site has not been elucidated, but if it is in the BIR domain, that would suggest TBK1 may interact similarly with survivin.

Ubiquitination

Regulation of survivin could also involve the ULD of TBK1, which has established functions in regulating IRF 3 activity in humans and in binding DIAP1 in *Drosophila* (Kuranaga *et al.*, 2006; Ikeda *et al.*, 2007). K-63 linked polyubiquitination has been shown to target survivin for proteasome degradation and so there is potentially a role for TBK1 in mediating this (Zhao *et al.*, 2000). Under oncogenic conditions the mutated KRas pathway, of which TBK1 is a vital part, can prevent proteasome degradation of survivin, so a role for TBK1 in regulating survivin ubiquitination can be hypothesised (Barbie *et al.*, 2009; Sebti, 2013).

4.1.4 Future experiments

Confirming TBK1 inhibition

There were limitations to the methodologies used here, primarily that for the microscopy data there was no verification that the TBK1 inhibitor had worked. A phospho-TBK1 antibody was acquired but was not fully optimised due to time constraints so although the western blot in Figure 16 was probed for phospho-TBK1, the results were inconclusive. As shown in Figure 13 and Figure 14, TBK1 was probed for on microscopy but there was no change in expression after BX795 treatment because the inhibitor does not affect the amount of endogenous TBK1, rather it just prevents its activation (Clark *et al.*, 2009). Any expansion on the experiments detailed here would require staining for phospho-TBK1 on fixed microscopy and on western blot in order to definitively link TBK1 inhibition to reduced survivin expression.

Microscopy of non-transfected cells

Whilst the effects of BX795 on survivin expression in non-transfected HeLa cells were able to be studied by western blotting, this was not corroborated with microscopy data as survivin in those cells is not tagged with a fluorophore. To study this, fixed cells could be stained with a survivin antibody however this would have to be applied to the transfected cells as well and as they were already fluorescing in the blue, green, red and far red channels it was deemed impractical for this set of experiments. To continue these experiments with a survivin antibody, the mitochondrial stain could be replaced with a red channel secondary antibody or alternatively, future research could focus solely on non-transfected cells in which case survivin could be probed for in the green channel.

It must also be accounted for that fixing the cells could quench the GFP signal. Paraformaldehyde was chosen as the fixative as it is less likely to affect the signal than methanol, but it could still be influencing the results. Live imaging could be used to mitigate this, although this would mean that non-transfected cells could not be studied.

Western blot repetition

The western blotting data requires replication in order to produce statistically significant results. As mentioned, an optimised phospho-TBK1 antibody would be needed to prove that lowered survivin expression is correlated with reduced TBK1 activity. The finding that TBK1 inhibition does not alter expression of survivin$_{1\text{-}10}$ also requires corroboration through western blotting.

Other cell lines

For all of these experiments, there must be consideration that HeLas are an immortalised cell line and their age, as well as their unusual karyotype of 76-80 chromosomes, means that they are not always an accurate analogue for in vivo conditions. All experiments would need to be repeated in other cancer lines to

ensure that the results are not HeLa-specific. Ideally, the experiments would also be repeated in non-cancerous cell lines to ascertain whether the changes are also cancer-specific. It wouldn't be possible to study this using the methods set out in this thesis as the level of endogenous survivin expression in normal cell lines is too low, but it could potentially be studied using a tetracycline-inducible expression system to account for this (Das, Tenenbaum and Berkhout, 2016). However, these systems come with the caveat of impaired mitochondrial function, which could affect survivin expression.

4.2 Survivin and c-Src

4.2.1 Basis for studying an interaction between survivin and c-Src

The relationship between survivin and c-Src, two proteins that are emerging as potential cancer biomarkers, is beginning to be established in the scientific literature. It has been found that survivin's N-terminus can immunoprecipitate c-Src and is obligatory for survivin to be able to regulate the activity of phosphorylated c-Src (Dunajová *et al.*, 2016).

Equally, a role for activated c-Src in regulating survivin has also been suggested, with decreased survivin expression seen in multidrug-resistant myeloma cells treated with drugs that inhibit the activation of c-Src (Tsubaki *et al.*, 2014). A potential mechanism for this lies in c-Src's functions at adherens junctions. c-Src phosphorylates β-catenin which decreases its ability to bind and stabilise E-cadherin, leading to the disintegration of cell-cell junctions (Roura *et al.*, 1999). Survivin upregulation in canine kidney cells transformed with activated c-Src was directly linked to this junction breakdown (Capra and Eskelinen, 2017).

Conversely, c-Src may also be able to downregulate survivin expression through phosphorylation of the tumour suppressor Fhit (Pekarsky *et al.*, 2004).

Phosphorylated Fhit regulates the PI3K pathway by inhibiting Akt and, subsequently, survivin (Semba *et al.*, 2006; Zhao *et al.*, 2010).

Survivin and c-Src are linked through several different pathways and mechanisms, but to-date most of the research has been on how c-Src regulates survivin. Following the evidence that the N-terminus of survivin can regulate c-Src (Dunajová *et al.*, 2016), the experiments detailed here were an attempt to establish how that interaction might occur.

4.2.2 Co-immunoprecipitation of survivin and c-Src requires survivin phosphorylation at T5

Here, we have shown that the interaction between survivin and c-Src is dependent on phosphorylation of survivin at T5. This was underlined by the absence of an interaction between c-Src and both the wild-type and the T5A survivin constructs. Mechanistically, this seems counter-intuitive as phosphorylation confers a negative charge on survivin which should theoretically reduce its affinity for the negatively charged SH3 domain of c-Src (Ahmad, Gu and Helms, 2008). Mutation to alanine, a hydrophobic residue, could also be predicted to aid the interaction between survivin and c-Src through hydrophobic interactions with the SH3 domain. It is still likely that the interaction is direct due to the strong affinity between PPII helices and SH3 domains but it should also be considered that the binding of T5-phopshorylated survivin and c-Src may either require cofactors or occur outside of c-Src's SH3 domain.

4.2.3 Implications of a phosphorylation-dependent interaction between survivin and c-Src

Phosphorylation at T5 could be necessary for c-Src regulation

The requirement for survivin to be T5-phosphorylated in order to bind c-Src suggests that the previously studied regulation of c-Src by the N-terminus of

survivin is also dependent on this phosphorylation. This raises the possibility that oncogenic control of c-Src activity through cyclical phosphorylation and dephosphorylation of survivin at T5 could be used to regulate cancer cell adhesion, invasion and motility. The data from Dunajová *et al.*, (2016) suggest that this would present phenotypically as rapid cell growth and loss of adhesion when activated, which in cancer cells would presumably then lead to migration and metastasis, based on the typical cellular changes associated with increased c-Src activity. The potential for survivin T5 phosphorylation as an on/off switch for metastatic transformation of cancer cells through regulation of c-Src could have wide oncotherapeutic implications.

Other effects of T5 phosphorylation

Preliminary research from the Wheatley Lab has suggested roles for T5 phosphorylation in inhibiting mitochondrial targeting of survivin, disrupting survivin's localisation in mitosis, and promoting dimerisation. It must be considered that these alterations in survivin's function could be due to the interaction between T5 and c-Src.

4.2.4 Future experiments

Elucidation of the T5 kinase and phosphatase

The mechanism of survivin T5 phosphorylation requires further study, with the unpublished findings that the kinase involved is TBK1 in need of verification. This could be done through treating cells expressing survivin$_{1\text{-}10}$ with BX795 and then blotting with an antibody for Phospho-Threonine. Similarly, dephosphorylation at this site needs to be examined. Computational analysis or high-throughput screening would be needed to discover candidate phosphatases, which could then be examined for interaction with survivin via the dephosphorylation assay described by Peck (2006).

Examination of a direct interaction

The experiments performed here do not differentiate between direct and indirect interactions, so this must be further examined. The co-immunoprecipitation should be repeated with in-vitro translated c-Src used in place of whole cell extracts to study this. The domain of c-Src at which the interaction occurs remains to be elucidated. This could also be studied by co-immunoprecipitation through examining whether constructs of the SH2 and SH3 domains can pull down survivin from HeLa whole cell extracts.

Structural changes induced by T5 phosphorylation

The study by Dunajová *et al.*, (2016) on the differences in cell adhesion in HeLa cells transfected with survivin$_{1\text{-}10}$-GFP and survivin$_{11\text{-}142}$-GFP could be expanded upon through the use of survivin T5A-GFP and survivin T5E-GFP constructs instead to assess whether the structural changes seen are dependent on T5 phosphorylation.

Other cell lines

As with the TBK1 experiments, it must be considered that HeLa cells are unusual and therefore all of the studies suggested must be repeated in other cancer cell lines. This is especially pertinent when looking at c-Src because of the various ways it is activated in different cancers, as described in 1.5.4.

4.3 Conclusions

The data presented in this thesis provide evidence that TBK1 can regulate survivin expression, although the differing results in transfected and non-transfected cells means that the exact nature of the relationship is still to be established. It was also found that phosphorylation of survivin at T5 by TBK1 is confers the ability to bind c-Src. From this, we suggest novel roles for survivin and TBK1 in regulating cancer cell adhesion dynamics.

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6 Appendix

Appendix A

Kinase data from the Chellappan Lab at the Moffitt Cancer Centre shows that TBK1 increases the expression of a phospho-tag in survivin$_{1-10}$, indicating that it is the kinase for survivin T5.

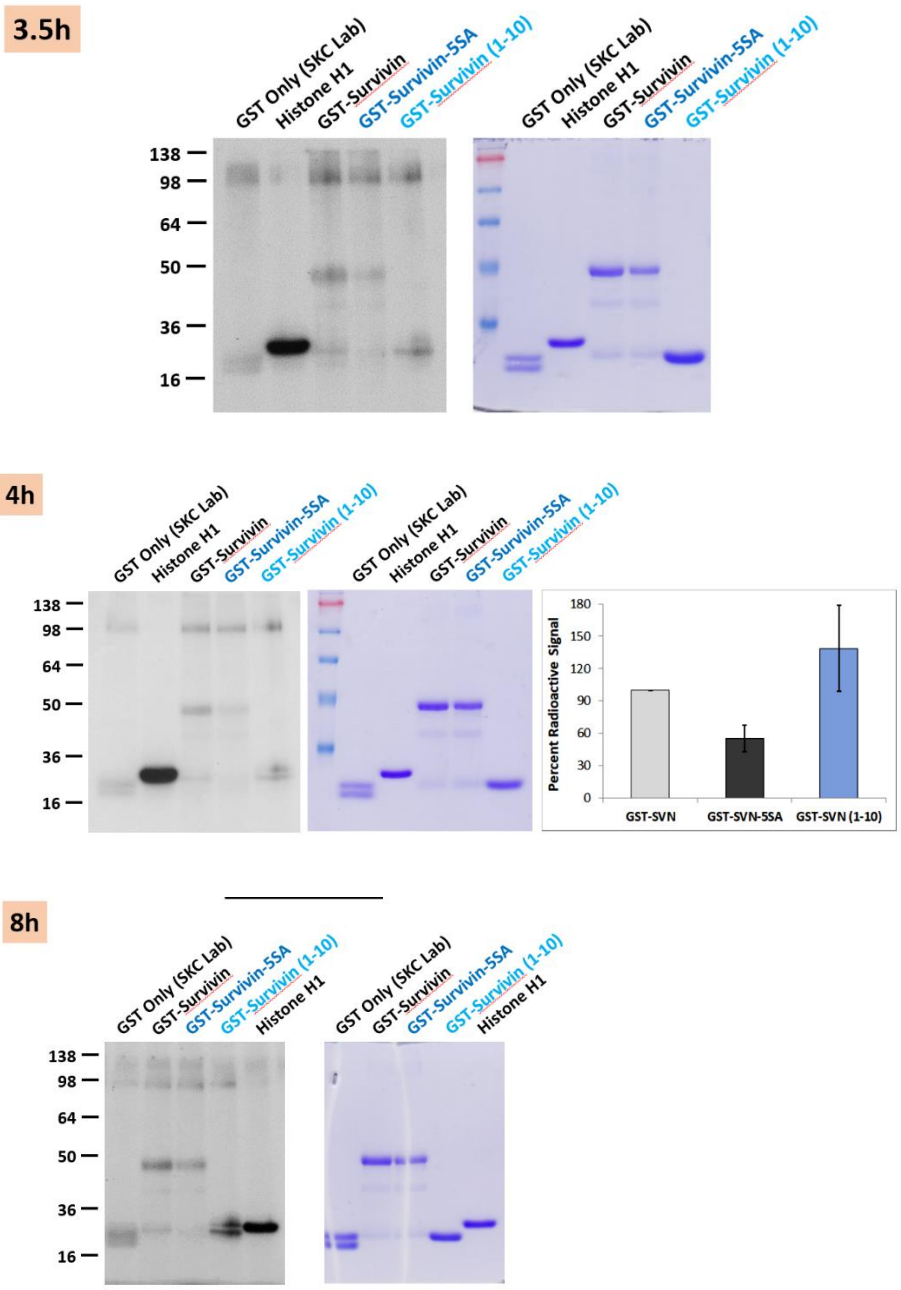


Figure 19 - Kinase data showing that after 3.5,4 and 8 hour incubations with TBK1, expression of a phospho-tag is markedly increased in survivin$_{1-10}$.

Appendix B

The western blot detailed in 3.4.2 was done in triplicate, with the two further repeats shown here.

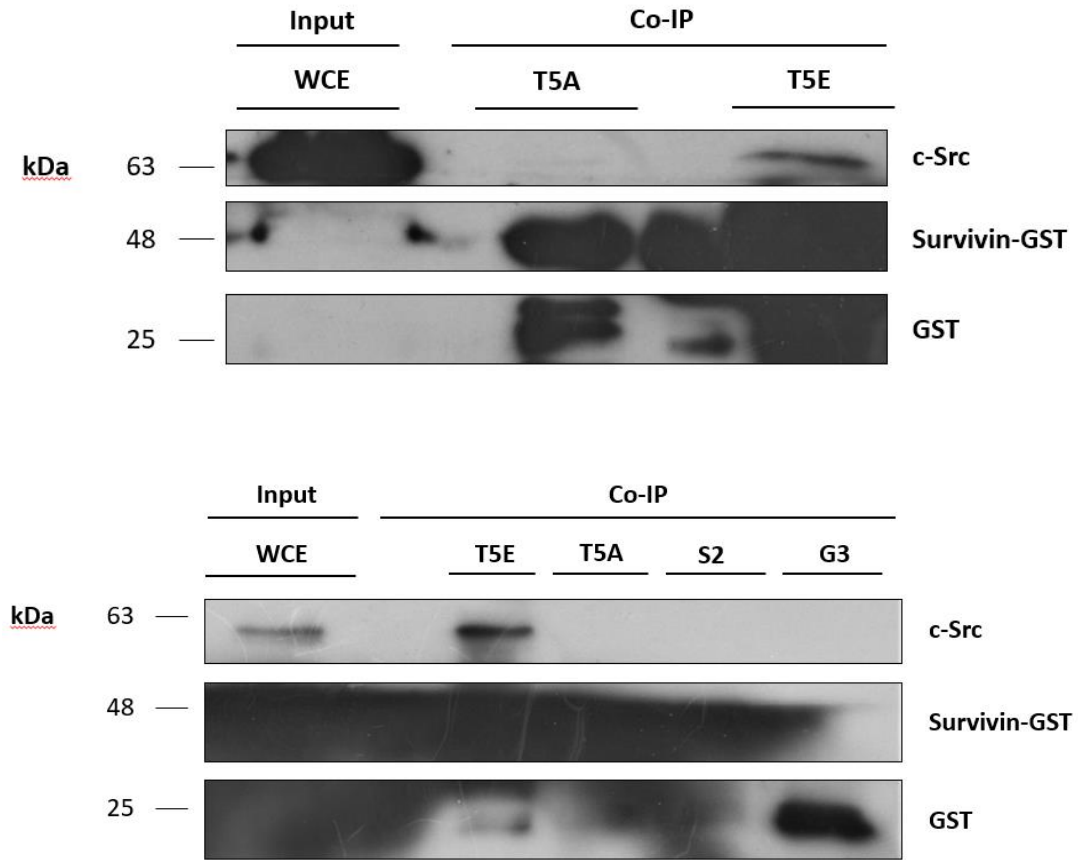


Figure 20 - Repeats of the pull-down assay of survivin mutants and c-Src in HeLa cells.