SIGNALLING TOWARDS IRES

By

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Abstract

XIAP and Bcl-xL are critical anti-apoptotic molecules that directly inhibit caspases and block mitochondrial membrane permeabilization, respectively. In addition to preventing apoptosis, both XIAP and Bcl-xL can be generated by cap-independent translation via the utilization of an IRES in the 5'-UTR of their mRNAs. In recent years it has been shown that activation of S6K2 induces the translational upregulation of these two apoptotic regulators. Here I have determined that activation of S6K2 enhances IRES-mediated translation of XIAP and Bcl-xL by inducing the degradation of PDCD4, which I have identified as a novel regulator of XIAP and Bcl-xL IRES elements. Furthermore, I have shown that PDCD4 is a positive modulator of the Apaf-1 IRES element. The concurrent regulation of XIAP, Bcl-xL and Apaf-1 by PDCD4 suggests a model in which the level of PDCD4 expression alters the apoptotic threshold by specifically impacting IRES-mediated translation of the XIAP, Bcl-xL and Apaf-1 mRNAs.

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List of Abbreviations

7-mG – 7-methylguanosine

β-gal – β-galactosidase

AIF – apoptosis inducing factor

AP-1 – activating protein 1

Apaf-1 – apoptotic protease activating factor 1

Bag-1 – Bcl-2 associated athanogene

Bcl-2 – B-cell lymphoma protein 2

BSA – bovine serum albumin

CAT – chloramphenicol acetyl transferase

c-IAP1 – cellular inhibitor of apoptosis protein 1

ct – cycle threshold

DIABLO – direct inhibitor of apoptosis-binding protein with a low isoelectric point

DISC – death-inducing signalling complex

DMEM – Dulbecco's modified Eagle medium

eIF – eukaryotic initiation factor

eIF4E-BP – eIF4-binding protein

ELISA – enzyme linked immunosorbent assay

EMCV – encephalomyocarditis virus

EndoG – endonuclease G

ER – endoplasmic reticulum

FADD – Fas-associated death domain protein

FAG – Fragment of Apoptotic Cleavage of eIF4G

FGF-2 – fibroblast growth factor 2

HEK293 – human embryonic kidney 293 cells

HIF1α – hypoxia inducible factor 1 α

hnRNP A1 – heterogeneous nuclear ribonucleoprotein A1

hnRNP C1/C2 – heterogeneous nuclear ribonucleoprotein C1/C2

HuR – Hu antigen R

IAP – inhibitor of apoptosis protein

IBM – IAP binding motif

IRES – internal ribosome entry site

ITAF – IRES *trans*-acting factor

IVT – *in vitro* trancription

miR-21 – microRNA-21

OMI/HTRA2 – OMI/High Temperature Requirement A 2

PBS – phosphate buffered saline

PDCD4 – programmed cell death gene 4

PKCε – protein kinase C ε

PMSF – phenylmethylsulfonyl fluoride

RIPA – radioimmunoprecipitation assay

rpS6 – ribosomal protein S6

S6K1 – ribosomal protein S6 kinase 1

S6K2 – ribosomal protein S6 kinase 2

SCLC – small cell lung cancer

SD – standard deviation

SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Smac – second mitochondria-derived activator of caspases

TNFRI – tumour necrosis factor receptor I

UTR – untranslated region

VEGF – vascular endothelial growth factor

XIAP – X-linked inhibitor of apoptosis protein

X-DC – X-linked dyskeratosis congenita

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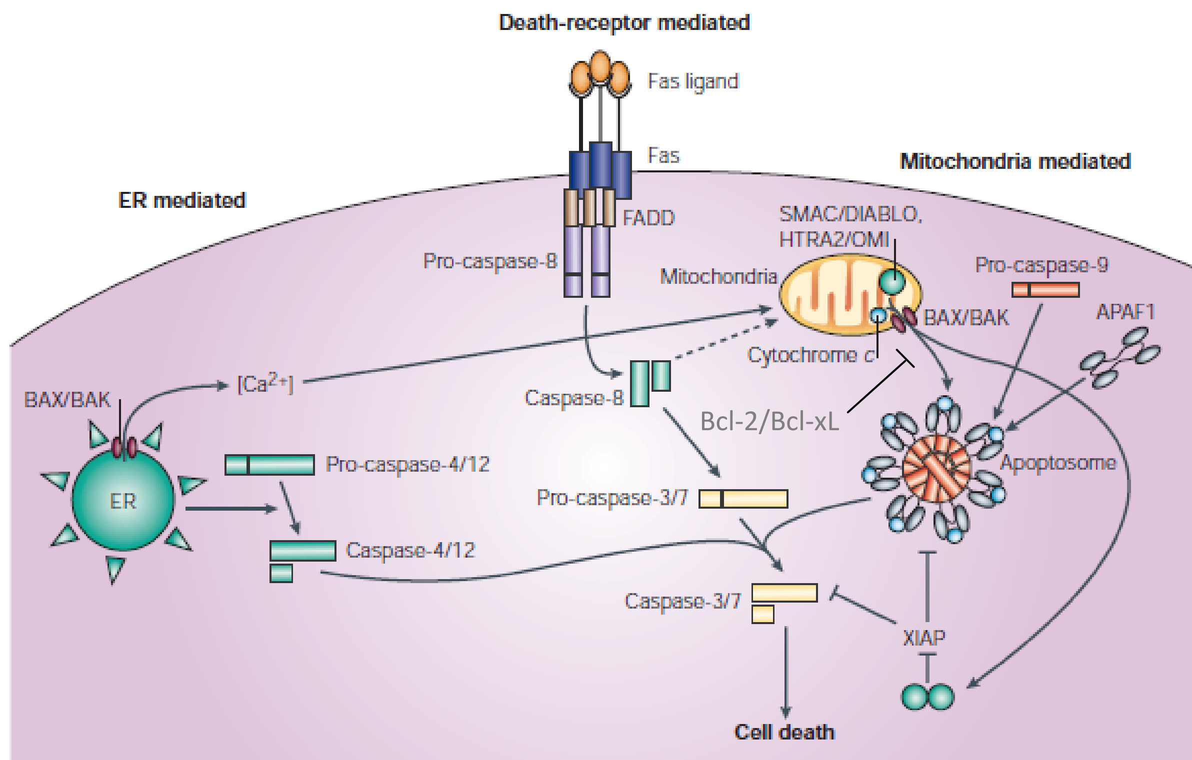
Chapter 1: Introduction

1.1 Programmed Cell Death

Programmed cell death, or apoptosis, is a crucial cellular process necessary for proper cell turnover in certain tissue types, correct development during embryogenesis, and for the removal of cells that have acquired pathogenic properties (Kerr et al. 1972, Vaux and Korsmeyer 1999). Apoptosis differs from necrosis - an unregulated form of cell death resulting from overwhelming cellular stress, as well as other forms of cell death that fall outside of the apoptosis-necrosis paradigm, in that it arises from the cell's decision to die in response to external or internal cues (Degterev and Yuan 2008). Initiation of apoptosis can occur following the activation of the death receptor pathway, or following the activation of the intrinsic pathways; irrespective of the pathway triggering the onset of apoptosis, ultimately all signals converge to activate caspase cascades (see Figure 1; Debatin and Krammer 2004).

Caspases comprise a specific class of cysteine proteases that exist in normal cells as inactive enzymes; however, during apoptosis they must undergo cleavage in order to become catalytically active (Shi 2002). The caspases involved in apoptosis can be separated into two distinct categories – initiator caspases, and the effector caspases (Shi 2002). Extracellular ligand binding to members of the death receptor family, such as CD95 or tumour necrosis factor receptor I (TNFRI), induces clustering of the receptors and subsequent formation of a death-inducing signalling complex (DISC) (Hengartner 2000). The DISC complex will then recruit a number of procaspase-8 molecules via the adaptor

Figure 1. Apoptotic pathways in eukaryotes. Apoptosis can be triggered by both extrinsic and intrinsic signals, which evoke different cellular pathways, but ultimately converge to activate caspases; the activation of caspases is required to initiate the destruction of the cell. There are a number of proteins that regulate apoptosis to either ensure that apoptosis is carried out or prevent cell death, and are appropriately termed pro- or anti-apoptotic proteins respectively. Pro-apoptotic molecules include cytochrome c and Apaf-1, both of which are necessary for the formation of the apoptosome. During intracellular stress, other pro-apoptotic factors including Smac/DIABLO and OMI/HTRA2 are also released from the mitochondria to effect the apoptotic programme. The release of these factors is facilitated by the pro-apoptotic members of the Bcl-2 family of proteins, including Bax and Bak, which act to modulate the outer mitochondrial membrane and the endoplasmic reticulum (ER). To counteract apoptosis there are two families of proteins: the inhibitor of apoptosis proteins (IAP), including the potent caspase inhibitor XIAP, and anti-apoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-xL, which inhibit membrane pore formation in the mitochondria or ER. Figure from Nature Reviews Molecular Cell Biology, volume 6, page 323, April 5, 2005. Reprinted with permission from Nature Publishing Group.



Death-receptor mediated
Fas ligand
Fas
ER mediated
Mitochondria mediated
FADD
SMAC/DIABLO,
HTRA2/OMI
Pro-caspase-8
Mitochondria
Pro-caspase-9
BAX/BAK
APAF1
Cytochrome c
[Ca2+]
BAX/BAK
Caspase-8
Bcl-2/Bcl-xL
ER
Pro-caspase-4/12
Apoptosome
Pro-caspase-3/7
Caspase-4/12
Caspase-3/7
XIAP
Cell death

molecule FADD (Fas-associated death domain protein), which results in the activation of caspase-8 (Srinivasula et al. 1996, Boldin et al. 1996). Alternatively, intracellular stress such as DNA damage will trigger the intrinsic apoptotic pathway, which is classically characterized by the release of cytochrome c from the mitochondria. The cytoplasmic pool of cytochrome c associates with Apaf-1 (apoptotic protease activating factor 1) and procaspase-9 to form the apoptosome, which then proceeds to activate caspase-9 (Cain et al. 2000, Li et al. 1997, Zou et al. 1997). Lastly, endoplasmic reticulum (ER) stress can also trigger the intrinsic apoptotic pathway or an ER-specific pathway, which results in the activation of caspase-2 and -4 (Cheung et al. 2006, Dahmer 2005, Hitomi et al. 2004, Rao et al. 2004). Regardless of the initial apoptotic stimuli or subsequent pathway that is activated by that insult, the now catalytically active initiator caspases converge to promote the activation of the effector caspases, including caspase-3 and -7 (Boatright and Salvesen 2003, Degterev et al. 2003); the activation of the effector caspases ultimately induces the ordered dismantling of the cell.

Given the many important roles of apoptosis in mammalian biology, it is evident that there must be strict control over this process to ensure that the apoptotic program is not inappropriately activated or inhibited. The inhibition of apoptosis is mediated by two distinct families of proteins: the inhibitor of apoptosis proteins (IAPs) and the Bcl-2 (B-cell lymphoma protein 2) family of proteins. Conversely, pro-apoptotic members of the Bcl-2 family of proteins and Apaf-1, along with a number of apoptogenic molecules released from the mitochondria, including cytochrome c, Smac/DIABLO (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with a low isoelectric

point), OMI/HTRA2 (OMI/High Temperature Requirement A2), Endonuclease G (EndoG) or AIF (apoptosis inducing factor), promote apoptosis. These proteins are discussed to a greater extent in the next section.

1.2 Apoptosis Regulating Proteins

The Bcl-2 family of proteins is comprised of both pro- and anti-apoptotic protein members, all of which function to regulate mitochondrial membrane homeostasis. Pro-apoptotic members of this family, such as Bad, Bax, Bid or Bak, are thought to act by interacting via common structural motifs to permeabilize the outer mitochondrial membrane allowing for the release of cytochrome c and other intermembrane proteins. Anti-apoptotic members of this family, which includes Bcl-2, Bcl-xL, Bcl-w, and Mcl-1, prevent apoptosis by binding to the pro-apoptotic members of the family to block this pore formation (Gross et al. 1999, Sharpe et al. 2003, Tsujimoto 2003). If the pro-apoptotic Bcl-2 family molecules are successful in permeabilizing the mitochondrial membrane, apoptotic signalling is potentiated via the release of various apoptogenic molecules. Included in this set of proteins is OMI/HTRA2, a serine protease capable of binding to and inhibiting IAPs (Martins et al. 2002, Vaux and Silke 2003, Verhagen et al. 2002), EndoG, a nuclease that causes fragmentation of oligonucleosomal DNA once translocated to the nucleus (Arnoult et al. 2003, Galuzzi et al. 2008), AIF, which also translocates to the nucleus to induce chromatin condensation and degradation of DNA (Arnoult et al. 2003, Susin et al. 1999), Smac/DIABLO, which promotes apoptosis by sequestering IAPs (Du et al. 2000, Vaux and

Silke 2003, Verhagen and Vaux 2002), and cytochrome c, which as previously mentioned is a critical component of the apoptosome (Garrido et al. 2006, Liu et al. 1996). As mentioned above, in addition to cytochrome c, a necessary component of the apoptosome is Apaf-1, a pro-apoptotic protein that oligomerizes in the presence of both cytochrome c and dATP (Cain et al. 2000, Zou et al. 1997). Apaf-1 has proven to be necessary for the progression of the intrinsic apoptosis pathway by activating caspase-9, and in fact, overexpression of Apaf-1 will sensitize cells to apoptosis inducing agents such as paclitaxel and etoposide (Perkins et al. 1998).

In addition to members of the Bcl-2 family of proteins, IAPs operate to ensure that apoptosis does not occur unnecessarily. Common to all members of the IAP family is the presence of at least one BIR (baculovirus IAP repeat) domain, which are important structural components required for the anti-apoptotic activity of most IAPs and in some instances, mediate direct IAP-caspase binding (Deveraux et al. 1997, Eckelman et al. 2008, Huang et al. 2001, Roy et al. 1997). It should be noted however, that not all members of the IAP family are capable of binding to caspases, and a significant numbers of studies have demonstrated that in addition to their roles in apoptosis, IAPs also function as signalling molecules or ubiquitylases (Li et al. 2002, Rothe et al. 1995, Suzuki et al. 2001, Yamaguchi et al. 1999). Moreover, recent studies have shown that the binding of an IAP to a caspase does not necessarily ensure caspase inhibition; it appears that XIAP (X-linked IAP) is the only IAP that prevents apoptosis through direct caspase inhibition (Eckelman and Salvesen 2006). XIAP has been proven to directly bind and potently inhibit caspase-9 as well as the effectors caspases, caspase-3, and -7, rendering XIAP the most effective IAP in terms of inhibiting

apoptosis (Huang et al. 2001, Riedl et al. 2001, Scott et al. 2005, Shiozaki et al. 2003, Srinivasula et al. 2001, Sun et al. 2000).

The expression of these apoptosis-regulating molecules must be tightly controlled as they regulate the balance between life and death for a cell. Therefore it is not surprising that mutations in or uncontrolled overexpression or loss of these proteins severely impairs the proper functioning of the apoptotic program. This is of particular importance for cancer, as the gained ability to avoid apoptosis is a hallmark of cancer (reviewed in Hanahan and Weinberg 2000) and the dysregulation of the apoptotic signalling pathways is frequently implicated in both tumourigenesis and drug resistance (Fesik 2005, Kabore et al. 2004, Pommier et al. 2004). For example, a number of studies attribute chemoresistance exhibited by pancreatic cells to the overexpression of XIAP, c-IAP2 or Bcl-xL (Bai et al. 2005, Li et al. 2006, Lopes et al. 2007), while decreasing expression of XIAP is sufficient to enhance the chemosensitivity of various cancer cell lines (Gagnon et al. 2008, McManus et al. 2004, Tong et al. 2005). Likewise, in a screen of a panel of cell lines collected from various human tumours and their sensitivity to chemotherapeutic drugs it was found that the cell lines expressing high levels of Bcl-xL were relatively resistant to approximately 70,000 different cytotoxic agents (Amundson et al. 2000). As a result of these studies, numerous small molecule drugs targeting the expression of antiapoptotic proteins have been developed for use in cancer therapy. For instance, ABT-263 is a Bcl-2 family inhibitor that displays a high affinity for Bcl-2, Bcl-xL and Bcl-w and is currently in early clinical trials for the treatment of small cell lung cancer, a number of lymphoid malignancies, and chronic lymphocytic leukaemia (reviewed in Vogler et al. 2009). On the other hand, a number of

small molecule inhibitors targeting the IAP family have also been developed by Genetech, Inc., Novartis, Aegera Therapeutics/Human Genome Sciences, Ascenta Therapeutics, Tetralogic Therapeutics, and Joyant Therapeutics among others; these drugs are currently in phase I/II clinical trials for the treatment of solid tumours and various cancers, including non-Hodgkin lymphoma, glioma, melanoma and prostate cancer (Call et al. 2008, Dynek and Vucic 2010).

Alternatively, the loss of pro-apoptotic molecules is also implicated in cancer biology; for instance, the loss of Apaf-1 is a marker for malignant progression of melanoma (Baldi et al. 2004, Soengas et al. 2001), and is a common feature in human leukaemia (Fu et al. 2003). Similarly, functional loss of pro-apoptotic members of the Bcl-2 family, such as Bax, significantly contributes to tumour progression as well as apoptotic resistance (Ionov et al. 2000, Pepper et al. 1996, Rashmi et al. 2005, Shibata et al. 1999).

Clearly the expression of these apoptotic proteins must be tightly controlled under normal cellular conditions, given the detrimental outcome following the dysregulation of their expression – particularly in the case of anti-apoptotic proteins; therefore specific mechanisms unique to apoptotic proteins ought to exist to regulate their relative concentrations in the cell. When cells encounter an apoptotic trigger, one of the primary cellular responses is attenuation of global translation, which is a rapid means by which the cell can both conserve energy and preclude the synthesis of unnecessary proteins (Rudra and Warner 2004, Warner 1999). However, a number of different proteins are needed to efficiently respond to apoptotic stimuli; thus, to avoid this translational block, several apoptotic effector molecules, including XIAP, c-IAP1, Bcl-2, Bcl-xL and Apaf-1, have evolved

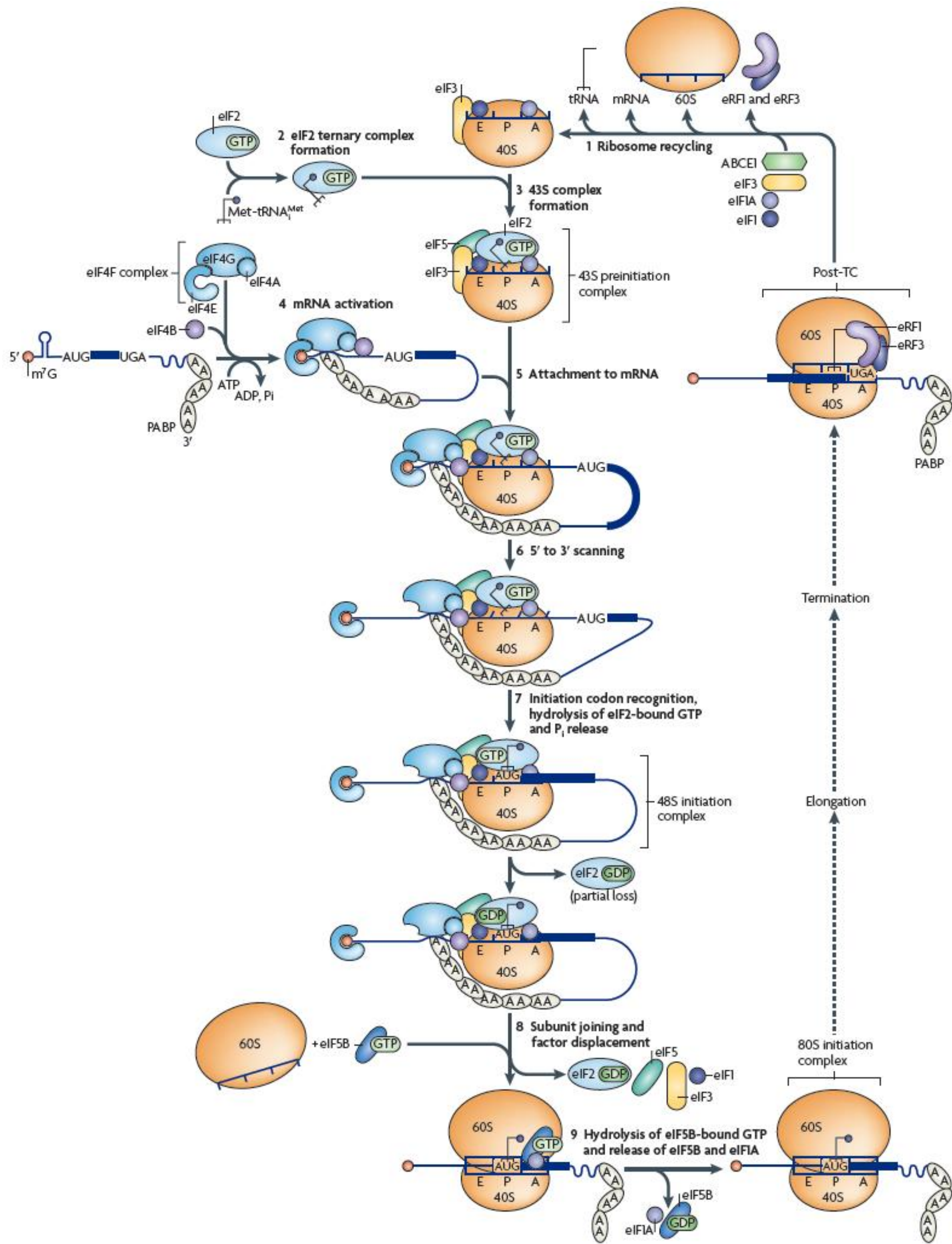
an alternative mechanism by which their expression is controlled during times of cellular stress. Though apoptosis compromises general protein synthesis machinery, the 5'-untranslated regions (UTRs) of the transcripts encoding the aforementioned proteins all contain an internal ribosome entry site (IRES), which allows for the generation of these proteins regardless of constraints restricting global translation (Coldwell et al. 2000, Holcik et al. 1999, Sherrill et al. 2004, Van Eden et al. 2004b, Yoon et al. 2006).

1.3 Translation Initiation in Eukaryotes

Though IRES elements were initially discovered in picornaviruses, the existence of additional viral IRESs and numerous cellular IRESs has since been uncovered (Hellen and Sarnow 2001, Jang et al. 1988, Pelletier and Sonenberg 1988). The majority of cellular transcripts are translated in a cap-dependent manner – meaning that they require the presence of a 7-methylguanosine (7-mG) cap at the 5' end of the transcript and the availability of the cap-binding protein, eukaryotic initiation factor 4E (eIF4E), whereas IRES-containing transcripts can be translated in a cap-*in*dependent fashion. The process of translation is composed of three stages: initiation, elongation and termination. The central difference between cap-dependent and -independent translation is at the stage of initiation; therefore to compare these two mechanisms of translation and emphasize the importance of IRES-mediated translation, cap-dependent translation initiation will first be reviewed.

Translation initiation is thought to be the rate limiting phase of translation, requiring over 25 proteins, in which a translationally competent 80S ribosome is ultimately positioned correctly at the initiation codon (AUG), therefore allowing the ribosome to synthesize the protein encoded for by the mRNA (See Figure 2; Gebauer and Hentze 2004, Pestova et al. 2001, Preiss and Henzte 2003). The initial step in this process is the formation of the 43S pre-initiation complex; this complex is formed by the small 40S ribosomal subunit, eukaryotic initiation factors (eIFs) (eIF1, eIF1A, eIF3, eIF5), GTP-eIF2 and Met-$tRNA_i^{Met}$. The 43S complex is then recruited to the 7-mG cap on the mRNA through bridging reactions between eIF3 and the eIF4F complex. The eIF4F complex consists of eIF4E, the cap-binding protein mentioned above, eIF4A, a DEAD box helicase, and eIF4G, which functions as a scaffolding protein by interacting with eIF4E, eIF4A and eIF3. Once the 43S complex has bound to the mRNA, eIF4A, along with its co-factor eIF4B, unwinds secondary structures present in the mRNA to allow the 43S complex to scan the 5'-UTR until it reaches the first initiation codon in the appropriate sequence context; eIF4F is essential for scanning to occur and is assisted by both eIF1A and eIF1, which also plays a significant role in selection of the correct initiation codon (Gebauer and Hentze 2004, Hinton et al. 2007, Kozak 1999, Pestova and Kolupaeva 2002, Rogers et al. 2001). Once the 43S complex has recognized the correct AUG, and the initiator tRNA base-pairs with the start codon, eIF5 catalyzes the hydrolysis of eIF2 bound GTP; at this point eIF2-GDP and most translation initiation factors are released from the complex (Asano et al. 2001, Gebauer and Hentze 2004). The ribosome then stimulates the hydrolysis of eIF5B bound GTP, thus releasing

Figure 2. Translation initiation in eukaryotes. The canonical pathway for eukaryotic translation initiation is a complex process involving numerous proteins. First, the 43S pre-initiation complex is formed by the 40S ribosomal subunit, eIFs (1, 1A, 3, 5), GTP-eIF2 and Met-tRNA$_i^{Met}$. This complex is then recruited to the mRNA cap structure via bridging reactions involving eIF3 and the eIF4F complex (eIF4E/4G/4A). Once the 43S complex has bound the mRNA, eIF4A and eIF4B act to unwind the secondary structure present in the mRNA to allow the 43S complex to scan the 5'-UTR until it reaches the first initiation codon in the appropriate sequence context. Upon this recognition event, the initiator tRNA base-pairs with the start codon and eIF5 catalyzes the hydrolysis of eIF2 bound GTP; eIF2-GDP and most translation initiation factors are then released from the complex, at which time the 60S ribosomal subunit joins the complex. The ribosome then stimulates the hydrolysis of eIF5B bound GTP, thus releasing eIF5B from the complex, and ultimately a translationally competent 80S ribosome is formed. Figure from Nature Reviews Molecular Cell Biology, volume 10, page 116, February 1, 2010. Reprinted with permission from Nature Publishing Group.



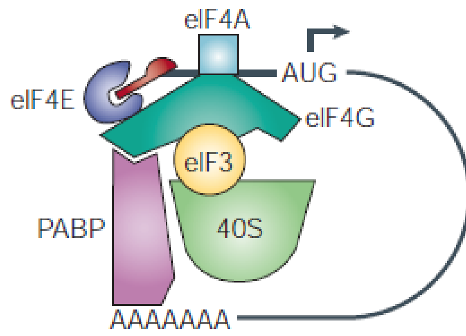
tRNA
mRNA
60S
eRF1 and eRF3
eIF3
E P A
40S
1 Ribosome recycling
ABCE1
eIF3
eIF1A
eIF1
eIF2
GTP
2 eIF2 ternary complex formation
Met-tRNAiMet
3 43S complex formation
eIF5
eIF3
43S preinitiation complex
eIF4F complex
eIF4G
eIF4A
eIF4E
4 mRNA activation
eIF4B
5′
m7G
AUG
UGA
ATP
ADP, Pi
PABP
3′
5 Attachment to mRNA
Post-TC
eRF1
eRF3
6 5′ to 3′ scanning
Termination
7 Initiation codon recognition, hydrolysis of eIF2-bound GTP and Pi release
48S initiation complex
Elongation
eIF2 GDP
(partial loss)
8 Subunit joining and factor displacement
+eIF5B
80S initiation complex
9 Hydrolysis of eIF5B-bound GTP and release of eIF5B and eIF1A
eIF5B
eIF1A
GDP

eIF5B; it is at this point that the large 60S ribosomal subunit joins to form a translationally competent 80S ribosome (Pestova et al. 2000, Lee et al. 2002).

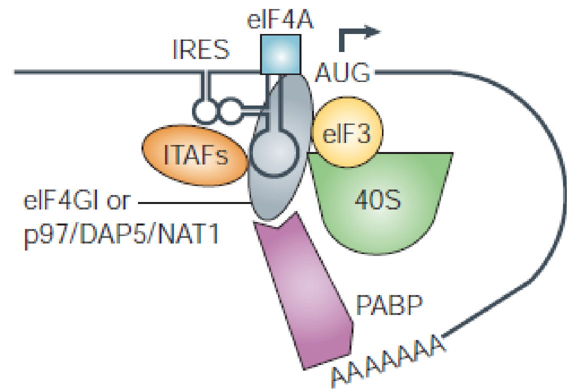
The process detailed above is applicable to the translation of most cellular mRNAs, however, as previously mentioned, an alternative mechanism of translation initiation exists that is not dependent on the formation of the eIF4F complex (see Figure 3). The notion that a cap-independent mechanism of translation existed stemmed from various observations regarding picornaviral translation. First, picornaviral RNAs found associated with polysomes did not have the traditional 7-mG cap that cellular mRNAs have, suggesting that the binding of eIF4E was not required for the recruitment of the ribosome (Nomoto et al. 1977, Wimmer 1982). It was hypothesized from this data that the mechanism of initiation must involve an internal signal; this was proven to be true when the 5'-UTRs of the encephalomyocarditis (EMCV) and poliovirus were inserted into intercistronic region of a bicistronic mRNA and were capable of mediating translation of the second cistron (Jang et al. 1988, Pelletier and Sonenberg 1988). Further experiments demonstrated that translation of picornaviral mRNAs is not inhibited upon sequestration or inhibition of eIF4E, which was assessed by a number of different approaches (Beretta et al. 1996, Canaani et al. 1976, Pause et al. 1994). Additionally, cap-dependent translation of host cell mRNAs is reduced upon infection with certain picornaviruses due to the overexpression of proteases that cleave eIF4G, thus eliminating its function as a scaffolding protein, or enhanced sequestration of eIF4E by eIF4E-BPs (Gingras et al. 1996, Gradi et al. 1998, Lamphear et al. 1993, 1995).

Figure 3. Cap-dependent versus cap-independent translation initiation. Most translation initiation occurs as outlined in Figure 2, whereby translation is initiated at the 7-mG cap structure, and recruitment of the ribosome to the mRNA is dependent on the formation of the eIF4F complex at the mRNA cap and the bridging reactions mediated by eIF4G and eIF3. Cap-independent translation however, does not require the cap structure or the cap-binding protein eIF4E; in this mode of translation initiation, the ribosome is recruited directly to the mRNA in close proximity of the start codon. The specific requirement for canonical initiation factors in cap-independent translation initiation is not well characterized, but it is known that this mode of translation is distinctly regulated by the action of IRES *trans*-acting factors (ITAFs), which can act in a positive and negative manner. As in the case of initiation factors, the cohort of ITAFs affecting an individual IRES element depends on the IRES in question. Figure from Nature Reviews Molecular Cell Biology, volume 6, page 320, April 5, 2005. Reprinted with permission from Nature Publishing Group.

Cap-dependent translation initiation



Cap-independent translation initiation



The regulation of IRES-mediated translation has been the focus of countless studies to date. The search for a common structural or sequence element shared by IRES elements that mediate translation initiation has yielded different data. While many viral IRESs have been shown to have similar sequence or structural elements, this has not proved to be the case for cellular IRESs (Baird et al. 2006). Additionally, the requirement for canonical initiation factors varies for viral and cellular IRES elements, with some viral IRESs requiring no initiation factors at all (Jan and Sarnow 2002, Schüler et al. 2006, Spahn et al. 2004, Spriggs et al. 2009). In addition to initiation factors, viral and cellular IRES activity is also controlled by accessory proteins termed IRES *trans*-acting factors (ITAFs), which can both positively and negatively regulate efficiency of IRES-mediated translation initiation (Costa-Mattioli et al. 2004, Hunt et al. 1999, Kaminski et al. 1995, Spriggs et al. 2005). Some ITAFs regulate a wide range of IRES elements, while others affect only a specific subset; additionally, ITAFs may function to augment the activity of some IRESs, while repressing the activity of others. For example, hnRNP A1 (heterogeneous nuclear ribonucleoprotein protein A1) has been shown to negatively regulate the XIAP and Bcl-xL IRES elements, while promoting FGF-2 (fibroblast growth factor 2) IRES activity (Bevilacqua et al. 2010, Bonnal et al. 2005, Lewis et al. 2007). Similarly, PTB (polypyrimidine tract binding protein) is required for the Apaf-1 IRES and stimulates the activity of the Bag-1 (Bcl-2 associated athanogene) IRES, yet negatively regulates the Bip and XIAP IRES element (Baird et al. 2007, Kim et al. 2000, Mitchell et al. 2001, Pickering et al. 2003). The ITAFs themselves are also under regulatory control; generally this regulation is exerted at the level of subcellular localization, however, they can also be regulated by post-transcriptional modifications such

as phosphorylation (Dobbyn et al. 2008, Lewis et al. 2007, Lewis and Holcik 2008, Lin et al. 2007, Shi et al. 2008). There is no unified model as to how ITAFs function to modulate IRES activity, but it is believed that they aid in recruiting the ribosome to the IRES element, either directly or by inducing a conformational change in the secondary structure that is amenable for ribosome binding; in contrast, ITAFs may exert negative regulation by binding to the IRES elements in order to preclude the binding of another ITAF that would otherwise promote the activity of that IRES, or hold the secondary structure of the mRNA in a translationally inaccessible conformation (reviewed in Spriggs and Bushell 2005).

Upon encountering various stress signals many facets of the translational machinery are modified such that global translation is attenuated; generally, these modifications alter translational components required for cap-dependent translation. Therefore, the ability to initiate translation in a cap-independent manner imparts a unique selective advantage to IRES containing mRNAs, which becomes increasingly important during situations of cell stress.

1.4 Translational Regulation and Stress

As outlined in the previous section, translation initiation is a complex process involving a considerable number of protein factors. Therefore, it is not unexpected that control of this phase of translation is tightly regulated through various mechanisms as well, so as to ensure that aberrant translation does not occur and allow the cell to rapidly respond to any environmental changes. Inhibition of translation initiation is primarily

affected by the phosphorylation status of eIF2 and the availability of the cap binding complex, which can be influenced in a number of different ways.

Under normal conditions, eIF2 functions to catalyze the transfer of Met-$tRNA_i^{Met}$ to the 43S pre-initiation complex, and once the correct initiation codon is recognized, the GTP bound to eIF2 is hydrolyzed to GDP. This hydrolysis step occurs during each round of translation initiation and is necessary for the recycling of the ternary complex (reviewed in Gebauer and Hentze 2004, Proud 2005). During various cellular stress situations, such as amino acid starvation, viral infection, or ER stress, a number of kinases are activated that induce phosphorylation of eIF2 at Serine 51 on its α subunit (de Haro et al. 1996, Dever 2002); phosphorylation at this residue blocks the GTP hydrolysis reaction by preventing efficient dissociation of eIF2 from eIF2B (Rowlands et al. 1988). The inability of eIF2 to be recycled significantly impairs the formation of the pre-initiation complex, which inhibits global translation, yet allows the translation of specific transcripts (Dever et al. 1992, Kaufman, R.J. 2004).

The availability of eIF4E is also highly regulated to control rates of protein synthesis. First, the association of eIF4E with the 7-mG cap of mRNAs can be inhibited by the sequestration of eIF4E by eIF4E-BPs. eIF4E-BPs contain a protein binding domain similar to that which mediates the eIF4E-eIF4G interaction, and it is through this domain that eIF4E-BPs can disrupt binding of eIF4E to eIF4G, thus preventing the formation of the eIF4F complex (Lawrence and Abraham 1997, Mader et al. 1995, Pause et al. 1994). The ability of eIF4E-BPs to bind to eIF4E is controlled by its phosphorylation state, which is influenced by extracellular stimuli such as hormones, growth factors or cytokines. Under the

aforementioned conditions, which often coincide with enhanced cell growth, eIF4E-BPs are in a hyperphosphorylated state and cannot bind to eIF4E; however, cellular stresses such nutrient or growth factor deprivation, can induce eIF4E-BP hypophosphorylation, which in turn allows eIF4E-BPs to compete with eIF4G for binding to eIF4E and inhibit recruitment of the 43S pre-initiation complex to the cap structure (Braunstein et al. 2009, Fleurent et al. 1997, Gingras and Sonenberg 1997, Pause et al. 1994, Pyronnet et al. 1998). Though eIF4E-BPs are known to undergo caspase-mediated cleavage during apoptosis, one of the cleavage products of eIF4E-BP1 retains its ability to bind to eIF4E, thus maintaining repression of cap-dependent translation during stress (Bushell et al. 2000b, Tee and Proud 2000, 2003). Additionally, the phosphorylation of eIF4E itself also appears to influence translation and has been implicated in the regulation of translation initiation – though it is still unclear exactly how phosphorylation of eIF4E imposes regulation, if any, over this process (reviewed in Scheper and Proud 2000). Interestingly, it should be noted that the dephosphorylation of eIF4E has been linked to a switch to cap-independent translation (Dyer et al. 2003).

As indicated above, the association of eIF4E with the 7-mG cap structure is greatly facilitated by its interaction with eIF4G; however, like eIF4E-BPs, eIF4G is sensitive to cleavage during apoptosis and viral infection, which in turn affects the binding of eIF4E with the mRNA cap (Bushell et al. 2000b, Gradi et al. 1998, Kuo et al. 2002, Marissen et al. 2000, Morley et al. 2005). Similarly, the eIF4G family member p97/DAP5, which can bind eIF3 and eIF4A, but not eIF4E, is sensitive to caspase-3 mediated cleavage during apoptosis as well (Henis-Korenblit et al. 2000, Morley et al. 2005). The cleavage of eIF4GI during apoptosis

yields three major cleavage products: N-FAG (N-terminal Fragment of Apoptotic Cleavage of eIF4G), M-FAG (Middle-FAG), and C-FAG (C-terminal FAG); the cleavage causes the disassembly of the eIF4F complex as the fragments no longer retain the ability to bind eIF4E, eIF4A and eIF3 (Bushell et al. 2000a, Hundsdoerfer et al. 2005). While this cleavage suppresses the translation of a majority of cellular transcripts, a number of proteins can still be generated. Surprisingly, the eIF4G M-FAG alone has been demonstrated to support IRES-mediated translation of a number of mRNAs including p97/DAP5, XIAP and c-IAP1 (Hundsdoerfer et al. 2005, Nevins et al. 2003, Stoneley et al. 2000). Likewise, p97/DAP5 and its cleavage product p86/DAP5, generated during apoptosis by caspase-3-mediated cleavage, are able to selectively support cap-independent translation from the IRESs of p97/DAP5, c-Myc, Apaf-1, c-IAP1 and XIAP (Henis-Korenblit et al. 2000, 2002, Hundsdoerfer et al. 2005, Nevins et al. 2003).

The cellular response to stress signals evidently involves numerous modifications of protein synthesis machinery in order to suppress general translation, but promote the ongoing translation of particular transcripts. The switch to IRES-mediated translation under such conditions has been well characterized for a number of situations including heat shock, ER stress, hypoxia and during apoptosis (reviewed in Silvera et al. 2010). This switch allows the cell to adapt to cellular stress, which requires the ongoing expression of both pro- and anti-apoptotic proteins, in order to allow the cell to survive and recover from the stress or to fully execute the apoptotic program. However, the switch to IRES-mediated translation can sometimes be pathogenic. For example, as a tumour grows, the tumour microenvironment becomes increasingly more hypoxic. Under these hypoxic conditions

VEGF (vascular endothelial growth factor), FGF-2, Bcl-2 and HIF1α (hypoxia inducible factor 1α) are all overexpressed due to an upregulation of translation from their IRES elements; this selective translation is mediated by the overexpression of eIF4E-BPs and eIF4G and is particularly advantageous for cancer cells as VEGF, FGF-2, HIF1α and Bcl-2 are all significant factors in promoting tumour growth and survival (Bornes et al. 2007, Braunstein et al. 2007, Conte et al. 2008, Lang et al. 2002, Schepens et al. 2005, Stein et al. 1998). Conversely, in X-linked dyskeratosis congenita, a disease characterized by elevated rates of apoptosis of their hematopoetic progenitor cells, ultimately resulting in bone marrow failure, and predisposition to cancer, IRES-mediated translation of XIAP, Bcl-xL and $p27^{kip1}$ is significantly impaired, while global translation rates remain entirely unaffected (Yoon et al. 2006). X-DC is caused by a mutation in the DKC1 gene, which encodes for dyskerin, a pseudouridine synthase known to modify ribosomal RNA. It has been hypothesized that the symptoms manifested by X-DC patients result from the inability to generate XIAP, Bcl-xL and $p27^{kip1}$ in a cap-independent manner, thus linking IRES-mediated translation and oncogenesis directly for the first time (Yoon et al. 2006). Additionally, this study provided the first evidence that modifications of the ribosome could be of crucial importance to IRES-mediated translation initiation events. It has recently been established that mutation of the DKC1 gene actually impairs the formation of the 48S pre-initiation complex mediated by the p27 IRES element, though it remains to be seen if this is the mechanism by which IRES-mediated translation of XIAP and Bcl-xL are impacted (Bellodi et al. 2010).

The concerted regulation of the VEGF, HIF1α and FGF-2 IRES elements during hypoxia, or XIAP, Bcl-xL and $p27^{kip1}$ IRES elements in X-DC patient cell, are just two

examples of a common mechanism whereby specific triggers can modify the expression of precise cohorts of proteins via their IRES-mediated translation.

1.5 S6K2 and Translational Regulation of XIAP and Bcl-xL

Over the past few years a series of studies by Pardo and colleagues have characterized a signalling cascade initiated by FGF-2 that contributes to the inherent chemoresistance displayed by small cell lung cancer (SCLC) cells, which is one of the foremost problems in the treatment of this disease (Pardo et al. 2001, 2002, 2003, 2006). The stimulation of SCLC cells with FGF-2 significantly increases their proliferative capacity and provides protection against etoposide-induced cell death. It was discovered that FGF-2 triggers the formation of a multiprotein complex involving B-Raf, PKCε (Protein Kinase C ε) and S6K2 (ribosomal protein S6 Kinase 2). Furthermore, this was the first time a specific role for S6K2 had been established, as S6K1 (ribosomal protein S6 Kinase 1) was neither incorporated into the multiprotein complex, nor could it substitute for S6K2. The most intriguing finding, however, is that FGF-2-mediated activation of S6K2 led to the specific translational upregulation of four anti-apoptotic proteins: XIAP, Bcl-2, Bcl-xL and c-IAP1. As in the case of hypoxia or X-DC where translation of specific transcripts is affected, the mutual upregulation of these four proteins is fascinating because they can all be generated as a result of IRES-mediated translation.

The IRES-mediated translation of both XIAP and Bcl-xL is affected similarly under two other scenarios – through the subcellular relocalization of hnRNP A1, which negatively

regulates IRES-mediated translation XIAP and Bcl-xL, and by a mutation in the DKC1 gene, which also suppresses their IRES-dependent translation. The XIAP IRES element has been thoroughly characterized in terms of the stresses and ITAFs that regulate its activity. For instance, in addition to hnRNP A1, the XIAP IRES is negatively regulated by PTB; conversely, binding of La autoantigen, hnRNP C1/C2, MDM2 or HuR (Hu antigen R) to the XIAP IRES enhances its activity (Baird et al. 2007, Durie et al. 2011, Gu et al. 2009, Holcik and Korneluk 2000, Holcik et al. 2003, Lewis et al. 2007). In fact, the binding of hnRNP C1/C2 to the XIAP IRES has been implicated in protection during ischemia and staurosporin induced cell death, while HuR-mediated cytoprotection is thought to result from its enhancement of IRES-driven XIAP expression (Durie et al. 2011, Spahn et al. 2008). Given its critical role in cell survival, it is not surprising that the XIAP IRES is activated in response to a number of other stresses, including gamma irradiation and serum deprivation, where the corresponding increase in XIAP protein expression provides a protective effect (Holcik et al. 1999, 2000, Riley et al. 2010). In contrast, relatively little is known about the specific regulation of the Bcl-xL IRES by ITAFs or stressors, other than what has been mentioned thus far. And while the secondary structure of the XIAP IRES has been determined (Baird et al. 2007), it has not been solved for the Bcl-xL IRES, therefore no inferences can be made from structural elements in their IRES sequences as to how or why they may be similarly regulated via these structures; though as mentioned prior, sequence and structural data for IRES elements are not necessarily reliable predictors of function or regulation. It is clear however, particularly from the study by Yoon and colleagues (Yoon et al. 2006), that the

IRES-mediated translation of both XIAP and Bcl-xL can be uniquely regulated simultaneously.

The deleterious effects of dysregulation of either XIAP or Bcl-xL expression has been covered briefly, but it should be emphasized that these two proteins are particularly important in cell survival and have been linked numerous times in cancer to degree of malignancy, progression of the disease and chemoresistance. For instance, XIAP overexpression is found in ovarian, breast, renal, prostate, gastric, colon and lung cancers, is a contributing factor in chemoresistance in lung cancer, prostate cancer, and is even elevated over time during disease progression, as in the case of melanomas and leukaemia (Berezovskaya et al. 2005, Danson et al. 2007, Dasgupta et al. 2006, Hofmann et al. 2002, Kluger et al. 2007, Krajewska et al. 2003, Ramp et al. 2004, Tamm et al. 2000, Wu et al. 2005, Yamamoto et al. 2004). On the other hand, Bcl-xL is found to be overexpressed in leukaemia and breast and ovarian cancer, for example, and plays a role in their chemoresistant phenotype, as well as that of malignant pleural mesothelioma (MPM) (Brotin et al. 2009, Konopleva et al. 2002, Krajewski et al. 1999, Varin et al. 2010, Villedieu et al. 2007, Williams et al. 2005).

It stands to reason therefore, that understanding the mechanisms governing the concurrent translational regulation of both XIAP and Bcl-xL, particularly via their respective IRES elements, will provide invaluable insight for the development of new therapeutics to target their expression. Therefore, my research focuses on a potential mechanism by which XIAP and Bcl-xL are simultaneously upregulated.

1.6 Hypothesis and Objectives

I hypothesize that in response to activation of S6K2, the expression of XIAP and Bcl-xL is elevated through a translational mechanism that enhances IRES-mediated translation initiation at their respective IRES elements. The objectives of my thesis are to establish that S6K2 signalling does in fact enhance IRES-mediated translation of both XIAP and Bcl-xL, and also to identify the downstream target of S6K2 that mediates this effect.

Chapter 2: Materials and Methods

2.1 Reporter Constructs

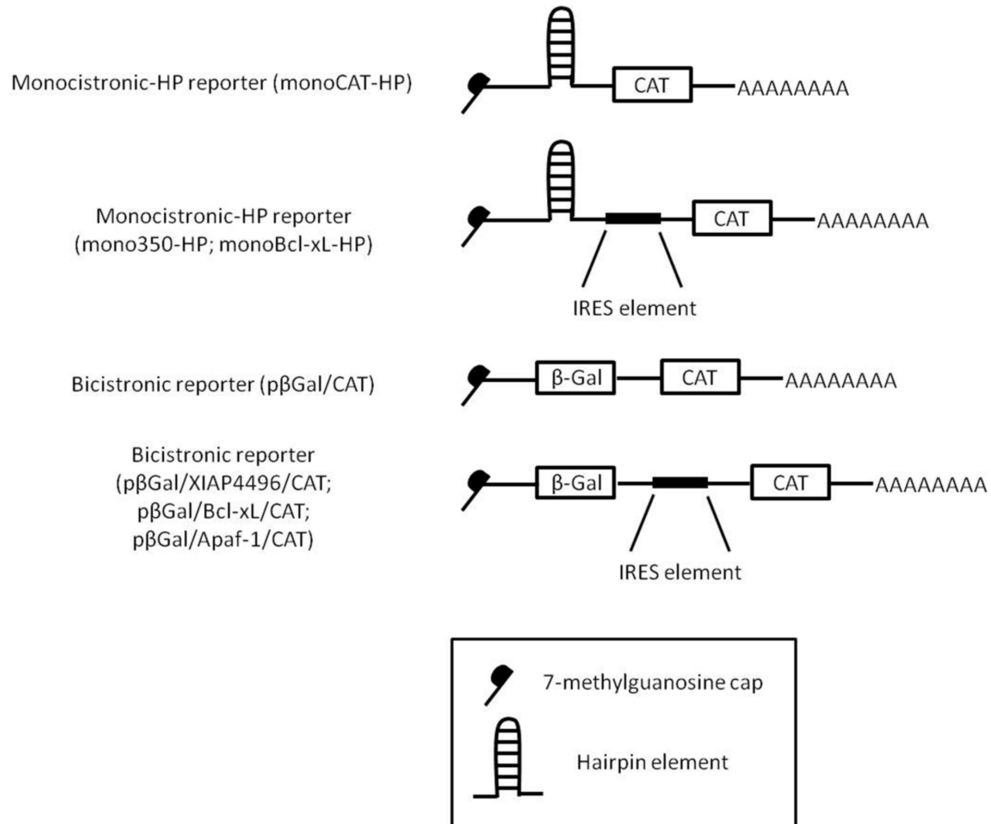
The bicistronic DNA reporter constructs pβgal/XIAP4496/CAT (containing the human XIAP IRES element), pβgal/Bcl-xL/CAT (containing the mouse Bcl-xL IRES element) and pβgal/Apaf-1/CAT (containing the human Apaf-1 IRES element) were previously described (Bevilacqua et al. 2010, Holcik et al. 1999, Nevins et al. 2003). The monocistronic hairpin constructs monoCAT-HP, mono350-HP (Riley et al. 2010) and monoBcl-xL-HP (kindly provided by Alura Riley) were also previously generated in the laboratory. A schematic representation of all bicistronic reporter mRNA or *in vitro* transcribed (IVT) monocistronic RNA is shown in Figure 4.

2.2 *In vitro* RNA Synthesis

DNA templates for the synthesis of reporter RNAs were generated from the corresponding 5'-UTR containing monocistronic hairpin constructs by polymerase chain reaction (PCR), using primers that have an incorporated T7 promoter sequence at the 5'-end to allow for RNA transcription. Reverse primers used in the PCR reactions include the 3'-end of the CAT gene as well as 31 T's, therefore providing the resultant PCR product with a polyT tail. All PCR products were purified by agarose gel electrophoresis and the UltraClean 15 DNA Purification kit (MO BIO Laboratories). IVT and capping were performed with the mMessage mMachine kit (Ambion). Newly synthesized RNA was purified using a

Figure 4. Representative schematic of reporter mRNAs

Representative schematic of bicistronic reporter mRNAs and *in* vitro transcribed monocistronic HP RNAs used to assess the relative IRES activity of the XIAP, Bcl-xL and Apaf-1 IRES elements. Legend: β-Gal - β-Galactosidase cistron; CAT – chloramphenicol acetyl transferase cistron.


Monocistronic-HP reporter (monoCAT-HP)
CAT
AAAAAAAA
Monocistronic-HP reporter
(mono350-HP; monoBcl-xL-HP)
CAT
AAAAAAAA
IRES element
Bicistronic reporter (pβGal/CAT)
β-Gal
CAT
AAAAAAAA
Bicistronic reporter
(pβGal/XIAP4496/CAT;
pβGal/Bcl-xL/CAT;
pβGal/Apaf-1/CAT)
β-Gal
CAT
AAAAAAAA
IRES element
7-methylguanosine cap
Hairpin element

Megaclear column (Ambion). Prior to transfection, a small volume of the purified RNAs were heated to 60°C and run on an RNase-free agarose gel in order to confirm that the RNAs were of correct size. The concentration of the isolated RNA was determined using the ND-1000 spectrophotometer (Thermo Scientific).

2.3 Cell Culture and Transfection Reagents

Tetracycline inducible kinase active S6K1 and S6K2 cells (TOKAS6K1 and TOKAS6K2 cells, respectively; kindly provided by Dr. Michael Seckl; Pardo et al. 2006) were maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) (Fisher) containing 1% tetracycline-free fetal calf serum (Fisher), 1% L-glutamine (Fisher), 100,000 U/L penicillin (Fisher) and 100 µg/L streptomycin (Fisher). Human embryonic kidney (HEK293) cells were maintained at 37°C, 5% CO_2 in DMEM containing 1% fetal calf serum (Diamed), 1% L-glutamine, 100,000 U/L penicillin and 100 µg/L streptomycin.

For experiments requiring the induction of either kinase active S6K1 or S6K2, cells were seeded 24 hours prior to addition of doxycycline (Sigma) to the media at 5×10^5 cells/2ml/well (6-well plates; Fisher). The cells were serum deprived for six hours prior to the addition of doxycycline to the media by removing the media from each well, gently washing the cells with warm phosphate buffered saline (PBS), and then adding 2 ml DMEM supplemented with only L-glutamine, penicillin and streptomycin. Following the serum deprivation, media in control wells was replaced with 2 ml tetracycline-free complete DMEM, while the media in wells to be used for S6K induction was replaced with 2 ml

tetracycline-free complete DMEM containing 1 μg/ml doxycycline; after 24 hours, media was removed and cells were harvested in either radioimmunoprecipitation assay (RIPA) buffer for protein content analysis or Stratagene Lysis Buffer for RNA extraction. For experiments done using MG132 (Sigma), tetracycline inducible S6K2 cells were seeded 24 hours prior to addition of media containing doxycycline, or doxycycline and MG132, at 5 x 10^5 cells/2ml/well (6-well plates). The cells were serum deprived for six hours as described above, after which the media was replaced with 2 ml tetracycline-free complete DMEM containing 1 μg/ml doxycycline or 2 ml tetracycline-free complete DMEM containing 1 μg/ml doxycycline and 2 μM MG132. After 18 hours, media was removed and cells were harvested in RIPA buffer for protein content analysis.

For DNA transfections of tetracycline inducible cells, cells were seeded 24 hours prior to transfection at 2.5 x 10^5 cells/2ml/well (6 well plates). Transient transfection of the cells with 2 μg plasmid DNA per well was performed using Lipofectamine 2000 (Invitrogen). Briefly, a master mix containing the appropriate volumes of OPTI-MEM and Lipofectamine 2000 reagent was prepared (12-well reactions: 100 μl OPTI-MEM + 5 μl Lipofectamine 2000 /well; 6-well reactions: 250 μl OPTI-MEM + 10 μl Lipofectamine/well) alongside tubes containing the appropriate volume of OPTI-MEM and IVT RNA or DNA to be transfected (12-well reactions: 100 μl OPTI-MEM + IVT RNA/well; 6-well reactions: 250 μl OPTI-MEM + DNA/well). Both mixes were incubated at room temperature before adding the appropriate volume of master mix to the DNA containing tubes (12-well reactions: 100 μl/tube; 6-well reactions: 250 μl/tube). These mixtures were then incubated at room temperature for 20 minutes. The media in each well to be transfected was then changed to contain fresh media

(12-well reactions: 1 ml/well; 6-well reactions: 2 ml/well). Following the 20 minute incubation, the correct volume of the Lipofectamine 2000/OPTI-MEM/DNA reaction was then added to the wells of cells to be transfected (12-well reactions: 200 µl/well; 6-well reactions: 500 µl/well). 18 hours following transfection, the cells were serum starved as described above for six hours, after which the media in the wells was replaced with either 2 ml tetracycline-free complete DMEM or 2 ml tetracycline-free complete DMEM containing 1 µg/ml doxycycline. Cells were harvested 24 hours later in CAT Lysis Buffer (Roche) for protein analysis.

For siRNA transfection of HEK293 cells, cells were seeded at 2 x 10^5 cells/2ml/well (6-well plates). 24 hours after seeding, cells were transfected with control siRNA (Qiagen) or with a PDCD4 targeted siRNA (Bitomsky et al. 2008; See Appendix for sequence information) using Lipofectamine RNAiMAX (Invitrogen). Briefly, a master mix containing 250 µl OPTI-MEM + 5 µl RNAiMAX per well was prepared alongside tubes containing 250 µl OPTI-MEM and the correct volume of siRNA per well. 250 µl of the master mix was then added to the OPTI-MEM/siRNA containing tubes. These mixtures were then incubated at room temperature for 20 minutes. The media in each well to be transfected was then changed to contain 2 ml fresh media. Following the 20 minute incubation, 500 µl of the RNAiMAX/OPTI-MEM/siRNA reaction was added to the wells of cells to be transfected. Cells were harvested 48 hours later in either RIPA buffer for protein content analysis or Stratagene Lysis Buffer for RNA extraction. For siRNA combined with DNA transfection, HEK293 cells were seeded, 24 hours prior to transfection with either control of PDCD4 targeted siRNA, at 2 x 10^5 cells/2ml/well (6 well plates). Cells were transiently transfected

with 1 µg plasmid DNA per well 24 hours following siRNA transfection using Lipofectamine 2000 (Invitrogen). Cells were harvested 24 hours later in CAT Lysis Buffer (Roche) for protein analysis. For siRNA combined with RNA transfection, HEK293 cells were seeded, 24 hours prior to transfection with IVT RNA, at 0.5 x 10^5 cells/1ml/well (12 well plates; Diamed). Cells were transiently transfected with 1 µg IVT RNA per well 44 hours following siRNA transfection using Lipofectamine 2000 (Invitrogen). Cells were harvested 4 hours later in CAT Lysis Buffer (Roche) for protein analysis.

2.4 β-Galactosidase and CAT Assays

To assess levels of β-galactosidase (β-gal) or chloramphenicol acetyl transferase (CAT), cells were washed with PBS, and then lysed for 20 minutes at room temperature in CAT lysis buffer (Roche), followed by centrifugation at 13 000 *g*, at 4°C, for 10 minutes in a bench top centrifuge (Heraeus) to remove cell debris. For cells transfected with IVT RNA, only the levels of CAT were determined; for cells transfected with bicistronic DNA constructs, the level of β-gal and CAT were determined in order to calculate relative IRES activity. The β-gal assay was performed in a 96-well plate (Diamed) in Z-buffer (60 mM Na_2PO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) using ONPG (4 mg/ml in 0.1 M NaP buffer, pH 7.5) as a colourimetric substrate (MacGregor et al. 1991). The reaction was stopped using 1M Na_2CO_3, and the absorbance was read at 420 nm, using a Spectramax 340PC plate reader (Molecular Devices). The CAT ELISA (Roche)

ELISA was performed and absorbances read as per the manufacturer's protocol, using a Spectramax 340PC plate reader.

2.5 RNA Extraction and Quantitative PCR

Total RNA was isolated from cells using the Absolutely RNA® Miniprep kit (Stratagene) and quantified using the ND-1000 spectrophotometer (Thermo Scientific). Reverse transcription was carried out using 1 µg of total RNA and an equal mixture of NotI-d(T)18 and PdN6 primers and the First-Strand cDNA Synthesis kit (GE Biosciences). The quantitative polymerase chain reaction (PCR) was performed using PerfeCTa SYBR Green FastMix (Quanta Biosciences) along with gene specific primers for XIAP (QuantiTect Primer Assay, Qiagen), XIAP 323 (shorter 5'UTR), XIAP 4496 (longer 5' UTR), Bcl-xL (QuantiTect Primer Assay – Qiagen), PDCD4 (QuantiTect Primer Assay – Qiagen), Apaf-1, and GAPDH (see Appendix for primer sequence information) and analyzed on a Mastercycler® ep *realplex* (Eppendorf) using the associated realplex software. The following cycling conditions were used: 95°C x 20 seconds, 95°C x 3 seconds-55°C x 15 seconds-72°C x 10 seconds – x45 cycles.

2.6 Western Blot Analysis

To assess protein levels, cells were washed with PBS, and then lysed for 20 minutes at room temperature in RIPA buffer (50 mM Tris-Cl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1%

NP-40, 0.5% deoxycholic acid, 0.05% SDS) containing protease inhibitors (1 μM leupeptin, 1 mM PMSF; Sigma) and phosphatase inhibitors (5 μM β-glycerol phosphate, 50 mM NaF; Sigma), followed by centrifugation at 13 000 *g*, at 4°C, for 10 minutes in a bench top centrifuge (Heraeus) to remove cell debris. The supernatant was then collected and the protein concentration determined using the BCA (bicinchoninic acid) Protein Assay Kit (Thermo Scientific Pierce), as per the manufacturer's protocol. Equal amounts of protein were loaded on a 10% polyacrylamide gel and resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes using a semi-dry transfer protocol. Membranes were probed with antibodies to mouse anti-rpS6 (1:1000 in 5% milk in TBST; Cell Signaling Technology), rabbit anti-phospho-rpS6 (Ser240/244) (1:2000 in 5% BSA in TBST; Cell Signaling Technology), rabbit anti-Bcl-xL (1:1000 in 5% BSA in TBST; Cell Signaling Technology), XIAP [rabbit anti-GST-XIAP (1:2000 in PBST; Li et al. 2001) or rabbit anti-RIAP3 (1:5000 in PBST; Holcik et al. 2002)], rat anti-Apaf-1 (1:2000 in PBST; Chemicon), mouse anti-Nucleolin (1:1000 in 1% milk in PBST; C23; Santa Cruz Biotechnology, Inc.), or rabbit anti-PDCD4 (1:5000 in PBST; Rockland), and subsequently incubated with species-specific (rabbit or mouse) horseradish peroxidise-conjugated secondary antibodies (Cell Signaling Technology) or species specific Alexa-Fluor antibodies (Alexa-Fluor 680 goat anti-rat; LI-COR Biosciences). Antibody complexes were detected using either ECL Western Blotting Substrate (Pierce) or ECL Plus substrate (GE Biosciences) along with Hyperfilm (GE Healthcare) or Odyssey® Infrared Imaging System (LI-COR Biosciences), and were quantified using Odyssey densitometry software (LI-COR Biosciences).

2.7 Polysome Profiling

HEK293 cells from three 15 cm plates (Fisher) per condition were lysed for 30 minutes in cold polysome lysis buffer (15 mM Tris-HCl, pH 7.4, 15 mM MgCl2, 300 mM NaCl, 1% Triton X-100, 0.1 mg/ml cyclohexamide, 100 U/ml RNasin). Nuclei were pelleted by centrifugation at 5000 rpm, after which the supernatant was centrifuged at 13000 rpm in order to remove cell debris. Equal OD254 units (up to 800 µl) were loaded onto 10-50% linear sucrose gradients, while saving 200 µl for total RNA analysis, and centrifuged for 90 minutes at 39 000 r.p.m. at 4°C in a SW41Ti rotor in an Optima L-100 xP ultracentrifuge (Beckman Coulter). Gradients were then fractionated from the top using an Auto Densi-Flow (Labconco) and RNA/protein was monitored at 254 nm using a HPLC system (Åkta Explorer, GE Biosciences), with UV900 and P900 modules (Amersham Pharmacia Biotech) and the associated software (Unicorn 4.11; Amersham). Fractionation was done in 1 ml fractions and flash frozen in dry ice, and stored at -80°C. RNA was isolated from individual fractions by digesting each fraction with proteinase K, followed by phenol/chloroform extraction and recovery by ethanol precipitation. Equal volumes of RNA from each fraction were used to generate cDNA using reverse transcription, which was then used for qPCR, as previously described.

2.8 Statistical Analysis

All data are expressed as mean ± standard deviation (SD). Unless otherwise stated, all results reported were obtained through a minimum of three independent experimental

replications. Unless otherwise stated, for reporter assays, independent replicates consist of three biological triplicates. Unpaired t-tests were used to determine data significance.

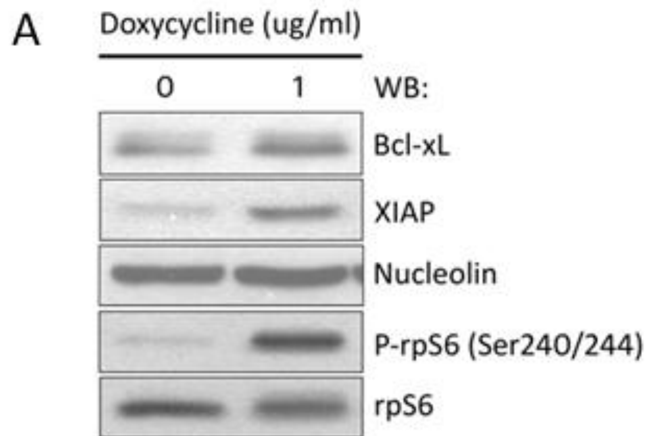
Chapter 3: Results

3.1 Activation of S6K2, but not S6K1, induces the translational upregulation of XIAP and Bcl-xL expression in a cap-independent manner.

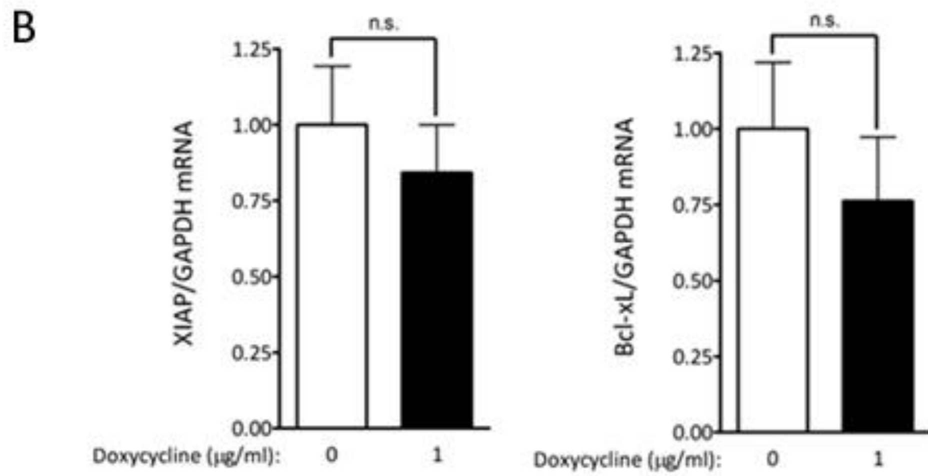
It has been previously determined that inducing the expression of a constitutively active form of S6K2, but not S6K1, in a tetracycline inducible cell line (TOKAS6K1 cells/TOKAS6K2 cells) will lead to the translational upregulation of both XIAP and and Bcl-xL (Pardo et al. 2006). However, the molecular mechanism of this upregulation has not been elucidated. Therefore, in order to examine the effect that S6K2 imparts on the XIAP and Bcl-xL IRES in this cell line, I first wished to verify that upon induction of kinase active S6K2 expression there was an increase in protein expression of XIAP and Bcl-xL, but that there was no impact at the transcriptional level. Western blot analysis showed that upon treating the TOKAS6K2 cells with media containing 1 µg/ml doxycycline for 24 hours, the expression of XIAP and Bcl-xL was elevated (Figure 5A), which is in agreement with previous studies (Pardo et al. 2006). The levels of phosphorylated rpS6 and total rpS6 were also examined by Western blot analysis, as the phosphorylation of rpS6 should be increased in the presence of activated S6K2. It can be seen in the representative Western blot (Figure 5A) that upon treating the cells with media containing 1 µg/ml doxycycline there is a notable increase in level of phosphorylated rpS6, therefore confirming that the overexpression of S6K2 was successful. RNA was then isolated from TOKAS6K2 cells treated with media containing 0 or 1 µg/ml doxycycline for 24 hours and used for quantitative RT-PCR. The levels of XIAP and Bcl-xL mRNA, relative to the levels of GAPDH mRNA, are not significantly altered when

Figure 5. Activation of S6K2, but not SK1, induces the translational upregulation of XIAP and Bcl-xL expression in a cap-independent manner. (A) Western blot analysis of endogenous XIAP and Bcl-xL protein levels in cell lysates from TOKAS6K2 cells treated with media containing 0 or 1 μg/mL doxycycline for 24 hours. Induction of expression of kinase-active S6K2 was confirmed by Western blot analysis for phosphorylated ribosomal protein S6 (Ser240/244). The membrane was reprobed with an antibody against nucleolin as a loading control. (B) Quantitative RT-PCR analysis of XIAP and Bcl-xL mRNA levels relative to GAPDH in TOKAS6K2 cells treated with 0 or 1 μg/mL doxycycline for 24 hours. Results were obtained using the ΔΔct method. Statistical significance was assessed using unpaired t-tests (non-significant [n.s.], $p>0.05$). Data obtained from cells treated with 0 μg/mL doxycycline were set to 1. (C) TOKAS6K1 or TOKAS6K2 cells were transiently transfected using Lipofectamine 2000 with 2 μg bicistronic construct DNA containing the XIAP or Bcl-xL IRES. 24 hours following transfection the cells were treated with media containing 0 or 1 μg/mL doxycycline for another 24 hours. The effect of over-expressing kinase-active S6K1 or S6K2 on IRES-mediated translation was determined by measuring the expression of β-gal and CAT, where relative IRES activity is the ratio of CAT/βgal. The activity of each construct in the absence of doxycycline was set as 1. Data is represented as mean ± SD. Statistical significance was assessed using unpaired t-tests (*, $p<0.05$).

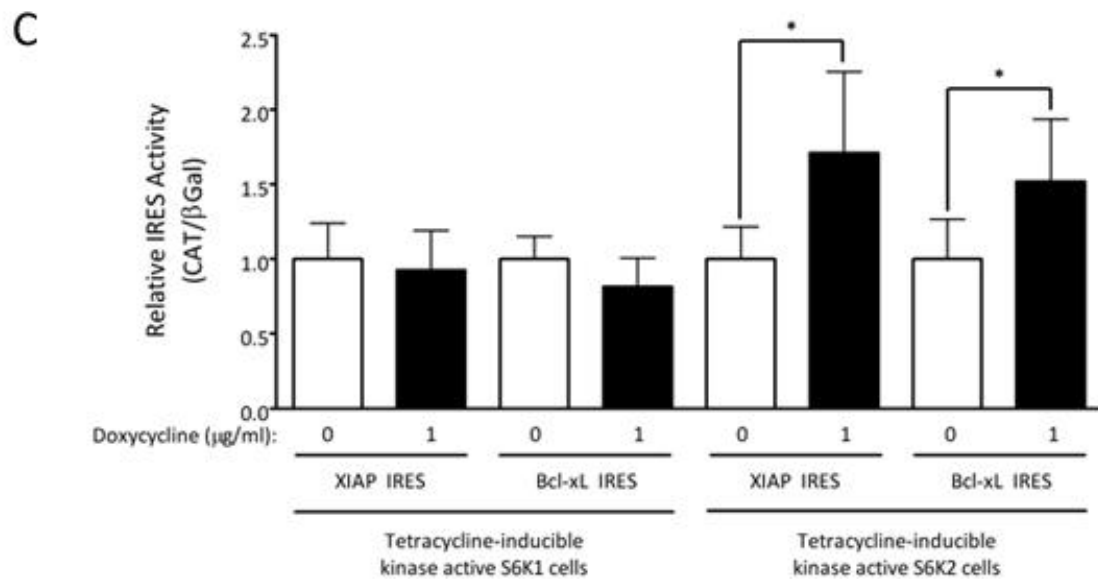
A



B



C



activated S6K2 is overexpressed (Figure 5B), therefore reaffirming that the induction of XIAP and Bcl-xL protein expression is due to post-transcriptional upregulation, and not a result of transcriptional activation or enhanced transcript stability.

I next sought to determine if the activation of S6K2, but not S6K1, would affect the relative IRES activity of both the XIAP and Bcl-xL IRES elements. In order to assess the effect these kinases may have on these IRES elements, TOKAS6K1 and TOKAS6K2 cells were transiently transfected with 2 μg plasmid DNA encoding bicistronic reporter constructs containing the XIAP IRES or the Bcl-xL IRES sequence. 18 hours later cells were serum deprived as outlined in the Materials and Methods for six hours, and then treated with media containing 0 or 1 μg/ml doxycycline for another 24 hours. Untransfected cells were also included as a control, and the level of phosphorylated and total rpS6 were assessed by Western blot to ensure that there was efficient induction of the activated kinases in cells treated with doxycycline. Cells were then lysed and the levels of β-gal and CAT were assessed. The relative activity of both the XIAP and Bcl-xL IRES in the presence of kinase active S6K1 were unchanged, yet the relative IRES activity of both IRESs were significantly enhanced in the presence of kinase active S6K2 (Figure 5C). This data suggests that signalling through S6K2, but not S6K1, leads to enhanced IRES-mediated translation of XIAP and Bcl-xL.

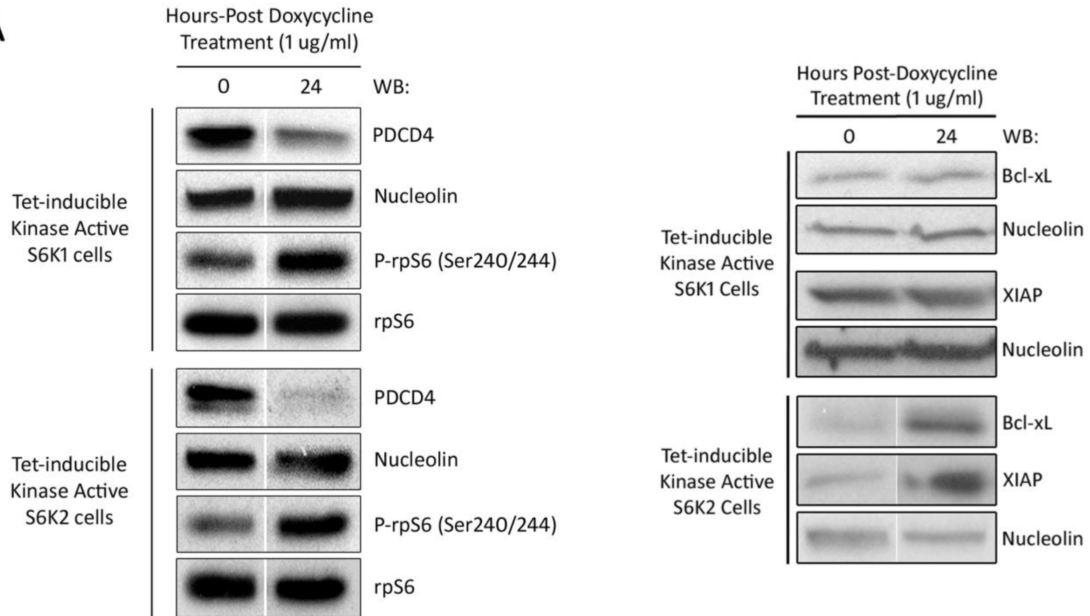
3.2 Kinase-active S6K2 induces the proteasomal degradation of PDCD4.

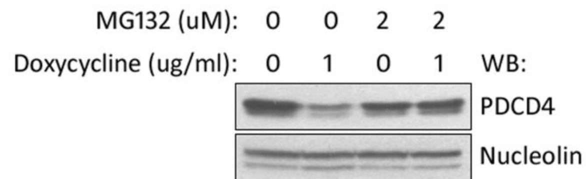
A few target substrates of S6K1 phosphorylation have been characterized thoroughly, and it is presumed that S6K2 will also phosphorylate these target substrates in the same manner, though a majority of the studies have focused on primarily dissecting the role of S6K1. Of all the target substrates of the S6Ks, the likeliest mediator of S6K signalling that would impact IRES-mediated translation would be a protein with a role in translation initiation. S6K signalling is known to result in the phosphorylation of ribosomal protein S6 (rpS6), eIF4B and programmed cell death gene 4 (PDCD4) (Dorrello et al. 2006, Krieg et al. 1988, Raught et al. 2004, Shima et al. 1998). The work here will focus on defining the potential role of PDCD4 as the primary effector molecule in this signalling pathway. PDCD4 is known to negatively impact translation as it binds to eIF4A and eIF4G; when eIF4A is bound to PDCD4 it is sequestered in an inactive conformation and is unable to be incorporated into the eukaryotic initiation factor 4F (eIF4F) complex (Göke et al. 2002, Loh et al. 2009, Yang et al. 2003). It has been demonstrated that S6K1-mediated phosphorylation of PDCD4 at Serine 67 ultimately results in the proteasomal degradation of PDCD4, however, this has yet to be established for S6K2 (Dorrello et al. 2006). Given that there is no commercially available antibody targeted against Serine 67 of PDCD4 specifically, the level of total PDCD4 was examined in the TOKAS6K1 and TOKAS6K2 cells in the presence and absence of doxycycline in order to determine if the activation of S6K2 is sufficient to induce the degradation of PDCD4, and to what degree, as compared to the degradation elicited upon expression of kinase active S6K1. To do so, the tetracycline inducible cells were serum starved as described in the Materials and Methods, at which

point half the cells were washed with PBS, pelleted and stored at -20°C. The cell lysates from these cells represent the 0 hour timepoint to which the 24 hour timepoint cell lysates will be compared. The other cells were then treated with media containing 1 μg/ml doxycycline for 24 hours. The cells from the 0 and 24 hour timepoints were lysed with RIPA buffer and the protein expression of PDCD4 and nucleolin were assessed by Western blot (Figure 6A). As a control to ensure that there was efficient induction of the activated kinases in cells treated with doxycycline, the levels of total rpS6 and phosphorylated rpS6 were also evaluated by Western blot analysis. As shown by the Western blots in Figure 6A, not only does activation of S6K2 induce the degradation of PDCD4, but the degree of degradation seen at 24 hours in the S6K2 inducible cell line is much greater than that seen in the S6K1 inducible cell line. Additionally, Western blot analysis shows that XIAP and Bcl-xL protein expression are both upregulated in response to the activation of S6K2, but not S6K1 (Figure 6A).

Prior studies have shown that S6K1 phosphorylation mediates the proteasomal degradation of PDCD4. Therefore, to determine if the downregulation of PDCD4 that is elicited by the activation of S6K2 is also dependent on proteasomal degradation, tetracycline inducible S6K2 cells were serum starved as described in the Materials and Methods, then treated with media containing 0 or 1 μg/ml doxycycline for 18 hours, or treated with media containing 0 or 1 μg/ml doxycycline in the presence of 2 mM MG132 for 18 hours. After cells were lysed in RIPA buffer, levels of PDCD4 and nucleolin were assessed by Western blot analysis (Figure 6B). As seen in Figure 6B, the inclusion of MG132 in the media blocked the previously observed degradation of PDCD4 protein expression, therefore

Figure 6. Kinase-active S6K2 induces the proteasomal degradation of PDCD4. (A) Western blot analysis of endogenous PDCD4, Bcl-xL and XIAP protein levels in cell lysates from TOKAS6K1 and TOKAS6K2cells treated with 1 µg/mL doxycycline for the time indicated. Induction of expression of kinase-active S6K1 or S6K2 was confirmed by Western blot analysis for phosphorylated ribosomal protein S6 (Ser240/244). The membrane was reprobed with an antibody against nucleolin as a loading control. (B) Western blot analysis of endogenous PDCD4 protein levels in cell lysates from TOKAS6K2cells treated with 0 or 1 µg/mL doxycycline in the presence or absence of the proteasome inhibitor MG132 for 18 hours. The membrane was reprobed with an antibody against nucleolin as a loading control.


A
Hours-Post Doxycycline
Treatment (1 ug/ml)
0
24
WB:
Tet-inducible
Kinase Active
S6K1 cells
PDCD4
Nucleolin
P-rpS6 (Ser240/244)
rpS6
Tet-inducible
Kinase Active
S6K2 cells
PDCD4
Nucleolin
P-rpS6 (Ser240/244)
rpS6
Hours Post-Doxycycline
Treatment (1 ug/ml)
0
24
WB:
Tet-inducible
Kinase Active
S6K1 Cells
Bcl-xL
Nucleolin
XIAP
Nucleolin
Tet-inducible
Kinase Active
S6K2 Cells
Bcl-xL
XIAP
Nucleolin


B
MG132 (uM): 0 0 2 2
Doxycycline (ug/ml): 0 1 0 1
WB:
PDCD4
Nucleolin

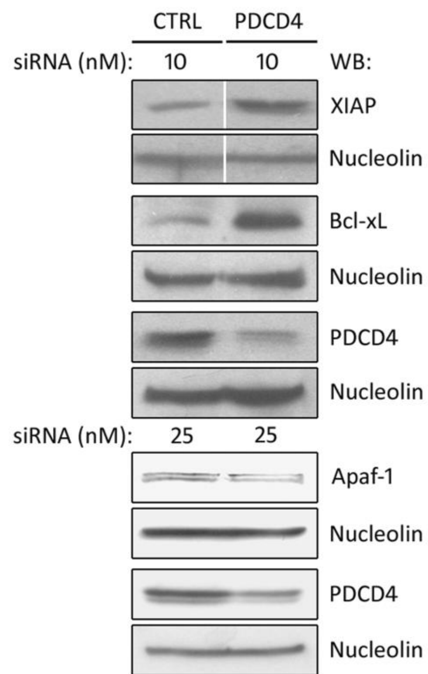
it can be concluded that S6K2 activation induces proteasome-mediated degradation of PDCD4.

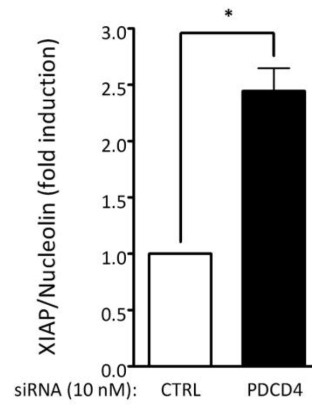
3.3 PDCD4 levels are sufficient to regulate the expression of XIAP, Bcl-xL and Apaf-1.

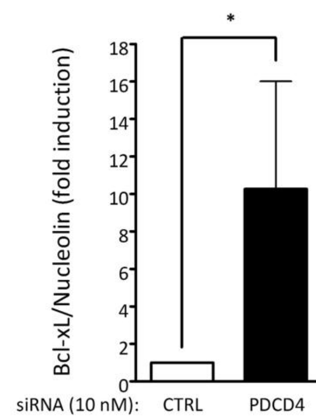
I next wanted to know if reducing the expression of PDCD4 specifically was sufficient to elevate XIAP and Bcl-xL protein levels. Therefore, HEK293 cells were transiently transfected using RNAiMAX with 10 nM non-targeting siRNA (CTRL) or siRNA targeting PDCD4 for 48 hours, after which the cells were lysed in RIPA buffer. The levels of PDCD4, XIAP, Bcl-xL and nucleolin were then assessed by Western blot analysis. Upon knockdown of PDCD4 it was evident that the expression of both XIAP and Bcl-xL was significantly enhanced, in agreement with my previous data (Figure 7, upper panel).

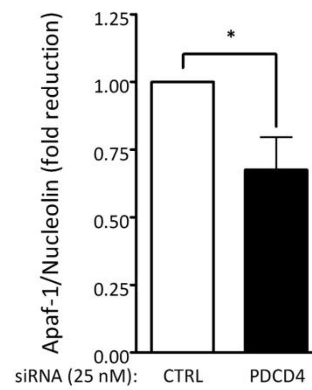
PDCD4 was first identified as a protein whose expression was elevated during apoptosis (Shibahara et al. 1995), and therefore the inverse correlation between the expression of PDCD4 and the expression of two critical antiapoptotic proteins could be expected. Interestingly, Apaf-1 is a key pro-apoptotic protein whose expression is controlled by an IRES element as well, therefore I wanted to determine if Apaf-1 protein expression is also regulated by PDCD4. To investigate this possibility, HEK293 cells were transiently transfected as described above using 25 nM siRNA, after which the cells were lysed in RIPA buffer and the expression of PDCD4, Apaf-1 and nucleolin assessed using Western blot analysis (Figure 7, lower panel). Upon the knockdown of PDCD4, the expression of Apaf-1 was also reduced.

Figure 7. **PDCD4 levels are sufficient to regulate the expression of XIAP, Bcl-xL and Apaf-1.** Western blot analysis of endogenous XIAP, Bcl-xL and Apaf-1 protein levels in cell lysates from HEK293 cells transfected with non-targeting siRNA (CTRL) or siRNA targeting PDCD4 for 48 hours. The membrane was reprobed with an antibody against nucleolin as a loading control. Graphs representing the fold change of each protein relative to nucleolin, as assessed using Odyssey densitometry software (LI-COR), from a minimum of three experiments are shown on the right. Densitometry values from siPDCD4 samples are represented as mean ± SD, relative to densitometry values from non-targeting siRNA samples, which were set to 1. Statistical significance was assessed using unpaired t-tests (*, $p<0.05$).


CTRL
PDCD4
siRNA (nM):
10
10
WB:
XIAP
Nucleolin
Bcl-xL
Nucleolin
PDCD4
Nucleolin
siRNA (nM):
25
25
Apaf-1
Nucleolin
PDCD4
Nucleolin


XIAP/Nucleolin (fold induction)
*
3.0
2.5
2.0
1.5
1.0
0.5
0.0
siRNA (10 nM):
CTRL
PDCD4

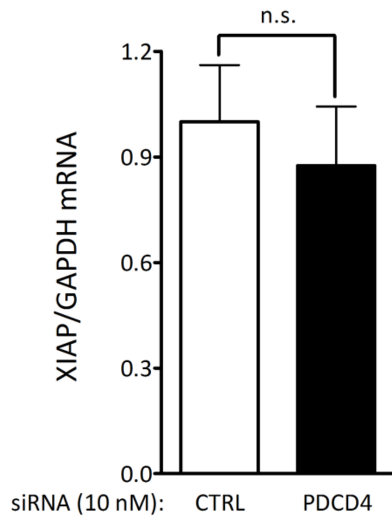

Bcl-xL/Nucleolin (fold induction)
*
18
16
14
12
10
8
6
4
2
0
siRNA (10 nM):
CTRL
PDCD4

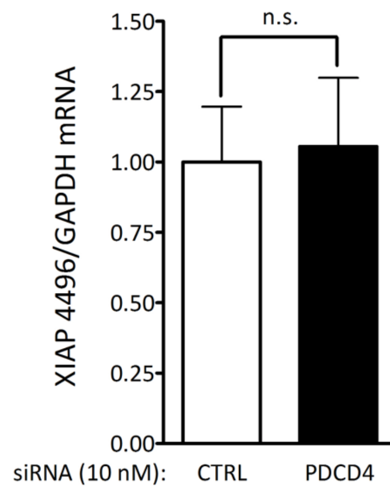

Apaf-1/Nucleolin (fold reduction)
*
1.25
1.00
0.75
0.50
0.25
0.00
siRNA (25 nM):
CTRL
PDCD4

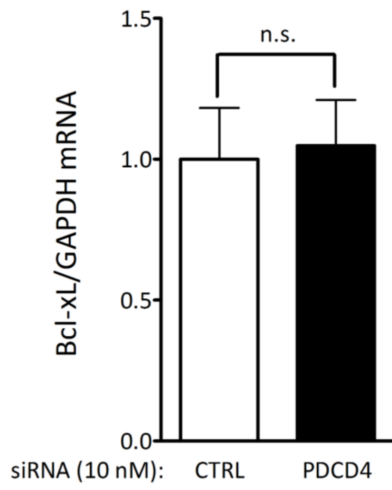
3.4 PDCD4 expression does not impact the steady state levels of XIAP, Bcl-xL and Apaf-1 mRNAs

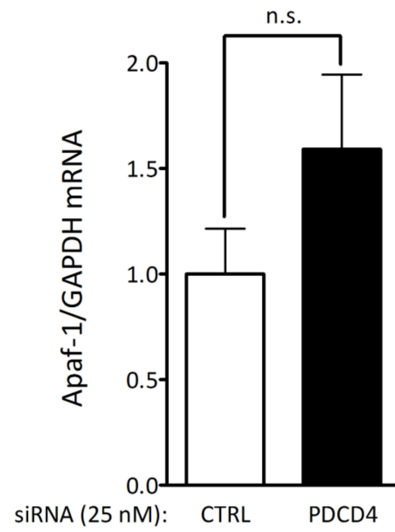
PDCD4 is also known to act as a transcriptional repressor (Bitomsky et al. 2004, Wang et al. 2008, Yang et al. 2001, 2006). Therefore to determine if the observed effects of PDCD4 knockdown on the expression of XIAP, Bcl-xL and Apaf-1 are not due to a change in the steady state levels of their respective mRNAs, HEK293 cells were transiently transfected with siRNA as described above for 48 hours. The RNA was then isolated from these cells and used for quantitative RT-PCR in order to determine the level of XIAP, Bcl-xL, Apaf-1 mRNA relative to the level of GAPDH mRNA in each sample. XIAP is known to be encoded by two distinct mRNAs that differ only in their 5' UTRs, which dictate the mode of XIAP mRNA translation. The shorter 5’ UTR (XIAP323) is responsible for cap-dependent, while the longer 5’ UTR (XIAP4496) drives the IRES-dependent translation of XIAP under distinct physiological conditions (Riley et al. 2010). Given that there are two XIAP transcripts, quantitative RT-PCR was also performed using primers specific for the XIAP transcript containing the longer, IRES containing 5' UTR (XIAP4496). Upon PDCD4 knockdown the levels of neither total nor IRES containing XIAP transcript, or the Bcl-xL and Apaf-1 transcripts were changed relative to GAPDH (Figure 8). From these data it can be concluded that PDCD4 does not affect the steady state levels of the XIAP, Bcl-xL or Apaf-1 mRNA transcripts.

Figure 8. PDCD4 expression does not impact the steady state levels of XIAP, Bcl-xL or Apaf-1 mRNAs. HEK293 cells were transiently transfected with non-targeting siRNA (CTRL) or siRNA targeting PDCD4 for 48 hours. The RNA from these cells was extracted and quantitative RT-PCR was performed to determine the levels of XIAP, Bcl-xL or Apaf-1 mRNA, relative to GAPDH. Results were obtained using the ΔΔct method. Data is represented as mean ± SD. Statistical significance was assessed using unpaired t-tests (non-significant [n.s.], $p>0.05$). Data obtained from cells transfected with non-targeting siRNA were set to 1.


n.s.
1.2
0.9
0.6
0.3
0.0
XIAP/GAPDH mRNA
siRNA (10 nM):
CTRL
PDCD4


n.s.
1.50
1.25
1.00
0.75
0.50
0.25
0.00
XIAP 4496/GAPDH mRNA
siRNA (10 nM):
CTRL
PDCD4


n.s.
1.5
1.0
0.5
0.0
Bcl-xL/GAPDH mRNA
siRNA (10 nM):
CTRL
PDCD4

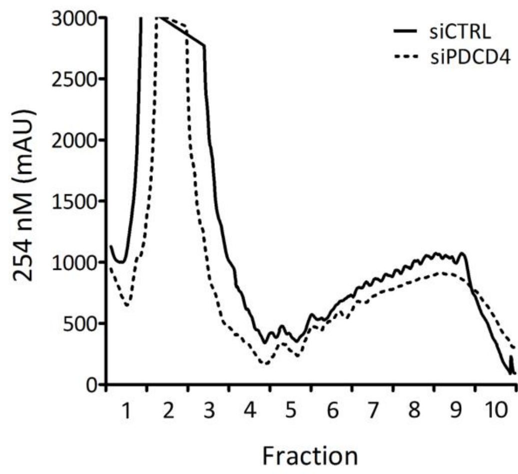

n.s.
2.0
1.5
1.0
0.5
0.0
Apaf-1/GAPDH mRNA
siRNA (25 nM):
CTRL
PDCD4

3.5 PDCD4 regulates the translation of XIAP, Bcl-xL and Apaf-1 mRNAs.

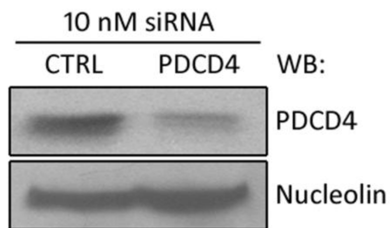
To determine if PDCD4 is specifically regulating the translation of XIAP, Bcl-xL and Apaf-1, HEK293 cells were transiently transfecting using RNAiMAX with 10 nM non-targeting siRNA (CTRL) or PDCD4-specific siRNA, and the ribosomal recruitment of XIAP, Bcl-xL and Apaf-1 mRNA investigated using a polysome profiling (Figure 9). Following a 48 hour knockdown of PDCD4, mRNA-ribosome complexes were loaded on a sucrose gradient column, after which the ribosome bound fractions were separated by ultracentrifugation. All fractions were then collected and the RNA extracted to be used for quantitative PCR. Transcripts in the high polysomal fractions are associated with a greater number of ribosomes than transcripts found in the monosome and low polysome fractions, and are therefore deemed to be undergoing a greater degree of translation. Cells were also transiently transfected with siRNA in parallel and used for Western blot analysis to analyze the expression of PDCD4 and nucleolin, to ensure that there was sufficient knockdown of PDCD4 expression.

As mentioned, XIAP is known to be encoded by two distinct mRNAs, I therefore performed quantitative RT-PCR analysis of the polysome fractions for the two distinct XIAP transcripts and found that upon knockdown of PDCD4 expression, there was no significant change in the distribution of the XIAP transcript with the shorter 5'-UTR (Figure 9), whereas the XIAP transcript with the longer 5'-UTR was recruited into the heavier polysomes (Figure 9). Furthermore, consistent with PDCD4 having a role in repressing the translation of Bcl-xL, I found that Bcl-xL mRNA is recruited into the heavier polysomes relative to GAPDH mRNA when PDCD4 expression is reduced (Figure 9). In addition, in concordance with previous

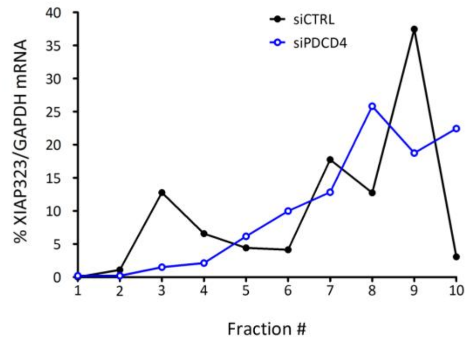
Figure 9. PDCD4 regulates the translation of XIAP, Bcl-xL and Apaf-1 mRNAs.
Representative polysome profiles from HEK293T cells transfected with non-targeting siRNA (CTRL) or siRNA targeting PDCD4 for 48 hours were generated, and the levels of XIAP, Bcl-xL and Apaf-1 mRNAs associated with the polysomes were determined using a quantitative RT-PCR approach. Results were obtained using the ΔΔct method. The ratio of XIAP, Bcl-xL or Apaf-1 mRNA relative to GAPDH mRNA for each fraction is represented as a percentage of entire polysome profile, where the sum of all fractions is equal to 100. A Western blot image is shown to demonstrate the level of PDCD4 knockdown in this experiment. The membrane was reprobed with an antibody against nucleolin as a loading control.



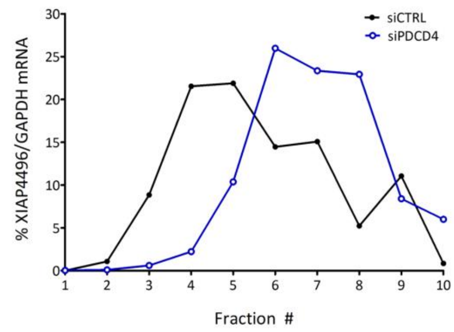
3000
2500
2000
1500
1000
500
0
254 nM (mAU)
siCTRL
siPDCD4
1 2 3 4 5 6 7 8 9 10
Fraction



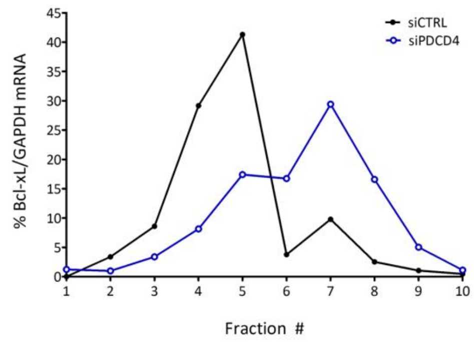
10 nM siRNA
CTRL
PDCD4
WB:
PDCD4
Nucleolin



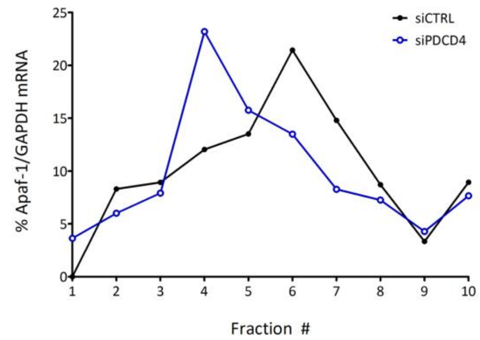
% XIAP323/GAPDH mRNA
siCTRL
siPDCD4
Fraction #



% XIAP4496/GAPDH mRNA
siCTRL
siPDCD4
Fraction #



% Bcl-xL/GAPDH mRNA
siCTRL
siPDCD4
Fraction #



% Apaf-1/GAPDH mRNA
siCTRL
siPDCD4
Fraction #

data indicating that loss of PDCD4 represses Apaf-1 protein expression, the Apaf-1 mRNA shifted out of the heavy polysome fractions when PDCD4 expression was reduced (Figure 9).

These data provide evidence that PDCD4 specifically negatively regulates the translation of XIAP and Bcl-xL, but positively regulates the translation of Apaf-1. Moreover, the distinct regulation of the two XIAP transcripts would suggest that PDCD4 normally functions to inhibit the IRES-mediated translation of XIAP, and not cap-dependent translation. Further, the overall polysome profile is not significantly altered upon knockdown of PDCD4, which would indicate that PDCD4 does not play an essential role in global translation inhibition. Given that translation of Bcl-xL and Apaf-1 is also regulated by an IRES element in their 5'-UTRs, my data identifies PDCD4 as a potential specific regulator of IRES-mediated translation.

3.6 PDCD4 regulates the cap-independent translation of XIAP, Bcl-xL and Apaf-1 mRNAs.

The observation that loss of PDCD4 expression enhances expression of XIAP and Bcl-xL protein levels and increases recruitment of the Bcl-xL and IRES-containing XIAP mRNAs to the heavy polyribosomes, suggest that loss of PDCD4 allows for these transcripts to be preferentially translated, possibly in a cap-independent manner. It was also observed that the loss of PDCD4 expression decreases expression of Apaf-1 and causes the Apaf-1 mRNA to shift out of the heavy polyribosomes, suggesting that opposite to its effect on XIAP and Bcl-xL, PDCD4 may normally function to enhance IRES-mediated translation of Apaf-1 and

that the loss of PDCD4 decreases the functionality of the Apaf-1 IRES in initiating cap-independent translation.

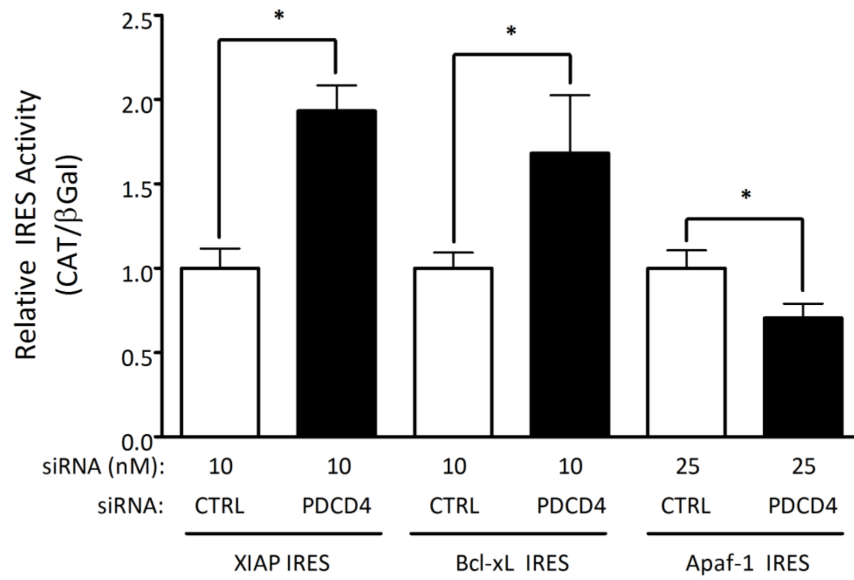
To investigate this hypothesis I transiently transfected HEK293 cells using RNAiMAX with non-targeting siRNA (CTRL) or PDCD4-specific siRNA for 24 hours, after which the cells were transiently transfected using Lipofectamine 2000 with 1 μg of pβgal/XIAP4496/CAT, pβgal/Bcl-xL/CAT or pβgal/Apaf-1/CAT bicistronic reporter constructs (see Figure 4) for 24 hours. Cells were then lysed and the levels of β-gal and CAT were assessed. I found that reducing the expression of PDCD4 significantly enhanced the relative IRES activity of both XIAP and Bcl-xL, indicating that PDCD4 inhibits IRES-mediates translation of XIAP and Bcl-xL (Figure 10A). However, unlike the XIAP and Bcl-xL IRES elements, I found that the relative IRES activity of Apaf-1 was significantly reduced in the absence of PDCD4, suggesting that PDCD4 positively regulates the Apaf-1 IRES (Figure 10A).

To confirm the results obtained using the bicistronic reporter constructs we transiently transfected HEK293 cells using RNAiMAX with 10 nM non-targeting siRNA (CTRL) or PDCD4-specific siRNA for 44 hours, after which the cells were transfected with 1 μg translationally competent *in vitro* transcribed RNA (monoCAT-HP, monoXIAP4496-HP, monoBcl-xL-HP; see Figure 4) for 4 hours, after which cells were then lysed and the levels of CAT and total protein were assessed. All *in vitro* transcribed RNAs contained a nucleotide cap, were polyadenylated, and contained a hairpin structure to block cap-dependent translation. The insertion of an IRES element downstream of the hairpin, but upstream of the CAT cistron allows the measuring of relative IRES activity by normalizing the level of CAT

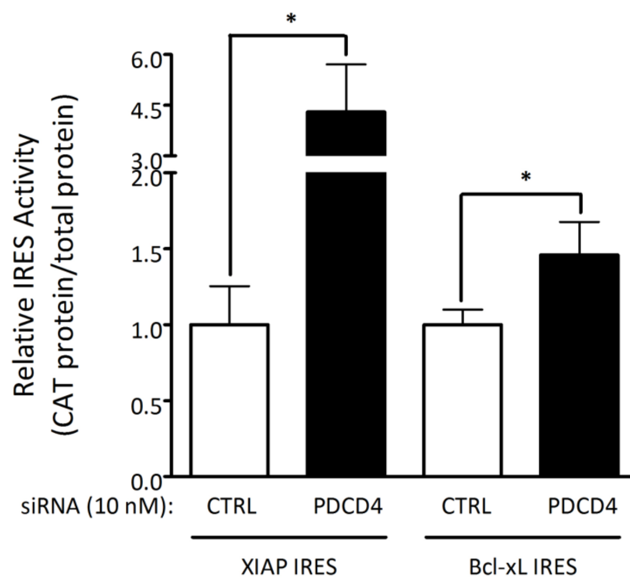
Figure 10. PDCD4 regulates the cap-independent translation of XIAP, Bcl-xL and Apaf-1 mRNAs.

(A) HEK293 cells were transiently transfected with 10 or 25 nM non-targeting siRNA (CTRL) or siRNA targeting PDCD4 for 24 hours, then transfected using Lipofectamine 2000 with 1 µg plasmid DNA encoding the bicistronic constructs containing the XIAP, Bcl-xL or Apaf-1 IRES for 24 hours. The effect of knocking down PDCD4 on IRES-mediated translation was determined by measuring the β-gal levels relative to CAT protein levels. The activity of each construct in cells transfected with non-targeting siRNA was set as 1. Data is represented as mean ± SD. Statistical significance was assessed using unpaired t-tests (*, $p<0.05$). (B) HEK293 cells were transiently transfected with 10 nM non-targeting siRNA (CTRL) or 10 nM siRNA targeting PDCD4 for 44 hours, then transfected using Lipofectamine 2000 with 1 µg of *in vitro* transcribed (IVT), capped and polyadenylated RNA transcripts encoding the monocistronic hairpin constructs containing the XIAP or Bcl-xL IRES for 4 hours. The effect of knocking down PDCD4 on IRES-mediated translation was determined by measuring the CAT protein levels relative to total protein levels. The activity of each construct in cells transfected with non-targeting siRNA was set as 1. Data is represented as mean ± SD. Statistical significance was assessed using unpaired t-tests (*, $p<0.05$).

A



B



protein to total protein. It was found that when the expression of PDCD4 was knocked down the relative IRES-activity of XIAP and Bcl-xL was significantly enhanced (Figure 10B), thus replicating the results obtained with the DNA constructs. It should be noted that for all experiments done using the bicistronic or monocistronic hairpin reporter mRNA, additional cells were also included that were transfected with non-targeting or PDCD4-specific siRNA alone and analyzed by Western blotting to ensure that PDCD4 was efficiently knocked down in the PDCD4-specific siRNA transfected cells as compared to control cells.

Collectively, these data confirm that PDCD4 acts as a negative regulator of the XIAP and Bcl-xL IRES elements, but positively regulates the Apaf-1 IRES under normal growth conditions.

Chapter 4: Discussion

A vast majority of cancers display a significant degree of chemoresistance, which represents a major hurdle in the treatment of these diseases. In many of these instances the enhanced rates of cell survival can be attributed to the dysregulated expression of apoptotic molecules, therefore elucidating the mechanisms by which the expression of these proteins is modulated is of great importance. In the case of SCLC, chemoresistance is due to the activation of S6K2 and the concomitant translational upregulation of a number of anti-apoptotic proteins, including XIAP, Bcl-xL, Bcl-2 and c-IAP1 (Pardo et al. 2002, 2003, 2006); however, the mechanism by which the expression of these four proteins are specifically upregulated is unknown. Interestingly, all four anti-apoptotic proteins can be generated through cap-independent translation. The concerted regulation of specific IRES-containing transcripts, but not others, has been demonstrated for some IRES elements – including the XIAP and Bcl-xL IRESs (Bevilacqua et al. 2010, Lewis et al. 2007, Yoon et al. 2006), indicating that there are subsets of IRES-containing transcripts whose translation could be regulated by single signalling pathway(s) or ITAFs. This research focuses on a S6K2-dependent signalling pathway whose activation ultimately results in the proteasomal degradation of PDCD4 and concurrent regulation of IRES-mediated translation of three specific transcripts encoding XIAP, Bcl-xL and Apaf-1.

I have shown here that in agreement with previous studies, the activation of S6K2, but not S6K1, leads to the upregulation of XIAP and Bcl-xL protein expression (Figure 5A), with no effect on the XIAP or Bcl-xL mRNA transcript level (Figure 5B). To demonstrate that

S6K2, but not S6K1 activation, enhances the IRES activity of XIAP and Bcl-xL, I used a bicistronic assay in the context of the tetracycline inducible S6K cell lines, where the relative IRES activity was compared between induced and non-induced cells. These experiments established that S6K2 signalling alone was capable of upregulating the relative IRES activity of XIAP and Bcl-xL (Figure 5C).

My data thus far indicated that S6K2 signalling enhances the IRES-mediated translation of XIAP and Bcl-xL; therefore the downstream target of S6K2 in this signalling pathway must affect the step of translation initiation, given that this is the unique property of IRES-mediated translation. There are two established downstream targets of S6K2 implicated in the regulation of translation initiation: ribosomal protein S6 (rpS6) and eukaryotic initiation factor 4B (eIF4B). S6K1 and S6K2 are both known to phosphorylate ribosomal protein S6 (rpS6), a component of the 40S subunit of the ribosome, at five evolutionarily conserved serine residues – Ser235, Ser236, Ser240, Ser244 and Ser247 (Krieg et al. 1988, Shima et al. 1998). It should be noted that S6K2 functions as the primary kinase in the phosphorylation of rpS6, which is much more significantly hypophosphorylated in S6K2$^{-/-}$ cells as compared to S6K1$^{-/-}$ cells (Pende et al. 2004). The definitive physiological role of rpS6 phosphorylation is still unclear, but it is agreed that phosphorylation of rpS6 has a role in regulating cell size, cell proliferation, glucose homeostasis and muscle strength (Ruvinsky et al. 2005, 2009). Researchers were led to believe that rpS6 affects translation initiation or efficiency of protein synthesis as rpS6 has been localized to the interface between the two ribosomal subunits where it interacts with mRNA, initiation factors and tRNA (Nygård and Nilsson 1990). However, studies examining

this hypothesis primarily generated conflicting data, with some studies showing a strong correlation between degree of rpS6 phosphorylation and overall translation, while others have shown this relationship to be untrue or even an increase in global translation levels when the five phosphorylatable serine residues are mutated to alanines; it is now generally accepted that rpS6 phosphorylation does not enhance overall levels of translation (Montine and Henshaw 1990, Ruvinsky et al. 2005, Stolovich et al. 2002). eIF4B, a cofactor of eukaryotic initiation factor 4A (eIF4A) (Lawson et al. 1989, Rogers et al. 1999, 2001), is another known substrate of S6K1 and S6K2 (Raught et al. 2004). The phosphorylation of eIF4B by S6K1 or S6K2 at Serine 422 appears to be significant for the function of eIF4B in translation, and is hypothesized to have a role in the co-activation of eIF4A, as Serine 422 resides in an RNA binding region of eIF4B required to promote eIF4A helicase activity (Methot et al. 1994). Due to the controversy regarding the role, if any, that rpS6 plays in translation initiation and the fact that knockdown of eIF4B elicits a 50% drop in total translation (Shahbazian et al. 2010), thus underlying its crucial role in cap-dependent translation, neither proteins seemed likely targets of S6K2 in the regulation of cap-independent translation. Therefore, I chose to focus on PDCD4 as the mediator of S6K2 signalling to the XIAP and Bcl-xL IRES elements.

S6K1 has been shown to phosphorylate the tumour suppressor PDCD4 (Dorrello et al. 2006). PDCD4 affects translation by interfering with the eIF4A-eIF4G interaction; eIF4A, eIF4G and PDCD4 all contain MA-3 domains, which can mediate protein-protein interactions. Via this shared binding domain, PDCD4 can bind to eIF4A and the central portion of eIF4G; when eIF4A is bound to PDCD4 it is sequestered in an inactive

conformation and is unable to be incorporated into the eukaryotic initiation factor 4F (eIF4F) complex (Göke et al. 2002, Loh et al. 2009, Yang et al. 2003). The specific cohort of canonical initiation factors required for IRES-mediated translation is not defined, and likely varies depending on the individual IRES element. However, recent data indicates that some cellular IRES elements, such as the L-, N-, or c-myc IRESs, may be enhanced by or have specific requirements for eIF4A, eIF4G, or both as a part of a larger complex (Hundsdoerfer et al. 2005, Spriggs et al. 2009). PDCD4 expression, and its ability to impede the eIF4G-eIF4A interaction, may therefore impact IRES mediated translation. Further, PDCD4 contains RNA binding domains that to date have no distinctly assigned function, except that recruitment of PDCD4 to the translation machinery is dependent on its RNA binding activity (Wedeken et al. 2010), arguing that there is a function for these binding domains in the selection of transcripts that are translationally regulated by PDCD4 that has yet to be determined. Additionally, the RNA binding capability of PDCD4 is not dependent on the presence of a 7-mG cap structure, which further supports a role for PDCD4 in cap-independent translation (Böhm et al. 2003).

My data suggests that the effector molecule in the FGF-2-stimulated signalling cascade responsible for XIAP and Bcl-xL upregulation is a unique substrate of S6K2, as S6K1 activation cannot perform the same role. However, I wanted to determine the effect of S6K2 signalling on PDCD4 as the previous studies examining phosphorylation of PDCD4 and its subsequent degradation focused solely on regulation contributed by S6K1. Additionally, given the potential for PDCD4 to act as a modulator of IRES-mediated translation through its interactions with eIF4A and eIF4G, and the fact that PDCD4 does not appear to regulate

global protein synthesis, this target substrate could not be disregarded (Böhm et al. 2003). Substrate redundancy has been described for the S6 kinases in previous reports (Krieg et al. 1988, Raught et al. 2004, Shima et al. 1998), and when I examined the effect of activated S6K2 on the expression of PDCD4 I found that S6K2 signalling also elicited substantial proteasomal degradation of PDCD4 (Figure 6B). Moreover, the level of PDCD4 downregulation seen in the TO-KAS6K2 cells exceeded that seen in the TO-KAS6K1 cells (Figure 6A). A possible caveat to comparing these cell lines directly is that I have not definitively determined that both kinases have equivalent activity. However, in previous studies using the tetracycline inducible cell lines it was determined that the level of kinase activity exhibited in the inducible cell lines is equivalent to the endogenous kinase activity elicited by the treatment of cells with FGF-2, which is comparable between S6K1 and S6K2 (Pardo et al. 2001, 2006). If the assumption is made that the activity of both kinases in the inducible cell lines are relatively equivalent, I propose that S6K2 activation causes a more robust phosphorylation-dependent degradation of PDCD4. Additionally, I hypothesize that this difference in degree of degradation of PDCD4 is responsible for the observed concomitant upregulation of XIAP and Bcl-xL protein expression.

If the significant degradation of PDCD4 in the S6K2 inducible cell line accounts for the elevated expression of XIAP and Bcl-xL, then it is reasonable to hypothesize that knocking down the expression of PDCD4 directly using siRNA should yield the same result. I have shown here that when PDCD4 expression is decreased by using PDCD4-specific siRNA that both XIAP and Bcl-xL protein levels are substantially elevated (Figure 7). Furthermore,

when examining the effect knocking down PDCD4 has on Apaf-1, whose translation is also IRES-driven, I find that the opposite is true and Apaf-1 expression is decreased (Figure 7).

PDCD4 is also known to act as a transcriptional repressor, and does so by reducing the transactivation of activating protein 1 (AP-1) responsive promoters (Bitomsky et al. 2004, Wang et al. 2008, Yang et al. 2001, 2006), inhibiting the transcription factors Sp1 and Sp3 (Leupold et al. 2007) and by also inhibiting β-catenin/Tcf-dependent transcription (Wang et al. 2008). The Bcl-xL and XIAP genes contain AP-1 binding sites in their promoter regions (Grillot et al. 1997, Lee et al. 2006, Sevilla et al. 2001) , while the XIAP and Apaf-1 gene promoter regions both contain putative Sp1 binding sites (Furukawa et al. 2002, Lee et al. 2006). Additionally, Bcl-xL expression has been shown to be responsive to β-catenin/Tcf-dependent transcriptional regulation (Xie et al. 2005); therefore it was important to determine if knocking down the expression of PDCD4 imparted regulation of XIAP, Bcl-xL or Apaf-1 mRNA levels. Despite previous studies describing the role of PDCD4 as a transcriptional repressor through various mechanisms, I find that in HEK293 cells, siRNA mediated reduction of PDCD4 expression does not trigger any significant changes in the steady state transcript level of XIAP, Bcl-xL or Apaf-1 (Figure 8); notably, the knockdown of PDCD4 expression does not alter the expression of the IRES-containing XIAP transcript either (Figure 8). It should be mentioned that the action of PDCD4 has proven to be relatively cell type specific (reviewed in Lankat-Buttgereit and Göke 2009); therefore it is possible that this proposed mechanism of regulation imparted by PDCD4 may be limited to certain cell types and not others. I have shown here that PDCD4 knockdown is sufficient to upregulate XIAP and Bcl-xL protein expression, and downregulate Apaf-1 protein

expression, with no significant change in the corresponding transcript levels, thus confirming that PDCD4 regulates XIAP, Bcl-xL and Apaf-1 post-transcriptionally.

Next, by employing the use of a polysome profiling technique, I have demonstrated here that siRNA-mediated knockdown of PDCD4 expression causes a profound change in the polysomal distribution of the IRES containing transcript of XIAP, as well as the Bcl-xL and Apaf-1 mRNAs relative to GAPDH mRNA (Figure 9). The most striking finding here is that when examining the distribution of the non-IRES containing XIAP transcript, I find that there is no change – yet the IRES-containing XIAP transcript shifts into the more heavily translated fractions of the profile (Figure 9). Also, in accordance with previous data shown here, upon PDCD4 knockdown the Bcl-xL transcript shifts into the heavy polysome fractions, where this transcript will be more efficiently translated, while the Apaf-1 transcript shifts out towards the monosomal fractions, where this transcript will undergo a lower rate of translation (Figure 9). These findings are significant for a number of reasons. To date the number of known translational targets of PDCD4 is considerably limited; these targets include carbonic anhydrase type II (Lankat-Buttgereit et al. 2004), the translational regulation of which was not very well characterized, and the transcription factor Sp1, which is also speculated to be a translational target of PDCD4 (Leupold et al. 2007), though no conclusive experimental data exists to support this hypothesis. Most importantly, this data shows that PDCD4 selectively regulates the translation of the IRES-containing XIAP transcript and the Bcl-xL transcript, indicating for the first time that PDCD4 plays a particular role in cap-independent translation versus cap-dependent translation. Lastly, the positive role that PDCD4 plays in

the translational regulation of Apaf-1 is also a novel finding, given that PDCD4 has only ever been described as a repressor of translation.

Using the aforementioned bicistronic reporter assay, I assessed the effect of knocking down PDCD4 expression on the relative IRES activity of the XIAP, Bcl-xL and Apaf-1 IRES elements. In concordance with the polysome profiling experiments, decreased expression of PDCD4 significantly augmented XIAP IRES activity, further attesting to the role of PDCD4 as a specific regulator of IRES-mediated translation of XIAP (Figure 10A). Additionally, reduced PDCD4 expression also elicited enhanced activity of the Bcl-xL IRES element, yet concurrently suppressed the relative IRES activity of the Apaf-1 IRES element (Figure 10A). The effect of PDCD4 knockdown on the translational efficiency of the transcripts as determined by polysome profiling is mirrored in the relative IRES activity of each IRES element resulting from diminished PDCD4 expression. These data strongly suggest that PDCD4 is capable of regulating specific transcripts via the modulation of IRES-mediated translation. As mentioned, the use of the βgal/CAT bicistronic reporter construct to measure IRES-mediated translation initiation has been criticized before (Baranick et al. 2008, Van Eden et al. 2004a), therefore to substantiate the data obtained using this methodology, IVT mRNA reporter constructs were used. The use of IVT RNA reporters to measure relative IRES activity bypasses the criticism that splicing is required for the previously observed IRES activity seen for either IRES as assessed using bicistronic reporter constructs. By transiently transfecting HEK293 cells with non-targeting or PDCD4 specific siRNA, followed by transfection with IVT reporter RNA containing the XIAP or Bcl-xL IRES sequence, I was able to corroborate the data found using the bicistronic reporter assay;

using RNA reporters I have shown that the activity of both the XIAP and Bcl-xL IRES elements was significantly enhanced when the expression of PDCD4 was reduced (Figure 10B). Unfortunately a monocistronic hairpin DNA construct bearing the Apaf-1 IRES element has not been successfully cloned, therefore there is no IVT RNA data to parallel that obtained using the bicistronic vector. Nevertheless, the elevation of XIAP and Bcl-xL IRES activity as assessed by direct RNA transfection upon knockdown of PDCD4 further validates PDCD4 as a novel regulator of IRES-mediated translation initiation.

A previous study examining the role of PDCD4 in the repression of translation ruled out any contribution PDCD4 might have in regulating IRES-mediated translation (Yang et al. 2003). This study, however, did not examine the effect of PDCD4 on a range of cellular IRESs, but instead focused on the ability of PDCD4 to inhibit translation driven by a single viral IRES (EMCV). The activity of EMCV IRES was unaffected by the overexpression of PDCD4 even though cap-dependent translation was reduced, so it was concluded that PDCD4 affects cap-dependent translation alone. It is possible that the level of PDCD4 expressed in the cell line they transiently transfected is sufficient to inhibit EMCV IRES activity – the basal level of PDCD4 expression is not shown. If the basal level of PDCD4 expression can suppress EMCV IRES activity, then overexpressing PDCD4 should not have any effect. Alternatively, if the expression of PDCD4 had been silenced, perhaps the activity of the EMCV IRES would have been enhanced; nevertheless, it is difficult to draw conclusions about regulation of cellular IRES elements in general based on data obtained through the examination of a single viral IRES, as there are differing requirements for the control of IRES-mediated translation initiation (reviewed in Hellen and Sarnow 2001, Spriggs

et al. 2005). Furthermore, I would speculate that PDCD4 does not regulate IRES activity in general. Both the inability of PDCD4 to affect the EMCV IRES as well as the negative regulation of the XIAP and Bcl-xL IRES elements, but stimulation the Apaf-1 IRES, support a model in which PDCD4 selectively regulates IRES-mediated translation of particular mRNAs.

The data shown here would indicate that the regulation exerted by PDCD4 is present under normal cellular conditions, given that the alteration in XIAP, Bcl-xL and Apaf-1 IRES activity is seen upon the reduction of PDCD4 expression in the absence of any other stimuli. This is interesting given that the regulation of IRES mediated translation by external factors is generally in response to a stress signal that causes the subcellular relocalization or change in activity or expression of specific ITAFs (reviewed in Lewis and Holcik 2008). The work presented here poses new questions: does PDCD4 exhibit selectivity for specific transcripts and how, does PDCD4 act directly or indirectly to regulate IRES-mediated translation initiation, and what is the biological significance of S6K2-dependent degradation of PDCD4?

What has not been examined thus far is the potential contribution of the RNA binding domains of PDCD4, which may govern the selection of transcripts that PDCD4 does control. PDCD4-mediated regulation of the IRES-mediated translation of the XIAP IRES-containing transcript and Bcl-xL and Apaf-1 mRNAs could depend on the ability of PDCD4 to bind to these transcripts in particular, within their IRES elements. Indeed, the RNA binding capacity of PDCD4 has been demonstrated to be required for its association with the 48S pre-initiation complex – although this association also relies largely on the ability of PDCD4 to interact with eIF4A (Wedeken et al. 2010). However, PDCD4 acts to negatively regulate

the XIAP and Bcl-xL IRES elements, while positively regulating the Apaf-1 IRES element; therefore if PDCD4 is exerting its effects via directly binding to these IRES elements it must somehow bind in such a way that it can act as both a repressor and promoter of cap-independent translation. The crystal structure of the PDCD4-eIF4A complex has been determined and proposes a model in which PDCD4 binds to eIF4A and prevents the association of eIF4A with the RNA in order to block its helicase function (Chang et al. 2009). This model suggests that PDCD4 may simply sequester eIF4A from the mRNA, but does not address whether or not this complex forms independently of mRNA, or if PDCD4 retains the capability to bind mRNA. Therefore in the case of XIAP and Bcl-xL, PDCD4 may prevent their IRES-mediated translation by binding to their IRES elements and inhibiting the action of eIF4A; for IRES-mediated translation, helicase function is critical for the unwinding of secondary structures in the 5'-UTR and therefore could reflect the requirement of eIF4A for efficient cap-independent translation of XIAP and Bcl-xL. Alternatively, if PDCD4 also binds the Apaf-1 IRES element, the sequestration of eIF4A into an inactive conformation by PDCD4 may allow for an alternative helicase to be recruited to the Apaf-1 IRES that would allow for more proficient cap-independent translation of Apaf-1.

In addition to eIF4A, PDCD4 is also able to bind the middle one third of eIF4GI (Yang et al. 2003). Though an initial report refutes the involvement of the middle fragment of eIF4GI in regulating the IRES activity of XIAP and Apaf-1 (Henis-Korenblit et al. 2002), a more recent study has demonstrated the ability of the middle fragment of eIF4GI to promote XIAP IRES mediated translation *in vitro* (Hundsdoerfer et al. 2005). Though these reports conflict, another role of PDCD4 in negatively regulating IRES mediated translation of

XIAP during cellular stress could be to bind and sequester the middle fragment of eIF4GI that is cleaved after the onset of apoptosis (Marissen and Llyod 1998); conceivably, this regulation could then be reversed by exogenous signals targeting PDCD4 for proteasomal degradation. Currently there is no data linking cleaved eIF4GI and the activation of the Bcl-xL IRES element, but it is intriguing to speculate that this is another level at which PDCD4 controls the IRES-mediated translation of Bcl-xL. There have been no recent reports disproving the initial finding that eIF4GI does not impact the activity of the Apaf-1 IRES element, but this is in agreement with my data demonstrating the positive effect of PDCD4 on the Apaf-1 IRES.

The functions of PDCD4 described above apply only when PDCD4 is cytoplasmic, yet PDCD4 contains two putative nuclear localization signals, and is known to shuttle between the nucleus and cytoplasm; currently, there are discrepancies regarding the subcellular distribution of PDCD4 in normal versus carcinogenic cells (reviewed in Lankat-Buttgereit and Göke 2009). For instance, one study examining PDCD4 expression in the progression of colorectal cancer showed a transition from nuclear and cytoplasmic staining to cytoplasmic staining alone from normal tissue, to adenomas, and finally carcinomas (Mudduluru et al. 2007). Conversely, in a study investigating the subcellular localization of PDCD4 in ductal carcinomas versus normal breast tissue found that PDCD4 was predominantly localized to the nucleus in neoplastic breast tissues, while it was largely cytoplasmic in normal breast tissue (Wen et al. 2007). The specific signals that regulate the shuttling of PDCD4 between the nucleus and cytoplasm have also not been well characterized, though it has been proposed that this shuttling is mediated by phosphorylation of PDCD4 (Böhm et al. 2003).

Currently there are two proposed models as to how ITAFs regulate IRES-mediated translation based on their subcellular localization (reviewed in Lewis and Holcik 2008). In one model ITAFs are sequestered in the nucleus, but upon receiving the appropriate stimuli they accumulate in the cytoplasm to exert their translational effect. On the other hand, some ITAFs are bound to their target mRNAs in the nucleus, and when prompted by signalling events move to the cytoplasm with their target mRNA and facilitate ribosome recruitment. It is possible that the subcellular localization of PDCD4 could contribute to the mechanism in which it regulates IRES-mediated translation as well. For instance, sequestration of PDCD4 in the nucleus would preclude its ability to interact with eIF4A or eIF4G, and therefore prevent inhibition of translation via its binding interactions with these initiation factors. Alternatively, specific transcript selection by PDCD4 might possibly take place in the nucleus; in this model, PDCD4 would shuttle to the cytoplasm bound to its specific target mRNAs, where it could act to promote or repress IRES-mediated translation of those transcripts. Given the cell type specific nature of subcellular localization of PDCD4 in normal cells, it is difficult to propose a mechanistic model as to how PDCD4 regulates IRES-mediated translation that incorporates a role for its subcellular localization that applies to all cell types; however, the shuttling capabilities of PDCD4 present potentially another facet to PDCD4-mediated translational regulation of XIAP, Bcl-xL and Apaf-1.

The ability of PDCD4 to modulate IRES-mediated translation of these three transcripts under normal and stress conditions is likely crucial to maintain cellular homeostasis and for mounting appropriate responses to apoptotic stimuli. Though under normal conditions XIAP is predominantly expressed from its non-IRES containing transcript,

upon encountering cellular stress the IRES-containing transcript of XIAP becomes increasingly important for the production of XIAP protein. As Bcl-xL expression is also regulated by IRES-mediated translation, similar to XIAP it is extremely important to control the activity of its IRES element as well. Interestingly, Apaf-1 is required for the intrinsic apoptotic pathway and the expression of this protein relies solely on the activity of its IRES element (Coldwell et al. 2000); therefore PDCD4 could be an essential factor in maintaining the expression of Apaf-1 in order to execute the intrinsic apoptotic pathway should the need arise.

The data presented here highlight the importance of S6K2 signalling and the tumour suppressor PDCD4, particularly in the context of human pathophysiological conditions, given the specialized nature of the downstream proteins affected. The overexpression of S6K2 has been documented in human breast tumours, endometrial adenocarcinomas and in a number small cell lung cancer (SCLC) cell lines (Filonenko et al. 2004, Lyzogubov et al. 2004, Pardo et al. 2001, Pérez-Tenorio et al. 2010). However, the extent of research investigating the aberrant expression of S6K2 in disease is not as comprehensive as the research done on S6K1; consequently the extent of dysregulation of S6K2-dependent signalling pathways in cancer is unknown. Similarly, the loss of PDCD4 has also been widely reported in a number of different cancer types, including lung, renal or tongue derived tumours, gliomas, hepatocellular carcinoma, skin cancer, breast carcinomas, ovarian cancer and colon cancer (Carinci et al. 2005, Gao et al. 2007, Jansen et al. 2004, Matsuhashi et al. 2007, Mudduluru et al. 2007, Wen et al. 2007, Wei et al. 2009, Zhang et al. 2006). Loss of PDCD4 expression is attributed not only to aberrant signalling targeting it for proteasomal

degradation, but also to microRNA-21 (miR-21) expression (Asangani et al. 2008, Lu et al. 2008) and CpG island methylation (Gao et al. 2009). S6K2 could contribute to the malignancy of cancers in which it is overexpressed by downregulating PDCD4 expression, thus enhancing XIAP and Bcl-xL expression, while suppressing Apaf-1 expression. Additionally, silencing of PDCD4 expression by miR-21 or via CpG island methylation would likely lead to deregulated translation of XIAP, Bcl-xL and Apaf-1 as well.

The coordinated regulation of these three apoptotic molecules would theoretically impart those cells with greater resistance to cytotoxic agents, rendering these tumours more resistant to many current cancer therapies that rely on cell death. In fact, both S6K2 and PDCD4 have been linked to chemoresistance in a number of cancers including small cell lung cancer, ovarian cancer, colon cancer and breast cancer (Allen and Weiss 2010, Bourguignon et al. 2009, Pardo et al. 2002, 2003, Shi et al. 2010, Zhang et al. 2010). Therefore, the expression level of S6K2 or PDCD4 could be invaluable as prognostic biomarkers for disease progression or degree of malignancy. The level of PDCD4 expression may also provide insight from a therapeutic standpoint, as to what therapies should be administered – given that the efficaciousness of a chemotherapeutic drug relying on apoptosis may be significantly reduced for cancers with low PDCD4 expression.

Conclusion

I propose a model (Appendix II) in which the activation of S6K2 signalling leads to the targeted proteasomal degradation of PDCD4. The significant downregulation of PDCD4 elicits the cap-independent translational upregulation of XIAP and Bcl-xL, and downregulation of Apaf-1, thus identifying PDCD4 as a novel regulator of IRES mediated translation.

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Kinase Kinase Kinase Kinase 1 Expression to Suppress Colon Carcinoma Cell Invasion. Mol. Cell. Biol. *26(4)*, 1297-1306.

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Contributions of Collaborators

Tetracycline-inducible kinase active S6K1 and S6K2 cell lines were generous gifts from Dr. Michael Seckl.

Plasmid encoding the monocistronic-hairpin Bcl-xL IRES construct was cloned by Alura Riley.

Appendix I

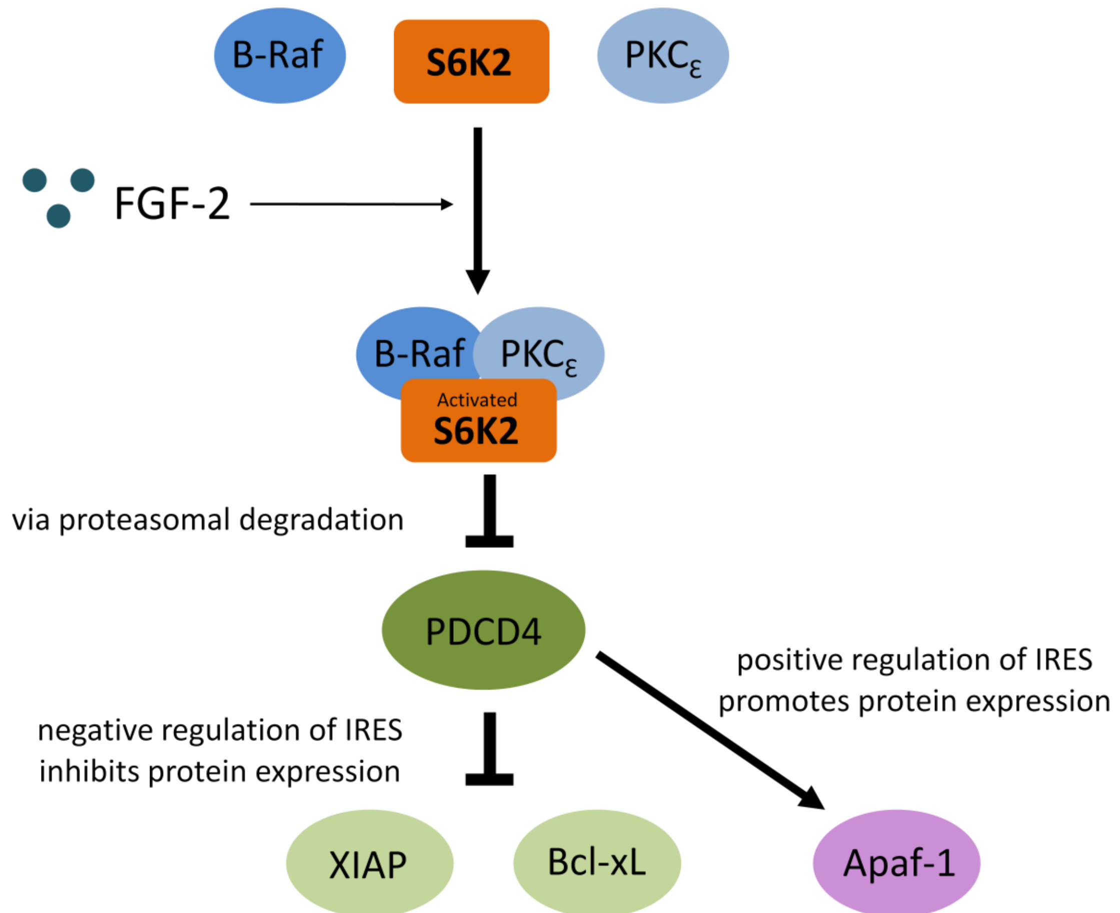
Oligonucleotide sequences:

q323-F: 5'-CTTCCGTTTCCTTCACCTA-3'
q323-R: 5'-TAGGACTTGTCCACCTTTC-3'
q4496-F: 5'-AGGGCACATGTATGTCATGG-3'
q4496-R: 5'-TAGAGGGTGGCTCAGGAAAA-3'
qApaf-1-F: 5'-CTTGAGCCCTGGAGTTTGAG-3'
qApaf-1-R: 5'-TGCATGAACTGCCATGAAAT-3'
qGAPDH-F: 5'-ACAGTCAGCCGCATCTTCTT-3'
qGAPDH-R: 5'-ACGACCAAATCCGTTGACTC-3'

siRNA sequences:

PDCD4: 5'-GUGUUGGCAGUAUCCUUAG-3'

Appendix II



Proposed model of PDCD4 regulation of XIAP, Bcl-xL and Apaf-1 protein expression.

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Curriculum Vitae

Education

September 2006 – present	M.Sc. Biochemistry, Specialization in Human & Molecular Genetics, University of Ottawa.
September 2002 – May 2006	B.M.Sc., Specialization Biochemistry, University of Western Ontario.

Research Experience

September 2006 – present	M.Sc. candidate. Apoptosis Research Centre, Children's Hospital of Eastern Ontario, Ottawa, Ontario.
September 2005 – May 2006	Honours research student. London Regional Cancer Centre, London, Ontario.
May 2005 – September 2005	Summer student. London Regional Cancer Centre, London, Ontario.

Awards and Honours

February 2010	AACR Meeting – Scholar-in-Training Award
May 2009 – November 2010	Frederick Banting and Charles Best Canada Graduate Scholarship – Doctoral Award (CIHR)
May 2009 – December 2010	University of Ottawa Excellence Scholarship
January 2008	The Dean's Scholarship, University of Ottawa
September 2002	Entrance scholarship, University of Western Ontario.

Graduate Classes Completed

BCH8103 – Advanced Topics in Molecular Biology of Human Disease I (A)

BCH8105 – Advanced Topics in Molecular Biology of Human Disease II (A-)

BCH8114 – Advanced Topics in Cell Cycle (A)

BCH8310 – Current Topics in RNA Molecular Biology (A)

Presentations

June 2010	15th Annual Meeting of the RNA Society – Poster presentation.
February 2010	AACR Special Conference on Protein Translation and Cancer – Oral/Poster presentation.
October 2009	2nd Annual Children's Hospital of Eastern Ontario Clinical Research Day – Poster presentation.
February 2009	Ontario Institute for Cancer Research Annual Scientific Meeting – Poster presentation.
September 2008	Cold Spring Harbour Translational Control Meeting – Poster presentation.

Publications

Riley, A., Jordan, L.E., and Holcik, M. (2010). Distinct 5' UTRs regulate XIAP expression under normal growth conditions and during cellular stress. Nucleic Acids Res. *38(14)*, 4665-74.

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