The function and regulation of PDCD4 – a novel inhibitor of selective translation initiation

Urszula LIWAK-MUIR

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Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

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Abstract

Internal ribosome entry site (IRES)-mediated translation is critical for the cell’s ability to respond to stress. Understanding how RNA binding proteins (IRES trans-acting factors; ITAFs) regulate IRESes is crucial to elucidating the mechanism of alternative translation initiation. Furthermore, determining how these ITAFs are regulated is central to understanding their functions in diseased states. I have identified the tumour suppressor programmed cell death 4 (PDCD4) as a novel ITAF of the XIAP and Bcl-xL IRES elements. I demonstrate that under normal conditions, PDCD4 acts to inhibit translation from these IRES elements by preventing formation of the 48S translation initiation complex. Furthermore, I show that in response to treatment with the pro-survival fibroblast growth factor-2 (FGF-2), S6 kinase 2 (S6K2) phosphorylates PDCD4 leading to its degradation and the subsequent de-repression of XIAP and Bcl-xL translation. Importantly, I demonstrate the clinical significance of this regulation in glioblastoma multiforme (GBM) tumours where the loss of PDCD4 expression correlates with an increase in Bcl-xL protein and poor patient outcome. Additionally, re-expression of PDCD4 down-regulates Bcl-xL and decreases cell viability, and direct inhibition of Bcl-xL by a small molecule antagonist ABT-737 sensitizes GBM cells to the chemotherapeutic doxorubicin. Finally, I demonstrate that PDCD4 can be regulated at multiple levels. Importantly, I identify the RNA binding protein HuR as a regulator of microRNA (miR) -21 induced silencing of PDCD4. I show that HuR can bind the PDCD4 3’UTR and prevent miR-21 binding, and that a loss of PDCD4 expression following H2O2 treatment is mediated via miR-21. These results provide novel insight into the role of PDCD4 as a tumour suppressor and highlight the importance of ITAFs in cancer progression.
Acknowledgements

I would like to express my deep gratitude to my supervisor Dr. Martin Holcik for his patient guidance, encouragement and insightful comments. His endless optimism and mentorship has encouraged me to succeed and fostered my love of research. I would also like to thank my thesis advisory committee – Dr. Ian Lorimer, Dr. Ken Dimock, and Dr. Robert Korneluk – for their input, comments, and direction. I am also grateful for the members of the Holcik lab and the researchers and support staff of the Apoptosis Research Center for their continued support. Finally, I would like to thank my friends and family for their many years of understanding, unconditional support and encouragement in all of my endeavours.
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<table>
<thead>
<tr>
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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease activating factor 1</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich element</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>Bcl-2-like 1</td>
</tr>
<tr>
<td>β-GAL</td>
<td>β-Galactosidase</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 homology domain</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculovirus IAP repeat</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>c-myc</td>
<td>Cellular myelocytomatosis oncogene</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>CAT1</td>
<td>Cationic amino acid transporter 1</td>
</tr>
<tr>
<td>Cdk1</td>
<td>Cyclin-dependent kinase 1</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Chk-2</td>
<td>Cell cycle checkpoint kinase 2</td>
</tr>
<tr>
<td>cIAP1</td>
<td>Cellular inhibitor of apoptosis protein 1</td>
</tr>
<tr>
<td>cIAP2</td>
<td>Cellular inhibitor of apoptosis protein 2</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>CrPV</td>
<td>Cricket paralysis virus</td>
</tr>
<tr>
<td>DIABLO</td>
<td>Direct IAP binding protein with low pi</td>
</tr>
<tr>
<td>DISC</td>
<td>Death inducing signaling complex</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic initiation factor</td>
</tr>
<tr>
<td>ELAV</td>
<td>Embryonic lethal abnormal vision</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain protein</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor -2</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma mutiforme</td>
</tr>
<tr>
<td>GCN2</td>
<td>General control non-derepressible-2</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione s- transferase</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney cells</td>
</tr>
<tr>
<td>hnRNP A1</td>
<td>Heterogeneous ribonucleoprotein A1</td>
</tr>
<tr>
<td>hnRNP C1/C2</td>
<td>Heterogeneous ribonucleoprotein C1/C2</td>
</tr>
<tr>
<td>HNS</td>
<td>Nucleoplasmic shuttling domain</td>
</tr>
<tr>
<td>HRI</td>
<td>Haem-regulated inhibitor</td>
</tr>
</tbody>
</table>
HRV    Human rhinovirus
HuR    Human antigen R
IAP    Inhibitor of apoptosis protein
IPTG   Isopropylthio-β-galactoside
IR     Ionizing radiation
IRES   Internal ribosome entry site
ITAF   IRES trans-acting factor
KSHV   Kaposi’s sarcoma-associated herpesvirus
La     La autoantigen
LB     Luria-Bertani
MAPK   Mitogen-activated protein kinase
miRNA  MicroRNA
mRNA   Messenger RNA
NF-κB  Nuclear factor-κB
NF45   Nuclear factor 45
OIS    Oncogene-induced senescence
Omi/HtrA2 OMI/High temperature requirement A2
ORF    Open reading frame
P-bodies Processing bodies
PABP   PolyA-binding protein
PAR-CLIP Photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation
PBS    Phosphate buffered saline
PCBP1  Poly (rC)-binding protein 1
PCR    Polymerase chain reaction
PDCD4  Programmed cell death gene 4
PERK   PKR-like endoplasmic reticulum kinase
PKC    Protein kinase C
PKR    Protein kinase activated by double-stranded RNA
PMSF   Phenylmethylsulfonyl fluoride
qRT-PCR Quantitative reverse transcription - polymerase chain reaction
REMSA  RNA electrophoretic mobility shift assay
RING   Really interesting new gene
RIPA   Radioimmunoprecipitation assay
RISC   RNA induced silencing complex
RRM    RNA recognition motif
rRNA   Ribosomal RNA
S6K1   Ribosomal protein S6 kinase 1
S6K2   Ribosomal protein S6 kinase 2
SCLC   Small cell lung cancer
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SG     Stress Granules
siRNA  Small interfering RNA
Smac   Second mitochondria-derived activator of caspase
TAP    Tandem affinity purification
TNF-α  Tumour necrosis factor- α
TNFR   Tumour necrosis factor receptor
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAIL</td>
<td>TNF-α-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>uORF</td>
<td>Upstream open reading frame</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet irradiation</td>
</tr>
<tr>
<td>X-DC</td>
<td>X-linked dyskeratosis congenita</td>
</tr>
<tr>
<td>XAF1</td>
<td>XIAP associated factor 1</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
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CHAPTER 1

General Introduction
1.1 Preamble

This chapter contains both unpublished (section 1.1-1.5) and published (section 1.6-1.14) sections. It begins with an introduction to apoptosis and the factors that inhibit apoptosis including XIAP and Bcl-xL. It further describes two main proteins involved in regulating these IRES-containing mRNAs including HuR and PDCD4. Section 1.5 “Translation Control in Apoptosis” is a review article published in the journal Experimental Oncology (Volume 34, October 2012). This review highlights key translational control mechanisms that are involved in the onset and progression of apoptosis, with a focus on the role of PDCD4 in mediating IRES dependent translation of XIAP and Bcl-xL. It also gives an introduction to the role of microRNA-mediated regulation of translation.

Author List for sections 1.6-1.14

Urszula Liwak, Mame Daro Faye, and Martin Holcik

Author Contribution for sections 1.6-1.14

UL, MDF, MH wrote the paper. UL wrote the sections: 1.8, 1.10.2, 1.11, 1.12.
1.2 Apoptosis

The cell’s ability to quickly respond to its environment is key to its survival. When an acute cellular insult occurs, the cell initially undergoes several processes to overcome the insult. However, severe or prolonged stress can lead to the activation of programmed cell death. This ensures that damaged cells are removed without causing harm to the neighboring cells or to the organism. When these protective mechanisms go awry, it can lead to the development of diseases such as cancer.

One key mechanism of programmed cell death is termed apoptosis, which is a controlled mechanism of cell dismantling characterized by cell shrinking, membrane blebbing, and DNA fragmentation. Apoptosis is regulated by cysteine proteases termed caspases (1). These caspases can be grouped into initiator and effector caspases that once cleaved and activated, will cleave other cellular proteins to undergo apoptosis. Apoptosis can be triggered from external cues that target the death receptors or internal cues such as DNA damage. Activation of the death receptors including the Tumour Necrosis Factor Receptor (TNFR), Fas, and TRAIL receptors results in the oligomerization of the receptors and formation of the death inducing signaling complex (DISC) which contains the adaptor protein FADD (Fas-associated death domain protein). The DISC recruits procaspase-8 molecules that once activated, process and activate caspases-3 and -7 (2). Apoptosis can also be initiated through an intrinsic route, for example by DNA damage. This route is characterized by the release of cytochrome c from the mitochondria. Once in the cytoplasm, cytochrome c binds to the apoptotic protease activating factor 1 (Apaf-1) and procaspase-9 to form the apoptosome leading to the activation of caspase-9, which further cleaves and activates caspases-3 and -7 (3, 4). Along with cytochrome c, other factors are released from the mitochondria including second mitochondria-derived activator of caspase (Smac)/direct
IAP binding protein with low pI (DIABLO) (5, 6) and Omi/HtrA2 (7) which bind to and inhibit anti-apoptotic inhibitor of apoptosis proteins (IAP), thus further promoting caspase activation (8).

Apoptosis is an important cellular event that is highly regulated to ensure the process is not inappropriately inhibited or activated. Any mis-regulation can have dire consequences including the formation of tumours; therefore, many proteins are involved in ensuring apoptosis is a controlled event. Two key families of proteins that are involved in the inhibition of apoptosis are the IAPs as well as the B-cell CLL/lymphoma 2 (Bcl-2) family of proteins. These proteins will be discussed in further detail below.

1.3 Apoptosis Regulating Proteins

1.3.1 Inhibitor of Apoptosis proteins (IAPs)

The inhibitor of apoptosis protein (IAP) family of proteins consists of eight members namely XIAP (BIRC4), cIAP1 (BIRC2), cIAP2 (BIRC3), survivin (BIRC5), livin (ML-IAP; BIRC7), NAIP (BIRC1), Bruce (BIRC6), and ILP2 (BIRC8). All IAP proteins are known to contain 1-3 baculovirus IAP repeat (BIR) domains and a few of the members, including XIAP, also contain a really interesting new gene (RING) domain, which contains E3 ubiquitin ligase activity (9). Although all members of the IAP family contain BIR domains, only a few are able to bind to caspases via this domain. However, binding to caspases does not necessarily mean caspase inhibition. Eckelman and Salvesen demonstrated that although cIAP1 and cIAP2 are able to bind to caspases, it does not appear that they inhibit caspase activity (10). XIAP, on the other hand, seems to be the only member that is effective at binding to and inhibiting caspases-3 and -7 (11-13).
1.3.2 X-linked inhibitor of apoptosis (XIAP)

As mentioned above, X-linked inhibitor of apoptosis (XIAP) is the most potent inhibitor of caspases of the IAP family. It is composed of three BIR domains and one carboxy-terminal RING domain. The third BIR domain of XIAP is responsible for binding to the initiator caspase-9 while the second BIR domain is important for binding the effector caspases-3 and -7 thus providing the anti-apoptotic role of XIAP. Three negative regulators of XIAP have been identified including XIAP associated factor 1 (XAF1) (14), Smac/DIABLO (5, 6) and Omi/HtrA2 (7). Nuclear XAF1 causes a relocalization of XIAP from the cytosol into the nucleus where it then binds to and inhibits XIAP, thus opposing its anti-caspase activity. Interestingly, XAF1 expression is lost in some cancer cells and is therefore no longer able to suppress XIAP’s anti-apoptotic activity (15). Smac/DIABLO and Omi/HtrA2 are two mitochondrial pro-apoptotic factors that are released during apoptosis that both act by binding to and inhibiting XIAP’s caspase binding ability. Smac/DIABLO has a high affinity for the BIR3 domain of XIAP thus preventing its binding to caspase-9 (16).

In addition to being regulated at the protein level, XIAP is also regulated at the level of translation initiation. The XIAP protein is translated from one of two transcripts. The shorter, more abundant transcript contains a 323 nucleotide 5'untranslated region (UTR) and is the primary source of XIAP protein under normal conditions. There is also a longer transcript variant that has been identified that contains a 1.7 kb 5'UTR. This longer UTR contains an internal ribosome entry site (IRES) region that mediates cap-independent translation under times of cellular stress (IRES-mediated translation is discussed further in section 1.10; (17)). Several proteins that bind to the IRES element of XIAP have been identified and include the autoantigen La (18), heterogeneous nuclear ribonucleoprotein
C1/C2 (hnRNP C1/C2; (19)), and hnRNP A1 (20). These proteins, termed IRES \textit{trans}-acting factors (ITAFs), can have repressive roles as is the case for hnRNP A1 or enhancing roles as is the case for La and hnRNP C1/C2, and are important for regulating the levels of IRES-mediated XIAP protein. The mechanism of IRES-mediated translation is still poorly understood and it is thought that several other proteins may be involved in regulating IRES translation. We demonstrate that the RNA binding protein Human antigen R (HuR) also binds to and enhances translation from the XIAP IRES, which contributes to an increase in cytoprotection against etoposide (Appendix B; (21)). On the other hand, data in chapter 2 demonstrates that the tumour suppressor programmed cell death 4 (PDCD4) represses translation from the XIAP IRES under normal conditions (22).

\textbf{1.3.3 Bcl-2 family of proteins}

The B-cell CLL/lymphoma 2 (Bcl-2) family of proteins consists of both pro- and anti-apoptotic proteins that regulate the mitochondrial pathway of apoptosis. These proteins are defined by the presence of the Bcl-2 homology domains (BH1 to BH4). The pro-survival proteins include Bcl-2, Bcl-xL, Bcl-W, Mcl-1, Bcl2A1, and Bcl-B which all contain the BH1, BH2, BH3, and BH4 domains. The pro-apoptotic members include the Bax family (Bax, Bak, Bok) that posses the BH1, BH2, and BH3 domains, and the BH3-only members (Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma, Blk, BNIP3, Spike) (23-25). Once activated by BH3-only proteins, Bax and Bak homo-oligomerize on the mitochondrial membrane causing permeabilization and release of cytochrome c and other pro-apoptotic proteins including Smac. The anti-apoptotic Bcl-2 members function to prevent this oligomerization by sequestering BH3-only proteins or by binding Bax and Bak directly thus preventing pore formation. This in turn prevents cytochrome c release and inhibits apoptosis (26).
1.3.4 Bcl-2-like 1 (Bcl-xL)

Bcl-2-like 1 (Bcl-xL) is an important regulator of apoptosis so it is not surprising that its expression is tightly regulated. The main transcript, Bcl-x is alternatively spliced generating two isoforms: Bcl-xS, which promotes apoptosis and Bcl-xL, which has anti-apoptotic effects (27). The shorter isoform antagonizes the effects of Bcl-2 and Bcl-xL and the high ratio of Bcl-xL to Bcl-xS observed in cancer cells points to the importance of Bcl-xL in apoptosis and disease (28-30).

Bcl-xL is also regulated at the level of translation initiation. Yoon et al (31) demonstrated the presence of an IRES element in the Bcl-xL 5'UTR. They showed that a mutated DKC1 gene, that acts to modify ribosomal RNA found in X-linked dyskeratosis (X-DC) patients, correlates with an impairment in IRES mediated translation of XIAP, p27kip1, and Bcl-xL. Using a bicistronic vector construct, they identified a bona fide IRES element in Bcl-xL. Similar to XIAP, as mentioned above, the Bcl-xL IRES is regulated by a variety of ITAFs. For example, the cytoplasmic accumulation of hnRNP A1 during osmotic shock suppresses Bcl-xL IRES activity (32). Likewise, we demonstrate in chapter 2 that PDCD4 reduces Bcl-xL IRES activity under normal conditions (22), while fibroblast growth factor-2 (FGF-2) treatment leads to a reduction of PDCD4 thus relieving this repression and leading to an increase in Bcl-xL protein expression. Furthermore, HuR has also been shown to bind directly to the 5'UTR of Bcl-xL to repress its translation. A reduction in HuR removes this repression resulting in an increase in Bcl-xL protein which affects mitochondrial morphology (33).

1.4 IRES trans-acting factors (ITAFs)
Many protective mechanisms are available to overcome a variety of stresses including the heat shock response, the unfolded protein response, a response to DNA damage or a response to oxidative stress. Although the mechanisms vary widely; generally, the cell’s initial response is to shut down cap-dependent translation in order to preserve cellular energy and to prevent a build up of unnecessary proteins. (A detailed review of translation regulation is provided in section 1.6). Although cap-dependent translation is attenuated, some proteins are still translated in order to manage the stress. (IRES-mediated translation is described further in section 1.10). IRES-mediated translation is governed by IRES trans-acting factors (ITAFs) which can enhance translation by facilitating recruitment of the ribosome or in some cases, ITAFs can inhibit binding of the ribosome to repress translation initiation. Two key ITAFs that are a focus of the following studies are Human antigen R (ELAV-like protein 1; HuR) and programmed cell death 4 (PDCD4).

1.4.1 Human antigen R (HuR)

Human antigen R (HuR) is part of the Hu family of RNA binding proteins that includes the neuronal HuB, HuC, and HuD (34). HuR is a ubiquitously expressed, nucleo-cytoplasmic shuttling protein that is involved in many cellular processes including mRNA splicing, export, stability and translation (21, 35-37). These functions are mediated through HuR’s three RNA recognition motifs (RRMs). RRM1 and RRM2 are important for HuR’s role in stability, splicing and translation, whereas RRM3 is important for polyadenylation and protein-protein interactions (38). Furthermore, the hinge region between RRM2 and RRM3 harbours a HuR nucleo-cytoplasmic shuttling domain (HNS) necessary for HuR to shuttle to the cytoplasm. Several signaling pathways regulate HuR’s shuttling ability in response to external cues. Cyclin-dependent kinase 1 (Cdk1), AMP-activated protein kinase
(AMPK), protein kinase C (PKC), and the mitogen-activated protein kinase (MAPK) p38 all phosphorylate HuR within or near the HNS to alter localization (39). This cytoplasmic localization is necessary for HuR’s ability to affect translation of some messenger RNAs (mRNAs). Moreover, a role for HuR in microRNA (miRNA)-mediated regulation is also starting to emerge (40). (A detailed description of miRNA regulation is provided in section 1.11). HuR has been shown to either compete with miRNAs resulting in stabilization of mRNAs or to act in cooperation with miRNAs to suppress mRNA translation. In a competitive setting, HuR binding prevents binding of miRNAs either through direct inhibition or a conformational change. For example, Bhattacharyya and colleagues (41) showed that amino acid starvation causes a relocalization of HuR into the cytoplasm where it relieves repression of the cationic amino acid transporter 1 (CAT1) mRNA by miR-122. HuR binding causes a transfer of the mRNA from processing bodies (p-bodies) into translationally active polysomes to enhance translation of CAT1. On the other hand, HuR has also been shown to cooperate with miRNAs to further destabilize mRNAs. For instance, Glorian and colleagues (42) demonstrated that the binding of HuR to the RhoB mRNA enables the binding of miR-19 leading to repression of RhoB after UV stress. Similarly, we demonstrate in chapter 4 that a loss of HuR results in a loss of PDCD4 protein expression that is dependent on miR-21. Furthermore, we show that hydrogen peroxide (H₂O₂) treatment causes a reduction in PDCD4 protein via miR-21 and we speculate that post-translational modifications of HuR after H₂O₂ treatment result in a loss of HuR’s ability to bind the PDCD4 mRNA thus promoting miR-21 binding.

1.4.2 Programmed Cell Death 4 (PDCD4)
The tumour suppressor programmed cell death 4 (PDCD4) is an RNA-binding protein that has been linked to the development of many human tumours including lung, colon, liver, breast, and brain (43-47). It was originally identified as a protein that increases during apoptosis (48) and has since been implicated in a variety of pathways. PDCD4 has been suggested to regulate global translation via its ability to bind to and inhibit eukaryotic initiation factors (eIFs) 4A and 4G (49, 50). Binding of PDCD4 to eIF4A sequesters it in an inactive conformation thus preventing it from incorporating into the eIF4F cap-binding complex (49, 51). However, although PDCD4 has been implicated in regulating general translation through eIF4A binding we, and others, have demonstrated that in fact PDCD4 regulates a subset of mRNAs (22, 52) including Bcl-xL and XIAP (22). PDCD4 protein levels are regulated through several mechanisms including phosphorylation by S6 kinase 1 (S6K1) leading to its βTRCP-mediated degradation (53). We further demonstrate in chapter 2 that in response to FGF-2 treatment, a complex forms between BRaf, PKCepsilon, and S6K2 resulting in the activation of S6K2. Activated S6K2 phosphorylates PDCD4 leading to its degradation, which relieves the repression of XIAP and Bcl-xL IRES translation. This in turn provides protection against apoptosis. Furthermore, the loss of PDCD4 contributes to the highly aggressive nature of Glioblastoma multiforme (GBM) tumours since it correlates with an increase in Bcl-xL and therefore an increase in evasion of apoptosis (chapter 3; (47)). Additionally, miR-21 post-transcriptionally downregulates tumour suppressor PDCD4 and stimulates invasion, intravasation and metastasis in colorectal cancer (54), breast (55), and glioblastoma (56) tumours. We further demonstrate in chapter 4 that HuR may also play an important role in the regulation by miR-21, which may have important implications for cancer research.
1.5 Hypothesis and Objectives

The role that ITAFs play in IRES-mediated translation is crucial to understanding the mechanism of alternative translation initiation. Furthermore, understanding how these ITAFs are regulated themselves is central to elucidating their functions in diseased states. Using the XIAP and Bcl-xL IRES elements as a model, I have hypothesized that there are specific regulators of IRES elements that act as repressors or enhancers of IRES activity and the levels and action of these regulators are tightly controlled to prevent disease. The main objectives of this dissertation were to (1) uncover regulator(s) of the XIAP and/or Bcl-xL IRES elements (2) determine how/why this regulation is important in diseased states, particularly glioblastoma multiforme tumours, and (3) determine how the expression of this regulator(s) is maintained.

1.6 Translation Control in Apoptosis

1.7 Abstract

Regulation of protein synthesis, although known for many decades, has only recently begun to be recognized as a critical control mechanism for the maintenance of cellular homeostasis and cellular stress response. One of the key advantages of translational control is the ability of cells to rapidly reprogram the protein output in response to internal or external triggers. This is particularly important during cellular response to stress that may lead to apoptosis by providing cells with a fine tuning mechanism that tips the balance between cell survival or apoptosis. In the following review we highlight several distinct mechanisms of translation
control and provide specific examples of translational control during apoptosis. This article is part of a Special Issue entitled ‘Apoptosis: Four Decades Later’.

1.8 Mechanisms of Translation Initiation

The regulation of gene expression occurs at many levels including the transcriptional and translational steps. In order for a cell to quickly respond to its changing environment, control of gene expression at the translational level is ideal since it allows for rapid and immediate changes in protein levels required to respond to the particular stress. Protein translation can be separated into three main steps including initiation, elongation, and termination. Translation initiation is often regarded as the rate-limiting step and thus it is highly regulated by several mechanisms including modifications of the initiation factors involved in the process as well as regulation by microRNAs.

The principal method of translation initiation occurs by means of a cap-dependent scanning mode, which is the primary source of de novo synthesized cellular proteins under normal growth conditions (Figure 1.1). This process requires the involvement of many eukaryotic initiation factors (eIFs), which themselves can be regulated to control rates of protein synthesis, as will be discussed below. In brief, the cap-dependent process involves the recognition of the 5' m^7G cap structure, invariably present on all mature cellular messenger RNAs (mRNAs), by the eIF4F complex, comprised of the cap binding protein eIF4E, the scaffold protein eIF4G, and the RNA helicase eIF4A. Separately, the formation of the 43S pre-initiation complex occurs through the association of the 40S ribosomal subunit with eIF3, eIF1A, and eIF2 bound to the initiator methionyl transfer RNA (Met-tRNA_i^Met). The 43S pre-initiation complex is then recruited to the mRNA through the interaction
Figure 1.1  Schematic diagram outlining the key points of regulation during translation initiation. For simplicity, not all initiation factors are shown. Initiation factors that are described in this review are indicated with asterisks. (Adapted from [123])
between eIF3 and eIF4G and is believed to subsequently scan the mRNA until it locates the initiation codon, typically AUG, in an appropriate context. Subsequently, joining of the 60S ribosomal subunit occurs, which forms the translationally competent 80S ribosome, while the eIFs are released and recycled for the next round of initiation. Furthermore, poly(A)-binding protein (PABP) associates with the poly(A) tail on the 3’ terminus of mRNA and is thought to interact with eIF4G causing circularization of the mRNA to enhance translation as well as to protect the mRNA from degradation (see Figure 1.1 for details, reviewed in [1]).

The process of translation consumes a significant amount of cellular energy (estimated to be as much as 50%, depending on the organism [2]). It is therefore not surprising that exposure of cells to majority of environmental stressors such as hypoxia, irradiation, or nutrient deprivation leads to modifications of the eIFs involved in the regulation of cap-dependent translation, ultimately resulting in attenuation of global protein synthesis. In addition to saving cellular energy, the attenuation of translation prevents synthesis of unwanted proteins that could obstruct the cellular stress response. Under these conditions, cells are able to cope with the stress or, if the damage to the cell is beyond repair, to initiate apoptosis. To facilitate the decision making process, some proteins, in particular those required for the stress response, are selectively translated even though cap-dependent translation is attenuated. It is the relative levels of these pro- and anti-apoptotic proteins that are important in tipping the balance in favour of survival or cell death. The question of how is a cell able to translate proteins when the required eIFs for cap-dependent translation are not available is at the centre of investigations in many laboratories, and a subject of this review.
One important mechanism that has acquired recent attention is the internal ribosome entry site (IRES) mediated translation initiation process that utilizes specialized RNA elements to selectively recruit ribosomes to mRNA without a need for the cap structure [3]. IRES elements are found in the 5' untranslated region (UTR) of mRNAs and were initially discovered in RNAs of picornaviruses [4]. Although the RNAs of these viruses do not contain a m$^7$G cap, they are still effectively translated. In addition, many viruses encode proteases that cleave several canonical eIFs in order to block translation of host proteins. For example, upon infection of cells with polio virus, the virus-encoded protease 2A specifically cleaves eIF4G thus inactivating the eIF4F complex and effectively preventing ribosome recruitment to capped cellular mRNAs. This ensures that the host cell's translational machinery is now available for virus protein translation [5]. Importantly, the polio virus IRES element is able to utilize the cleaved eIF4F complex and recruit the ribosome for efficient translation of its own proteins. In other viruses, such as the hepatitis C virus (HCV), the presence of eIF4F is not required at all and the IRES is able to recruit the ribosome in its absence [6]. Thus, even with the loss of some eIFs, the viral IRES elements are able to recruit the ribosome for efficient translation. These observations led researchers to study cellular mRNAs to determine if a similar mechanism(s) exists. In recent years, it has been proposed that an estimated 10% of all cellular mRNAs may contain IRES elements. Interestingly, many of these mRNAs encode proteins involved in processes such as cell proliferation and apoptosis, and are critical in determining the survival of a cell under physiological and pathophysiological stress conditions [3]. For example, IRESs have been identified in mRNAs encoding XIAP, cIAP1, Bcl-xL, Bcl-2, Bag-1, Apaf-1, p53, c-myc, DAP5, all proteins that are critically involved in the regulation of cell survival.
Although the mechanism of IRES-mediated translation is still poorly understood, it has become evident that not all cellular IRES elements act in a similar manner. That is, most cellular IRES elements require binding of some of the canonical initiation factors as well as for other protein factors termed ITAFs (IRES-trans acting factors) that modulate the IRES activity [3]. Most of the ITAFs identified thus far are RNA binding proteins that fulfill a variety of functions including involvement in mRNA splicing (for a review on splicing in apoptosis see [7]), export, stress granule formation, as well as important roles in translation initiation. The binding of ITAFs can either enhance or repress IRES activity; it is thought that the positive regulators act either as RNA chaperones that aid in the formation of the proper IRES structure, or directly recruit the ribosome. The precise mechanism of how the repressive ITAFs function is not clear. Interestingly, many ITAFs shuttle between the nucleus and cytoplasm and this shuttling is regulated by posttranslational modifications such as phosphorylation in response to a variety of triggers. Therefore, the cytoplasmic availability of positive or negative regulators can determine the extent of IRES translation (see below).

1.9 Global translation regulation during apoptosis: modifications of translation initiation factors

Induction of apoptosis is accompanied by a pronounced down-regulation of protein synthesis [8]. This inhibition in global translation rates is characterized by a decrease in polysome chains, suggesting that at least some regulation occurs at the translation initiation step [9]. Indeed, there is extensive evidence that apoptosis triggered by different stimuli leads to modifications in a defined set of canonical initiation factors that ultimately results in the
inhibition of translation initiation (reviewed in [8, 10]). These modifications generally consist of changes in phosphorylation status (e.g. eIF2α, eIF4E, eIF3, eIF4E binding proteins (4E-BPs)) or protein cleavage by caspases or viral proteases (e.g. eIF4G, eIF4B, eIF3 (Table 1.1; Figure 1.2)).

1.9.1 eIF2α

eIF2 plays a central role in translation initiation by bringing the initiator Met-tRNA to the 40S ribosomal subunit for the formation of the 43S pre-initiation complex. eIF2 is composed of three subunits (α, β and γ), of which the γ subunit is bound by GTP that is later hydrolyzed during translation initiation [1]. GDP to GTP exchange is necessary for regenerating active eIF2 and this process is catalyzed by eIF2B (Figure 1.1). However, in response to different stress stimuli, the α subunit of eIF2 is phosphorylated at serine 51 (Ser51), thus increasing its affinity for eIF2B and trapping the two in an inactive complex [11]. As a result, pre-initiation complex formation and global mRNA translation are inhibited. eIF2α phosphorylation is mediated by four different kinases that are activated by various stress triggers (reviewed in [1]): HRI (haem-regulated inhibitor) which is activated by iron deficiency, heavy metals, osmotic or oxidative stress and heat shock; PKR (protein kinase activated by double-stranded RNA) which is activated by double stranded RNA from viral infections or interferon-induced apoptosis; GCN2 (general control non-derepressible-2) which is activated by amino acid starvation; and PERK (PKR-like endoplasmic reticulum kinase) which is activated during the unfolded protein response (UPR).

The link between eIF2α phosphorylation and apoptosis is not straightforward, and eIF2α phosphorylation can either be a cause or consequence of the cell’s commitment to apoptosis. For instance, in MCF-7 breast cancer cells treated with TNFα or TRAIL (TNFα-
Table 1.1  *Modification of translation initiation factors during apoptosis.* Table showing selected eukaryotic translation initiation factors that are modified during apoptosis induced by different triggers. The type of modifications and their consequence for translation and cell survival, along with key references are shown on the right.
Table 1: Modifications and their consequence for translation and cell survival, along with key references are shown on the right.

<table>
<thead>
<tr>
<th>Translation initiation factors</th>
<th>Modifications</th>
<th>Effects</th>
<th>Apoptotic triggers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF2</td>
<td>Phosphorylation of eIF2α subunit at Ser51</td>
<td>Inhibition of GDP to GTP exchange on eIF2: • inhibition of global translation and apoptosis • translation of specific transcripts and cell survival</td>
<td>Iron deficiency, heavy metals, osmotic or oxidative stress, heat shock, double stranded RNA, amino acid starvation, unfolded protein response (UPR)</td>
<td>[1, 11]</td>
</tr>
<tr>
<td>eIF4E</td>
<td>De-phosphorylation</td>
<td>Global translation inhibition.</td>
<td></td>
<td>[20]</td>
</tr>
<tr>
<td>4E-BPs</td>
<td>De-phosphorylation</td>
<td>Competition with eIF4G on eIF4E. Global translation inhibition and apoptosis</td>
<td></td>
<td>[12, 16, 21, 22]</td>
</tr>
<tr>
<td>4E-BPs</td>
<td>Cleavage by caspases at Asp-24</td>
<td>Cleaved form strongly binding eIF4E and inhibition of cap-dependent translation</td>
<td>Staurosporine, etoposide, p53 activation</td>
<td>[21, 22]</td>
</tr>
<tr>
<td>eIF4GI and eIF4GII</td>
<td>Cleavage by caspase 3 at Asp-532 and Asp-1176 of eIF4GI, Cleavage by caspase 3 at Asp-560, 851, 978, 1162 and 1407 of eIF4GII.</td>
<td>Cleaved forms (except for M-FAG from eIF4GI) cannot bridge eIF4E, 4A and eIF3 together. Global translation and attenuation of anti-apoptotic response</td>
<td>UPR, Fas receptor activation</td>
<td>[3, 9, 16]</td>
</tr>
<tr>
<td>p97/DAP5/ NAT1</td>
<td>Cleavage by caspase 3 at Asp-790</td>
<td>p86 fragment with eIF4A and eIF3 binding sites but no eIF4E site. Inhibition of cap-dependent translation but stimulation of specific IRES-dependent translation</td>
<td></td>
<td>[18, 26–28]</td>
</tr>
<tr>
<td>eIF4B</td>
<td>Cleavage by caspase 3 at Asp-45</td>
<td>Fragment is still able to interact with eIF4F and eIF3 but lacks the region mediating PABP binding</td>
<td>Cycloheximide, Fas receptor activation</td>
<td>[16, 31–33]</td>
</tr>
<tr>
<td>eIF3j (p35)</td>
<td>Cleavage by caspase 3 at Asp-242</td>
<td>Reduced affinity of the eIF3 complex for the 40S ribosomal subunit. Inhibition of global protein translation</td>
<td>Cycloheximide, Fas receptor activation</td>
<td>[16, 29]</td>
</tr>
<tr>
<td>eIF3H (p47)</td>
<td>Phosphorylation</td>
<td>Enhanced association with the core subunits of eIF3. Inhibition of global protein translation</td>
<td>Staurosporine</td>
<td>[30]</td>
</tr>
</tbody>
</table>
**Figure 1.2** A model of the interconnectedness of translation and apoptosis. Only factors pertinent to this review are shown. The left side of the model shows regulation of cap-dependent translation; the right side depicts IRES-mediated translation. Green lines indicate positive, while red line negative interactions. Dotted line depicts indirect effect.
related apoptosis-inducing ligand) eIF2α phosphorylation by PKR is dependent on caspase 8 activity and happens several hours before the onset of apparent apoptosis [12, 13]. In contrast, in mouse embryonic fibroblasts treated with TNFα or deprived of serum, eIF2α phosphorylation is necessary to induce caspase 3 activation and subsequently apoptosis [14]. Interestingly, forced expression of a phosphomimetic S51D mutant of eIF2α is sufficient to activate caspase 3 and induce apoptosis in the absence of any other triggers, whereas expression of a non-phosphorylatable S51A mutant protects cells from TNFα or serum deprivation. It has also been reported that eIF2α itself can be cleaved in apoptotic cells, mainly by caspase 3, but also by caspases 6, 8 and 10 [15, 16]. The functional relevance of this cleavage product is not fully understood but it has been shown that its GTP exchange rate was higher, independent of eIF2B, and that it may contribute to translation inhibition [15].

In contrast to inhibiting global translation, eIF2α phosphorylation can up- or downregulate selective translation. For example, under hypertonic stress, eIF2α phosphorylation was shown to induce cytoplasmic accumulation of heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), a known ITAF, which in turn inhibited IRES-mediated translation of the anti-apoptotic proteins XIAP and Bcl-xL, thus sensitizing the cells to apoptosis [17]. Interestingly, in the context of the adaptive UPR, eIF2α phosphorylation can be a signal that promotes cell survival rather than apoptosis. In this context, eIF2α phosphorylation by PERK leads to the selective translation of transcription factors, such as ATF4 that controls the expression of pro-survival and anti-apoptotic proteins such as cIAP1 [3, 18].

1.9.2 eIF4E

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The cap-binding protein eIF4E is another initiation factor whose availability is regulated by phosphorylation during apoptosis. eIF4E is phosphorylated at Ser209 by the MAPK integrating kinases Mnk1 and Mnk2 in response to different stimuli such as treatment of cells with growth factors, anisomycin or UV, that activate the ERK and p38 MAPK pathways [19]. eIF4E phosphorylation reduces its affinity for the 5’ cap structure, thus stimulating translation initiation. Conversely, eIF4E dephosphorylation by protein phosphatase A leads to inhibition of global translation. However, the eIF4E binding to the 5’ cap is regulated mainly through the phosphorylation status of eIF4E binding proteins (4E-BPs). 4E-BP1, 2, and 3 are proteins that share structural similarities to the fragment of eIF4G responsible for binding to eIF4E. 4E-BPs are phosphorylated in normal growth conditions by the mTOR signaling pathway (reviewed in [20]). However, during apoptosis induced by DNA damage, TRAIL, the protein kinase inhibitor staurosporine, or the mTOR inhibitor rapamycin, there is a decrease in 4E-BPs phosphorylation [12, 16, 21, 22]. Hypophosphorylated 4E-BPs have a higher affinity for eIF4E and as a consequence, they competitively prevent eIF4G from binding to eIF4E, thus reducing the availability of the eIF4F complex and resulting in inhibition of translation (reviewed in [1]). Similar to eIF2α phosphorylation, loss of 4E-BPs phosphorylation occurs during the early phase of the apoptosis cascade [12, 16], leading to global translation inhibition and commitment to cell death. For example, ectopic expression of a non-phosphorylatable mutant of 4E-BP1 sensitizes multiple myeloma cells to dexamethasone-induced apoptosis [23]. Furthermore, apoptotic triggers such as staurosporine, etoposide or activation of p53 can lead to caspase-mediated cleavage of 4E-BP1 [21, 22], producing cleaved form that binds strongly to eIF4E and inhibits cap-dependent translation [24].
1.9.3 eIF4G

The availability and function of the initiation factors eIF4G1 and eIF4GII is regulated during apoptosis primarily through cleavage by caspases. Upon treatment of cells with apoptotic triggers such as TNFα, TRAIL, cisplatin or etoposide, eIF4G1 and eIF4GII are cleaved by caspase-3 at two different sites. This gives rise to three cleavage products named Fragments of Apoptotic cleavage of eIF4G (N-FAG, M-FAG and C-FAG [9, 16, 25]. The middle fragment, M-FAG, retains its ability to interact with eIF4A, eIF4E and eIF3, and supports cap-dependent translation during the early phase of apoptosis. However, M-FAG is degraded with prolonged exposure to stress resulting in the inhibition of cap-dependent translation and attenuation of the anti-apoptotic response. Interestingly, cleavage of the eIF4G-related protein factor p97/DAP5/NAT1 by caspases releases a p86 isoform that stimulates the IRES-mediated translation of apoptosis regulating factors such as XIAP, cIAP1, c-myc, APAF1 and p97/DAP5 itself [18, 26-28]. Hence, cleavage of eIF4G and p97/DAP5 proteins regulates the fate of the cell by tipping the balance between the translation of pro- and anti-apoptotic factors.

1.9.4 eIF3

eIF3 is a critical factor that bridges the binding between the 43S ribosome and eIF4F-bound mRNA. It has been reported that eIF3j (p35) is cleaved during apoptosis in BJAB cells treated with anti-Fas or cycloheximide [16]. eIF3j cleavage occurs in a caspase-3 dependent manner and results in reduced affinity of the eIF3 complex for the 40S ribosomal subunit and subsequent inhibition of global translation [29]. Similarly, the p47 subunit of eIF3, eIF3f, is phosphorylated by CDK11 during staurosporine-induced apoptosis of the human melanoma cell line A376. eIF3f phosphorylation results in its enhanced association
with the core subunits of eIF3 and sequestration in insoluble complexes, leading to an inhibition of protein synthesis and induction of apoptosis [30].

1.9.5 eIF4B

The initiation co-factor eIF4B which stimulates eIF4A helicase activity and ribosome binding to the mRNA, is also modified during apoptosis. eIF4B is cleaved in its C-terminal region by caspase 3 both \textit{in vitro} and in BJAB cells treated with anti-Fas or cycloheximide [16]. However, eIF4B can also be cleaved in a caspase-3 independent manner in apoptotic MCF-7 cells that lack caspase 3 [12]. The N-terminal fragment of eIF4B is still able to interact with eIF4F and eIF3 [16]; however, it lacks the region that mediates its interaction with PABP [31]. The effects of eIF4B truncation on translation and apoptosis are not well characterized. However, a substantial amount of work has been done on elucidating eIF4B's role in cell survival and proliferation (reviewed in [32]). For instance, eIF4B depletion from HeLa cells using RNA interference was shown to selectively reduce the translation of genes involved in cell proliferation (such as cdc25C, c-myc, and ornithine decarboxylase) and survival (such as Bcl-2 and XIAP). Moreover, eIF4B depletion caused a decrease in HeLa cells proliferation rates, enhanced apoptosis and sensitized these cells to camptothecin-induced cell death [33].

In general, modifications of translation initiation factors, whether they are a cause or a consequence of the initiation of the apoptotic cascade, are aimed at inhibiting global protein synthesis. This general inhibition of translation contributes to the shutdown of all cellular processes, and is believed to conserve cellular energy and prevent the synthesis of protein factors that could stall the apoptotic process. However, in cases of adaptive stress,
translation can be reprogrammed such that the translation of specific mRNA transcripts continues and influences the fate of the cell (Figure 1.2).

1.10 Selective translation via IRES

Despite the cessation of global protein synthesis during the early phase of apoptosis, selective translation of specific mRNAs can continue via the IRES mechanism, as described above. Several key regulators of cell death were shown to be translated via IRES elements; here we focus on the regulation of translation of cIAP1, XIAP and p53.

1.10.1 Cellular Inhibitor of Apoptosis 1 (cIAP1)

The inhibitor of apoptosis (IAP) family of proteins is comprised of eight members in mammals that regulate many key cellular processes including signaling, cell division, and apoptosis, and are the subject of another review in this special issue (D. Vucic, this issue). cIAP1 is a key regulator of nuclear factor-κB (NF-κB) dependent signaling and caspase-8 mediated cell death in mammalian cells. The abundance of cIAP1 in the cell is regulated at multiple levels: at the transcriptional level by the transcription factor NF-κB [34], at the protein stability level by autoubiquitination [35], and at the mRNA stability level by an AU-rich sequence (ARE) in its 3'UTR [36]. Importantly, cIAP1 expression in response to apoptotic triggers is mainly regulated at the level of protein translation. The cIAP1 mRNA has a long (1.2 kb) and highly structured 5'UTR that contains 23 AUG codons and two upstream open reading frames (uORF) which contribute to inhibition of its basal translation. In fact, the upstream ORF was shown to severely inhibit translation of the downstream ORF, thus explaining the low levels of cIAP1 observed under normal growth conditions [37]. Several studies have now established that cIAP1 expression is selectively upregulated in
response to apoptotic stress [18, 38-40]. Indeed, cIAP1 protein expression is upregulated via an IRES-dependent mechanism in response to tunicamycin- or thapsigargin-induced endoplasmic reticulum (ER) stress, in conditions where global protein synthesis is inhibited [18, 41]. The relevance of cIAP1 IRES-mediated translation to ER stress induced apoptosis was further shown by the fact that cIAP1 overexpression attenuated tunicamycin-induced death in HeLa cells, whereas cIAP1 depletion by RNA interference enhanced sensitivity to tunicamycin [18]. The importance of an IRES that drives cIAP1 expression to inhibit apoptosis was also demonstrated in the context of different apoptotic triggers such as etoposide or sodium arsenite treatment [38] and viral infection [40].

Interestingly, activation of the cIAP1 IRES in the context of ER stress is dependent on caspase activation and is accompanied by cleavage of eIF4GI and its homolog p97/DAP5/NAT1 during the early phase of the UPR [18]. As mentioned above, the p97/DAP5/NAT1 cleavage product, p86/DAP5 functions as an ITAF that stimulates the activity of several IRES elements including cIAP1 [18, 26-28]. Indeed, ectopic overexpression of a p86/DAP5 fragment, but not the full length p97/DAP5 protein, in both HEK293T and rabbit reticulocyte lysates was able to specifically drive cIAP1 IRES activity and increase cIAP1 endogenous protein levels [18]. It was later shown that both p97/DAP5/NAT1 and p86/DAP5 bind to the cIAP1 IRES, possibly through association with other accessory proteins [18, 41].

The structure of the cIAP1 IRES and the proteins that specifically interact with this IRES were characterized recently. One of these proteins, NF45, enhances cIAP1 IRES-dependent translation and mediates cIAP1 induction in response to thapsigargin-induced ER stress and UPR [39]. NF45 is an NFAT-related transcription factor that was first identified to regulate interleukin-2 transcription, together with its binding partner NF90 [42]. NF45 and
NF90 are involved in several cellular processes such as transcription [43], viral replication [44] and microRNA processing [45] and our study confirmed its role in IRES-mediated translation [39]. More recently, NF45 has been implicated in mitotic control in HeLa cells since depletion of NF45/NF90 complexes by RNA interference in these cells leads to the generation of large multinucleated cells, a result of impaired cytokinesis and cell growth due to defects in DNA break repair [46]. In line with this new role for NF45, we have recently discovered that NF45 preferentially regulates a cohort of AU-rich IRES-containing mRNAs including cIAP1 and XIAP, which are responsible for the multinucleated phenotype of NF45-deficient cells (MDF and MH unpublished observations). Loss of NF45 results in reduced IRES-mediated translation of XIAP and cIAP1 mRNAs. Interestingly, the resulting decrease in XIAP expression causes an increase in Survivin protein levels, likely due to Survivin protein stabilization [47]. Survivin, another member of the IAP family, plays an important role in microtubule spindle checkpoint regulation and its aberrant expression leads to cytokinesis defects [48], thus explaining the multinucleated phenotype of NF45-deficient cells. Similarly, through its control of cIAP1 translation NF45 regulates cyclin E expression. Nuclear cIAP1 was shown to transcriptionally regulate cyclin E [49], and we found that either NF45 or cIAP1 depletion caused a decrease in cyclin E expression that is rescued by NF45 re-expression. Coordinated changes in cyclin E and Survivin expression in NF45-depleted cells would lead to a block in cell cycle, mitotic catastrophe and defects in cytokinesis thus explaining the senescence-like phenotype of these cells. These observations uncovered a novel role for NF45 in controlling ploidy and highlight the importance of IRES-mediated translation in the regulation of mitosis, cell growth and apoptosis.

1.10.2 X-linked inhibitor of apoptosis protein (XIAP)
XIAP, a prototype member of the IAP family is a direct inhibitor of caspases 3, 7, and 9. Given XIAP’s key role in inhibiting caspases, it is not surprising that misregulation of its expression is associated with tumourigenesis and cancer. Importantly, elevated levels of XIAP, as is observed in many cancers, have been linked to enhanced chemo- or radiation resistance, whereas reduction of XIAP through chemical inhibitors can restore chemosensitivity [50].

Studies into the regulation of XIAP expression led to the discovery of an IRES element located in its 5'UTR region which mediates XIAP protein translation under conditions of cellular stress such as γ-irradiation or nutrient deprivation, thus providing the cell with protection against apoptosis [51]. Interestingly, XIAP protein is encoded by two mRNA splice variants that differ only in their 5'UTR regions [52]. The more abundant, shorter transcript produces the majority of XIAP protein under normal growth conditions by cap-dependent translation. However, during cellular stress, the longer transcript that contains the IRES element supports efficient translation even though global cap-dependent translation is attenuated [52]. The secondary structure of the XIAP IRES and its associated ITAFs has been determined [53]. Some of these, such as La autoantigen [54], hnRNP C1/C2 [55], and HuR [56] have been shown to enhance XIAP IRES translation, whereas others, such as hnRNP A1 [57], PTB [53] and PDCD4 [58] have been shown to repress XIAP IRES translation.

Interestingly, cytoplasmic localization of XIAP ITAFs appears to play a key role in the regulation of XIAP translation in response to stress. For example, osmotic shock causes an accumulation of hnRNP A1 in the cytoplasm by activating the mitogen-activated protein kinase kinase3/6-p38 signaling pathway resulting in phosphorylation of hnRNP A1, thus preventing its import into the nucleus [59]. Once in the cytoplasm, hnRNP A1 binds with the
XIAP IRES and inhibits protein expression [57]. Another example of an ITAF being regulated at the level of localization was shown by Gu et al. [60] in acute lymphoblastic leukemia (ALL) cells treated with ionizing radiation (IR). They observed that IR treatment resulted in the misregulation of the oncogene MDM2. MDM2 overexpression is observed in many cancers and correlates with poor patient outcome because it binds to and inhibits the activity of the tumour suppressor p53 [60]. It is known that the phosphorylation status of MDM2 dictates its localization such that survival signals promote nuclear localization and cell proliferation whereas cellular stress results in dephosphorylation of MDM2 and subsequent retention in the cytoplasm. However, the cytoplasmic function of MDM2 was not well understood. Upon treatment with IR, dephosphorylated MDM2 is retained in the cytoplasm and is no longer associated with its main target, p53 [60]. Instead, cytoplasmic MDM2 is able to directly and specifically bind to the XIAP IRES. It is interesting to note that many cancers express elevated levels of a mutated form of MDM2 that does not contain the N-terminal p53 binding domain [61] and it is this remaining C-terminal portion of MDM2 that is responsible for interacting with the XIAP IRES and upregulating its IRES-mediated translation. Importantly, the MDM2-mediated increase in XIAP expression leads to enhanced resistance to IR-induced apoptosis.

Similar to DNA damage or osmotic shock, cell proliferative stimulation also results in stimulation of IRES translation. For example, treatment of small cell lung cancer (SCLC) cells with the fibroblast growth factor (FGF) 2 protects them from etoposide induced cell death by upregulating the anti-apoptotic proteins XIAP and Bcl-xL. It was shown that a complex forms between S6 kinase 2 (S6K2), BRaf, and PKCɛ leading to activation of S6K2 in response to FGF 2 [62]. We have identified the target of activated S6K2 as programmed cell death 4 (PDCD4; [58]). PDCD4 is a known tumour suppressor and its loss has been
correlated with more aggressive and invasive tumours [63]. The FGF2-activated S6K2 phosphorylates PDCD4, leading to its proteasomal degradation. Furthermore, we identified XIAP and Bcl-xL as two novel translational targets of PDCD4. We showed that the N-terminal portion of PDCD4 was responsible for directly binding to XIAP and Bcl-xL IRES RNA both in vitro and in vivo and the loss of PDCD4 correlated with an increase in XIAP and Bcl-xL protein expression. This response to FGF-2 is a critical factor in tumour formation and resistance to apoptosis because mutations in cancer cells typically lead to an acquired ability of the cells to produce growth factors and stimulate proliferation through autocrine signalling (reviewed in [64]).

1.10.3 Tumour suppressor p53

p53 is a tumour suppressor that plays a major role in the regulation of cell cycle progression and apoptosis in response to cellular stress, mainly DNA damage and genomic instability [65]. p53 also plays a central role in the process of oncogenesis as its gene is mutated in more than 50% of all human cancers [66] and as such, p53 remains one of the most highly studied genes. It is now well established that p53 protein levels and activity increase in response to DNA damage and that regulation of this process occurs mainly at the level of protein stability by the ubiquitin ligase MDM2 [67]. However in recent years, there has been accumulating evidence that translational control is important in the induction of p53 expression in response to cellular stress (reviewed in [68]). Evidence of p53 translational control in response to cellular stress include, but is not limited to: (i) the fact that cycloheximide - a protein elongation inhibitor- prevents the increase in p53 protein levels normally observed after IR-induced [69] or etoposide-induced [70] DNA damage, (ii) the fact that there is an increase in p53 mRNA in polyribosomes upon IR exposure [71] or
etoposide treatment [70], and (iii) the fact that *de novo* protein synthesis rates of the p53 mRNA increase in response to DNA damage caused by IR [71, 72], UVC [73], etoposide [70], doxorubicin or in response to tunicamycin-induced ER stress [74].

In the past ten years, there has been more focus on understanding the mechanisms underlying p53 translation induction in response to cell stress. Yang *et al.* [70] were the first to report that the p53 mRNA can be translated in a cap-independent manner in MCF-7 breast cancer cells and subsequently identified an IRES within the p53 5'UTR that is induced more than 2-fold during etoposide-induced DNA damage. Moreover, a second study showed that the p53 5'UTR was able to direct *in vitro* translation of the p53 mRNA in the absence of a cap structure [75], further confirming the existence of a p53 IRES.

Interestingly, Ray *et al.* [75] proposed a model in which two different IRES structures control the translation of two different p53 isoforms, namely the full-length p53 protein (FL-p53) and the ΔN-p53 (p40/47) isoform. The ΔN-p53 protein is translated from an alternative initiation codon situated within the coding sequence, 40 nucleotides downstream of the FL-p53 translation start site [76, 77]. It has been suggested that ΔN-p53 acts as a dominant-negative form that antagonizes p53-mediated transcription and growth regulation [76]. However it appears that ΔN-p53 functions are much more complex since the protein does not contain an MDM2 binding site, is able to oligomerize with FL-p53 to induce different transcription patterns [77], and induces apoptosis when expressed in p53-null cells [76]. They further showed that expression of the two p53 isoforms is regulated in a cell-cycle dependent manner, *via* an IRES mechanism of translation [75]. In fact, the IRES driving FL-p53 protein expression is more active during the G2/M transition when p53 activity is required the most, whereas the IRES driving ΔN-p53 expression is active during the G1/S transition [75]. These findings are more consistent with ΔN-p53 being an antagonist of p53
activity where at the G1/S phase it would drive the expression of genes necessary for
transition through the cell cycle. Differential regulation of FL-p53 and ΔN-p53 via
translational control may also have an effect on cell sensitivity to apoptosis. For instance,
doxorubicin-induced DNA damage and tunicamycin-induced ER stress give rise to different
patterns of p53 isoform expression where H1299 lung carcinoma cells overexpressing the
ΔN-p53 are less sensitive to doxorubicin treatment and more sensitive to tunicamycin [74].
Thus, translational control via the IRES is an important mechanism by which p53 can
integrate and respond to the different apoptotic or proliferative cues the cell is exposed to.
Another layer of complexity is brought about by the different ITAFs that can bind to the p53
IRES and modulate its activity in response to stress. The ribosomal protein L26 [72], PTB
[78] and hnRNP C1/C2 [79] were all shown to enhance p53 expression, whereas nucleolin
was shown to repress it [72]. The La autoantigen, hnRNP U and p53 itself might also be
potential p53 IRES trans-acting factors [72]. PTB binds specifically to the p53 IRES
structure and a reduction in PTB protein levels by RNA interference leads to a decrease in
IRES activity and blunting of p53 isoforms induction in the presence of doxorubicin [78]].
Furthermore, treatment of A549 human lung carcinoma cells with doxorubicin causes PTB
translocation from the nucleus to the cytoplasm, corresponding with an increase in p53
expression. Interestingly PTB cytoplasmic levels are maximal at the G2/M phase and low at
the G1/M transition [80], suggesting that PTB might be the factor contributing to increased
p53 translation during the G2/M checkpoint [78]. These results further support the notion that
cell stressors can alter the expression level, cellular localization or status of different ITAFs
to modulate the output of p53 protein available to respond to the stress and decide the fate of
the cell.
Most of the work done on p53 in the context of carcinogenesis was aimed at characterizing the effects of p53 coding region mutations on the transcriptional activities of the protein. However, it has become apparent that mutations can also occur within p53 5'UTR and may have relevance to the pathology of cancer [81]. Indeed, a cancer-derived triple silent mutation at positions 185, 188 and 191 that was previously shown to alter MDM2 binding to the p53 mRNA [81], as well as a single silent mutation at position 200 of the p53 5'UTR [81] were found to alter p53 IRES activity, alter the profile of ITAFs binding to the IRES and blunt the IRES induction in response to doxorubicin [82]. Thus, it is possible that mutations within the p53 IRES may lead to carcinogenesis by decreasing p53 induction and protective activity in response to DNA damage. The recent characterization of the p53 IRES structure [82] may help in identifying more cancer-derived mutations that are relevant to p53 function and that could be used as predictors of response to certain cancer treatments.

Another aspect that may be relevant to the pathology of cancer was the recent finding that p53 IRES-dependent translation is impaired during oncogene-induced senescence (OIS) in DKC1<sup>m</sup> cells [83]. The DKC1 gene encodes the dyskerin protein which is responsible for modifying uridines in ribosomal RNA into pseudouridines, and mutations in DKC1 have been linked to the development of X-linked dyskeratosis congenita (X-DC). X-DC patients have increased susceptibility to cancer, as reflected by the fact that more than 50% of DKC1<sup>m</sup> mice develop tumours of different origin [84]. Interestingly, Yoon <i>et al.</i> [85] showed that DKC1-mutated cells are impaired in IRES-mediated translation, providing one of the first <i>in vivo</i> links between IRES-mediated translation and the onset of oncogenesis. In addition, during OIS, in which p53 translation is normally induced to counteract the oncogenic insult [86], p53 IRES-mediated translation is impaired both in DKC1<sup>m</sup> mice cells and in X-DC patient derived cells. This results in a significant decrease in p53 protein
induction and of its target genes p21 and MDM2 in response to etoposide treatment or γ-irradiation which correlated with a reduction in the number of apoptotic cells as compared to wild-type [83]. These results were corroborated by an independent group that showed that DKC1 knock-down in both MCF-7 breast cancer cells and in primary breast cancer cells caused a decrease in p53 IRES-mediated translation, which led to a decrease in p53 transcriptional activity and apoptosis upon doxorubicin treatment [87]. Together, these findings show that defects in p53 IRES-mediated translation are relevant not only to the OIS process but also to carcinogenesis, especially in the context of the X-DC pathology.

1.11 MicroRNA mediated regulation

MicroRNAs (miRNAs) are small, non-coding RNA sequences of approximately 21 nucleotides in length that regulate gene expression post-transcriptionally by binding to target mRNAs to silence their expression. miRNAs play a significant role in regulating processes as diverse as development, metabolism, cell proliferation, and apoptosis [88]. In humans, over 500 miRNAs have been identified so far and each miRNA has multiple targets, therefore it is thought that about 10,000 mRNAs could be regulated by miRNAs.

miRNAs are transcribed from the genome by RNA polymerase II or III as long, double-stranded hairpin transcripts containing a 5’cap and 3’ poly-A tail, termed primary miRNA (pri-miRNA) [89]. Pri-miRNAs are further processed into a smaller double stranded structure in the nucleus by the RNase III-like enzyme Drosha and DGCR8 to produce the precursor miRNA (pre-miRNA) [90] that are subsequently are exported into the cytoplasm by exportin-5 where they are further processed by the RNase III enzyme Dicer, yielding an approximately 22 nucleotide long, double-stranded product [91]. Only one of the miRNA
strands is then incorporated into the RNA-induced silencing complex (RISC) containing the argonaute (AGO) protein, while the other strand is degraded [89, 92]. Upon recognition of their target mRNA via near-perfect complementarity, miRNAs can direct the degradation of the target mRNA by the 5’-to-3’ mRNA decay pathway which involves deadenylation by the CAF1-CCR4-NOT deadenylase complex, followed by decapping by DCP2, and ultimately degradation by the exonuclease XRN1 [93, 94]. As well, miRNAs that bind with near perfect complementarity can direct endonucleolytic cleavage of their target mRNAs through the catalytically active Argonaute protein present in the RISC complex [95]. On the other hand, miRNAs can also bind their target mRNAs via imperfect complementarity resulting in a loss of protein product but no change in mRNA levels suggesting that an inhibition of translation occurs rather than degradation of mRNA. Early studies showed that these inhibited mRNAs were found associated with polysomes indicating that repression occurred at a post-initiation stage, likely during translation elongation [96, 97]. However, in recent years, it has been suggested that miRNAs inhibit translation initiation by interfering with the eIF4F and the poly-A binding complexes. Further evidence for miRNAs affecting the initiation step of translation was provided by Humphreys et al. [98] who showed that a construct containing the IRES element of cricket paralysis virus (CrPV) lacking a cap and poly-A structure was unaffected by miRNAs, suggesting that the initiation step was the target of miRNA regulation, since regulation of elongation or termination should still occur in the presence of the CrPV IRES. These data strengthen the link between miRNA function and translational control which is important not only during times of cellular stress, but also during the cell’s decision to undergo apoptosis. As mentioned above, many mRNAs that are involved in the cellular stress response contain IRES elements, which not only allow the mRNA to be translated during attenuation of cap-dependent translation, but may also aid in protecting the
mRNA from miRNA induced silencing. Furthermore, cytoplasmic processing bodies (P-bodies), which are involved in processes such as translation inhibition and mRNA degradation, have been suggested to be involved in retaining repressed mRNAs in the RISC complex thus preventing translation [99-101]. Interestingly, certain stressors can cause release of some repressed mRNAs from the P-bodies where they are able to re-enter into polysomes for efficient translation [102]. For example, in Huh7 hepatoma cells, the cationic amino acid transporter (CAT-1) mRNA is found in the P-bodies. However, upon exposure to amino acid starvation, the RNA binding protein HuR is relocalized from the nucleus to the cytoplasm where it binds the CAT-1 mRNA and releases it from the P-bodies. This allows translation of the mRNA to respond to the cellular stress. It is likely that this mechanism of release under stress occurs for other mRNAs in response to specific stressors and the combination of these mechanisms allows the cell to quickly respond to its changing environment by translating pro- or anti-apoptotic proteins that are crucial for deciding the fate of the cell. Although the exact mechanism of miRNA-mediated inhibition is still under debate, the result of inhibiting protein translation remains unchanged.

Interestingly, miRNAs have been identified as both tumour suppressors and oncogenes involved in tumour development, and mis-regulation of miRNA expression has been linked to cellular transformation. It has been suggested that as many as 50% of miRNAs are located in unstable regions of chromosomes that are prone to being amplified or deleted in many cancers [103]. Furthermore, proteins that are frequently mis-regulated or mutated in cancers can affect the levels of miRNAs. For example, upon DNA damage, p53 interacts with the Drosha complex to enhance the processing of select pri-miRNAs involved in apoptosis and cell proliferation [104]. However, inactive p53 mutants that are commonly
found in many cancers (for example p53 mutated at C135Y, R175H and R273H) prevent the interaction of p53 with the Drosha complex, therefore attenuating the processing of these miRNAs. Many miRNAs have been implicated in regulating expression of apoptotic proteins, thus altered levels of these miRNAs can have negative effects on the cells ability to respond to apoptotic cues, resulting in a lack of cell death and enhanced proliferation, ultimately leading to tumour growth and survival. (For a detailed review on miRNAs see [105, 106]

miRNA-21 is the most consistently up-regulated miRNA across many cancer types. Chan et al. [107] discovered that reducing miR-21 levels in glioblastoma cells increased apoptosis, which correlated with a decrease in tumour growth. Of the many targets of miR-21 [108], PDCD4 is an important target that is frequently down-regulated in a variety of cancers [109, 110]. The tumour suppressive function of PDCD4 stems from its ability to bind to and inhibit eIF4A, thus blocking cap-dependent translation and attenuating cell growth [111-113]. Upon loss of PDCD4, the cell loses this ability to regulate protein translation, leading to enhanced cell proliferation and increased tumour formation. However, we and others have shown recently that PDCD4 plays a more specific role in translation by regulating translation of a specific set of targets (as described above; [58, 114]. For example, PDCD4 can bind to and negatively regulate the expression of p53 under normal growth conditions. However, upon induction of DNA damage by ultraviolet irradiation (UV), PDCD4 is degraded, thus allowing for an up-regulation in p53 levels [115]. Similarly, PDCD4-dependent repression of XIAP and Bcl-xL in response to FGF 2 has been described above. This suggest that an increase in miR-21 leading to a loss in PDCD4 may not only result in an increase in overall translation through de-repression of eIF4A, but also to a
specific increase in expression of anti-apoptotic proteins, thus leading to enhanced resistance to apoptosis-inducing chemotherapeutics [58].

The Bcl-2 family of proteins has also been identified as being regulated directly by miRNAs. For example, the miR-15-16 cluster of miRNAs can induce apoptosis by inhibiting Bcl-2, an anti-apoptotic factor involved in maintaining mitochondrial membrane homeostasis. As is common for many miRNAs, this cluster is down regulated in many cancers. For example, the miR-15-16 cluster is deleted in B-cell chronic lymphocytic leukaemia (CLL; [116]), pituitary adenoma [117], and prostate carcinoma [118]. This down-regulation of miRNAs contributes to the increased expression of Bcl-2 that is often observed in many cancers, and promotes chemoresistance by inhibiting the release of mitochondrial cytochrome c required for activation of caspase 9.

Interestingly, apoptotic cues can also directly regulate the proteins involved in the miRNA process. For example, Matskevich et al. [119] demonstrated that the RNase III enzyme Dicer is cleaved by caspases in response to apoptotic cues, in particular inhibition of protein kinase C (PKC) as well as during HIV infection, resulting in an inhibition of the RNA interference pathway [119]. Furthermore, Nakagawa et al. [120] demonstrated that the C. elegans Dicer gene, DCR-1, is cleaved specifically by a caspase, CED-3. They identify a novel role for the remaining C-terminal fragment of Dicer that can no longer process double stranded RNA species instead, it gains a deoxyribonuclease activity where it can nick DNA leading to DNA degradation and enhanced apoptosis [120].

As mentioned above, many miRNAs play a large role in regulating genes involved in apoptosis or cell proliferation leading to development and progression of cancer. Recently, miRNA profiles have been generated that can be utilized as a tool for the identification and
classification of tumours with hopes that this information can help with disease prognosis and predictions of outcomes [121].

1.12 Conclusions

Regulation of translation can be both the consequence and the cause of apoptosis. We have chosen examples to illustrate how this process is highly dynamic and is crucial for the cell’s ability to respond to environmental cues (Figure 1.2). We have highlighted the critical points of control prior to and during the onset of apoptosis with the hope of convincing the reader that the ability to translate specific proteins in response to stress is essential to decide the fate of the cell. Both the IRES and miRNA-mediated control of translation initiation are emerging as key mechanisms that regulate selective translation. IRES mediated translation allows for a selective translation of a subset of mRNAs in times of attenuation of global cap-dependent translation by bypassing the requirement for canonical initiation factors that are subject to inhibitory modification during apoptosis. In contrast, miRNA-mediated control of translation may, in addition to regulating the expression of specific target mRNAs, protect IRES containing mRNAs from degradation. In combination, these examples demonstrate how misregulation of translation initiation plays a crucial part in tumourigenesis and chemoresistance through enhanced resistance to apoptosis.

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1.14 References


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CHAPTER 2

Tumour Suppressor PDCD4 Represses IRES-Mediated Translation of Anti-Apoptotic Proteins

and is Regulated by S6 Kinase 2
2.1 Preamble

“Tumour Suppressor PDCD4 Represses IRES-Mediated Translation of Anti-Apoptotic Proteins and is Regulated by S6 Kinase 2” is published in the journal Molecular and Cellular Biology (Volume 32, May 2012). This article describes how specific and direct binding of PDCD4 to the XIAP and Bcl-xL IRES elements prevents binding of the ribosome, thus inhibiting IRES-mediated translation. It further demonstrates that in response to the fibroblast growth factor-2 (FGF-2), S6 kinase 2 (S6K2) is activated where it subsequently phosphorylates PDCD4 leading to its degradation, thus relieving repression of IRES-mediated translation. This paper highlights the importance of PDCD4 in response to growth factors and describes XIAP and Bcl-xL as two novel and specific targets of PDCD4. Most importantly, it is shown for the first time that PDCD4 is a bona fide IRES trans-acting factor (ITAF).

Author List

Urszula Liwak, Nehal Thakor, Lindsay E. Jordan, Rajat Roy, Stephen M. Lewis, Olivier Pardo, Michael Seckl, and Martin Holcik

Author Contribution

UL and MH wrote the paper; U.L., O.P., M.S., and M.H. conceived the experiments; U.L., N.T., L.E.J., S.M.L., and R.R. conducted the experiments; UL designed and performed experiments presented in Figures 2.1, 2.2A,B, 2.4.
2.2 Abstract

Apoptosis can be regulated by extracellular signals that are communicated by peptides such as fibroblast growth factor-2 (FGF-2), which have important roles in tumour cell proliferation. The pro-survival effects of FGF-2 are transduced by the activation of the ribosomal protein S6 kinase 2 (S6K2), which increases the expression of the anti-apoptotic proteins X-linked inhibitor of apoptosis (XIAP) and Bcl-xL. We now show that the FGF-2 – S6K2 pro-survival signalling is mediated by the tumour suppressor programmed cell death 4 (PDCD4). We demonstrate that PDCD4 specifically binds to the Internal Ribosome Entry Site (IRES) elements of both the XIAP and Bcl-xL messenger RNAs and represses their translation by inhibiting the formation of the 48S translation initiation complex. Phosphorylation of PDCD4 by activated S6K2 leads to the degradation of PDCD4 and thus the subsequent de-repression of XIAP and Bcl-xL translation. Our results identify PDCD4 as a specific repressor of the IRES-dependent translation of cellular mRNAs (such as XIAP and Bcl-xL) that mediate FGF-2 – S6K2 pro-survival signalling, and provide further insight into the role of PDCD4 in tumour suppression.

2.3 Introduction

Apoptosis, or programmed cell death, is important for the normal development of organisms and maintaining tissue homeostasis. Misregulation of apoptosis may lead to neurodegenerative disorders when apoptosis is increased, and tumour formation when apoptosis is inhibited, thus the pathway is tightly controlled with both pro and anti-apoptotic factors playing a role. Of particular interest are members of the Inhibitor of Apoptosis (IAP) and Bcl-2 families of proteins that intercept virtually all apoptotic signals in the cell.
X-chromosome linked inhibitor of apoptosis (XIAP) is the most potent member of the IAP family; it directly interacts with and inhibits caspases 3, 7, and 9, and is therefore a key regulator of apoptosis (20). In contrast, Bcl-xL controls apoptosis by maintaining mitochondrial membrane homeostasis (14). Interestingly, both XIAP and Bcl-xL mRNAs contain an Internal Ribosome Entry Site (IRES) which allows them to be translated during cellular stress by a cap-independent mechanism when cap-dependent translation is inhibited, which is necessary for their protective roles in the cell (4, 11, 16, 24, 49). Under normal growth conditions, translation of cellular mRNAs occurs through a cap-dependent mechanism that requires interaction of specific initiation factors (such as eukaryotic initiation factor (eIF) 4E) with the 5′ cap of the mRNA, followed by recruitment of ribosomal subunits, recognition of the AUG start codon, and commencement of polypeptide chain elongation (reviewed in (26)). However, certain cellular stresses such as nutrient deprivation, hypoxia, or low dose irradiation cause attenuation of cap-dependent translation, yet during these conditions a sizeable proportion of cellular mRNAs, perhaps as much as 10%, have been shown to be translated by a cap-independent mechanism, such as through an IRES (23, 28, 39). IRES elements are located within the 5′ untranslated region (UTR) of some cellular mRNAs and are believed to recruit the ribosome directly, thereby bypassing the requirement for the mRNA 5′ cap and eIF4E. Moreover, while IRES-dependent translation requires some canonical translation initiation factors, most (if not all) cellular IRES elements require the activity of auxiliary RNA-binding proteins that function as IRES trans-acting factors (ITAFs) (reviewed in 28, 32). The mechanism by which ITAFs function is poorly understood, and since different IRESes require different sets of ITAFs, elucidating the identity of all known ITAFs proves challenging. Furthermore, it has been shown that ITAFs can function as either positive or negative regulators of IRES-mediated translation. For
example, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) was shown to act as a repressor of XIAP IRES-mediated translation (33), whereas La autoantigen (19), hnRNPC1/C2 (18), and HuR (11) have all been shown to have a stimulating effect on XIAP IRES-mediated translation.

We have previously demonstrated that treatment of small cell lung cancer (SCLC) cells with fibroblast growth factor (FGF) -2 results in enhanced cell survival which is due to increased expression of several anti-apoptotic proteins, including XIAP and Bcl-xL (35, 36). Mechanistically, this is due to a complex consisting of B-Raf, PKCε, and S6K2, which forms in response to FGF-2 and leads to the phosphorylation and activation of S6K2 by PKCε (37). Ribosomal protein S6 kinases (S6K) 1 and 2 are two closely related members of the AGC Group VI serine/threonine kinases that regulate several cellular processes, most notably protein synthesis via the phosphorylation of ribosomal protein S6 (rpS6) and eIF4B (reviewed in 13). Importantly, although S6K1 and S6K2 are usually assumed to regulate overlapping sets of cellular targets, we have shown that S6K1 cannot replace S6K2 in the complex with B-Raf and PKCε following FGF-2 treatment, thus identifying a novel function for S6K2.

We were interested in determining if the FGF-2 activated S6K2 modifies a specific ITAF(s) that is involved in the regulation of XIAP and Bcl-xL translation. We discovered that the tumour suppressor programmed cell death 4 (PDCD4) interacts with and is phosphorylated by S6K2 both in vitro and in vivo. We demonstrate that activation of S6K2 and subsequent phosphorylation of PDCD4 results in a loss of PDCD4 protein, which leads to enhanced translation of XIAP and Bcl-xL via their IRES elements. We further show that PDCD4 binds directly to XIAP and Bcl-xL IRES RNA both in vitro and in vivo and prevents
formation of a translationally competent 48S initiation complex on the IRES. Thus, our work identifies PDCD4 as a novel specific repressor of IRES-mediated translation of cellular IRESes. Our findings also indicate a novel role for S6K2 in the transmission of mitogenic signals (such as the FGF-2 signalling cascade) via PDCD4 and its effect on selective protein synthesis. Furthermore, we provide insight into the mechanism by which PDCD4 specifically regulates IRES-mediated translation. Given the tumour suppressor properties of PDCD4, and the key roles XIAP and Bcl-xL play in establishing apoptotic resistance of cancer cells, our work uncovers a new signalling axis that contributes to enhanced chemoresistance in cancer.

2.4 Materials and Methods

2.4.1 Cell culture, expression constructs and transfection

Tetracycline-inducible kinase-active S6K2 cells (TOKAS6K2) were described previously (37). TOKAS6K2 and human embryonic kidney (HEK293T) cells were maintained in standard conditions in serum- and antibiotic-supplemented Dulbecco's modified Eagle's medium (DMEM). The bicistronic DNA reporter plasmids pβgal/5'(-1007)/CAT (containing the XIAP IRES element), pBic-S (containing the XIAP shorter 5' UTR that does not contain an IRES), pBic (containing the 104 nt linker region from pcDNA3), pβgal/Bcl-xL/CAT (containing the Bcl-xL IRES element), and the HA-S6K2 and pcDNA3-Flag-hnRNP A1 expression plasmid were described previously (4, 21, 33, 37, 38). The pTripz-Kate plasmid was generated by replacing the shRNA cassette of pTripz (Open Biosystems) with mKate cDNA (Evrogen). PDCD4 mutant plasmids were generated by site-directed mutagenesis in which serines 67, 71, and 76 were mutated to alanine; His-PDCD4 was generated by cloning PDCD4 into the pTrcHis-B plasmid. The PDCD4<sup>RBD</sup> deletion mutant contains the putative
RNA binding domain of PDCD4 (aa 1-156); the PDCD4ΔRBD mutant lacks the RBD domain (aa 157-469). All the mutant constructs were verified by sequencing. Transient transfections were performed using LipofectAMINE 2000 according to the protocol provided by the manufacturer (Invitrogen). Briefly, cells were seeded at a density of $2.5 \times 10^5$ cells per well in 6-well plates and were transfected 24 hr later with 2 µg of plasmid DNA, or *in-vitro* transcribed RNA, per well. Cells were collected for analysis 4 hr (for RNA) or 24 hr (for DNA) post-transfection. siRNA transfections were performed using Lipofectamine RNAiMax according to the protocol provided by the manufacturer (Invitrogen). Briefly, cells were seeded at a density of $2.5 \times 10^5$ cells/well in 6-well plates and were transfected 24 hr later in serum-free DMEM with a 25 nM final concentration of PDCD4 siRNA (Dharmacon), S6K2 siRNA (Qiagen), or a non-silencing control siRNA (Qiagen). Cells were collected for analysis 48 hr or 72 hr post-transfection.

### 2.4.2 *In vitro* RNA synthesis

DNA templates for the synthesis of reporter RNAs were generated from the corresponding 5′ UTR-containing monocistronic hairpin construct (38) by PCR, using primers that have an incorporated T7 promoter sequence at the 5′ end to allow for RNA transcription. Reverse primers included the 3′ end of the CAT gene as well as 31 T residues, therefore providing the resultant PCR product with a poly T tail. All PCR products were purified by agarose gel electrophoresis and the UltraClean 15 DNA purification kit (MO BIO Laboratories). *In vitro* transcription and capping were performed with the mMessage mMMachine kit (Ambion). Newly synthesized RNA was purified using a MegaClear Column (Ambion). The concentration of the RNA was determined using the ND-1000 spectrophotometer (Thermo Scientific).
2.4.3 *In vitro* synthesis of $^{32}$P-labelled RNA

DNA templates were generated from the XIAP IRES sequence by PCR using primers that have an incorporated T7 promoter sequence at the 5′ end to allow for RNA transcription. RNA was generated using $^{32}$P-alpha UTP and the MAXIscript T7 kit (Ambion) as per the protocol provided by the manufacturer. The RNA was gel purified from a 5% acrylamide/8M urea denaturing gel.

2.4.4 RNA-protein complex immunoprecipitation

HEK293T cells were transiently transfected with pCDNA3-Flag, Flag-PDCD4, or Flag-hnRNP A1(F1) expression plasmids for 24 hr. RNA-protein complexes were crosslinked *in vivo* with 1% formaldehyde for 30 min at room temperature followed by 0.2 M glycine for 5 min to stop crosslinking. After washing with PBS, the cells were lysed in RIP buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% Na-deoxycholate, 1% Igepal, 1 mM sodium orthovanadate (Na$_3$VO$_4$), 1 mM NaF, 1 µg/ml each of Aprotinin, leupeptin, pepstatin, and 40 U/ml of RNAse inhibitor (Promega)) for 30 min at 4°C. Lysates were sonicated and treated with DNAse I for 30 min. Proteins were immunoprecipitated with anti-Flag M2 affinity gel (Sigma) for 2 hr at 4°C and RNA was extracted using phenol:chloroform purification and ethanol precipitation. cDNA was generated using the qScript cDNA SuperMix (Quanta Biosciences) and the products were PCR amplified using the GAPDH (Invitrogen), XIAP (QuantiTect Primer Assay, Qiagen) and Bcl-xL (QuantiTect Primer Assay, Qiagen) gene specific primers. PCR products were visualized on a 0.8% agarose gel by ethidium bromide staining.
2.4.5 UV-crosslinking of RNA-protein complexes

RNA-protein UV-crosslinking experiments were performed as previously described (33). Briefly, $^{32}$P-UTP labelled RNA was incubated with increasing concentrations of recombinant His-PDCD4 (0.5 µg, 1.0 µg, or 2.0 µg) or GST (2 µg) in RNA binding buffer (10 mM Tris-HCl, pH 7.4, 3 mM MgCl$_2$, 300 mM KCl, 1 mM DTT, 0.2 mM PMSF, 20 µg/ml leupeptin) for 30 min at room temperature. The complexes were UV crosslinked at 250 mJ/µm$^2$ in a Stratalinker, followed by treatment with RNase T1 (1 U/µl), RNase A (10 µg/ml) and heparin (5 mg/ml) for 10 min. The samples were mixed with 2X Laemmli buffer, boiled, separated on an SDS-PAGE gel, and exposed to X-ray film at -80°C overnight.

2.4.6 Nitrocellulose Filter Binding Assay

*In vitro* transcribed, $^{32}$P-labelled RNA was gel purified and incubated with recombinant proteins at room temperature for 10 min in RNA binding buffer. The reaction was spotted on a nitrocellulose membrane and drawn through the membrane using a vacuum manifold, washed twice with RNA binding buffer, dried for 30 min and the radioactivity was measured by a scintillation counter.

2.4.7 Kinase assay

HEK293T cells were transiently transfected with HA-S6K2 for 24 hr, treated with FGF-2 (10 ng/ml) for 3 hr, and lysed in co-IP buffer (25 mM Tris pH 7.5, 150 mM NaCl, 50 mM NaF, 0.5 mM EDTA pH 8.0, 0.5% Triton X-100, 5 mM beta-glycerophosphate, 5% glycerol, 1 mM DTT, 1 mM PMSF, and 1 mM Na$_3$VO$_4$) with sonication. Anti-HA-agarose beads
(Sigma) were incubated with the lysate for 1 hr at 4°C. Beads were washed 3 times with 1 ml of lysis buffer followed by a wash in 1X kinase buffer (20 mM Tris-HCl pH 7.5, 5 mM betaglycerolphosphate, 0.2 mM Na$_3$VO$_4$, 0.5 mM DTT). Kinase-bound beads, or recombinant GST-S6K2 (Invitrogen) were incubated with substrate in 1X kinase buffer in the presence of ATP (300 µM ATP, 66 mM MgCl$_2$, 33 mM MnCl$_2$) and 5 µCi of $^{32}$P-labelled gamma-ATP for 20 min at 30°C. Laemmli sample buffer was added and samples were separated by SDS-PAGE, transferred to PVDF membrane and exposed to X-Ray film. The membrane was subsequently analysed by Western blot.

2.4.8 β-galactosidase and CAT analysis

Transiently transfected cells were washed in 1 ml PBS and harvested in 300 µl CAT ELISA kit lysis buffer according to the protocol provided by the manufacturer (Roche Molecular Biochemicals). β-galactosidase (βgal) enzymatic activity was determined by spectrophotometric assay using o-nitrophenyl-β-D-galactopyranoside as previously described (34). CAT levels were determined using the CAT ELISA kit according to the protocol provided by the manufacturer (Roche Molecular Biochemicals). Relative IRES activity was calculated as the ratio of CAT/βgal.

2.4.9 S6K2 tandem affinity purification (TAP)

S6K2 open-reading frame was introduced into the GS-TAP vector (6) using gateway technology (Invitrogen) to express the Protein G and Streptavidin Binding peptide (SBP) as a N-terminal TAP fusion protein. TAP-S6K2 was transiently expressed in HEK293 cells and for each purification, ten 15 cm plates at 80% confluency were pooled to obtain cell lysates.
Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 125 mM NaCl, 5% glycerol, 0.2% NP-40, 1.5 mM MgCl₂, 1 mM DTT, 25 mM NaF, 1 mM Na₃VO₄, 1 mM EDTA, protease inhibitors) and lysates cleared by centrifugation. Cleared cell lysates were then incubated with rabbit IgG agarose (Sigma) for 2 hr at 4°C. Bound proteins were washed 3 times with the lysis buffer followed by twice with TEV cleavage buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.2% NP-40) and eluted by incubating the IgG beads with 100 units of TEV protease at 4°C for 2 hr. TEV cleaved lysates were incubated with Streptavidin beads (Pierce) for 2 hr at 4°C and washed 4 times with cleavage buffer (with protease and phosphatase inhibitors). Bound proteins were eluted by boiling in sample buffer and separated on a 4-15% polyacrylamide gradient gel (Invitrogen). The protein lane was then excised in 1 mm gel slices and digested with trypsin. Digested peptides were processed and analysed using an orthogonal acceleration quadrupole Tof mass spectrometer (SYNAPT HDMS, Waters, UK). LC/MS/MS data were then searched against a non-redundant protein database (UniProt 12.4) using the Mascot search engine programme (Matrix Science, UK) and analysed using Scaffold software.

2.4.10 HA-tagged Protein Immunoprecipitation

HEK293T cells were transiently transfected with HA-S6K2 for 24 hr followed by treatment with or without FGF-2 (10 ng/ml) for 3 hr. Cells were lysed in co-IP buffer (25 mM Tris pH 7.5, 150 mM NaCl, 50 mM NaF, 0.5 mM EDTA pH 8.0, 0.5% Triton X-100, 5 mM beta-glycerophosphate, 5% glycerol, 1 mM DTT, 1 mM PMSF, and 1 mM Na₃VO₄) with sonication. Anti-HA-agarose beads (Sigma) were incubated with the lysate for 2 hr at 4°C
followed by 3 washes with lysis buffer. Laemmli sample buffer was added and the samples were analysed by Western blot.

2.4.11 Endogenous Protein Immunoprecipitation

HEK293T cells were treated with FGF-2 (10 ng/ml) for 15 min and lysed in NP-40 buffer (50 mM HEPES pH 7.4, 120 mM NaCl, 0.5% NP40, 2 mM EDTA and 5% glycerol) and rotated for 30 min at 4°C. Lysates were centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was pre-cleared with Protein A/G agarose beads for 1 hr at 4°C. Protein A/G agarose beads were conjugated to 5 µg goat anti-S6K2 (SantaCruz Biotechnology) or rabbit anti-PDCD4 (Millipore) antibody overnight at 4°C in PBS, washed three times in cold PBS and used for IP. The IP was performed for 2 hr at 4°C, followed by three washes in cold lysis buffer. Laemmli sample buffer was added and the samples were analysed by Western blot using TrueBlot anti-Goat or anti-Rabbit HRP conjugated secondary antibodies (eBioscience).

2.4.12 Western blot analysis

Cells were washed in 1 ml PBS and lysed in 150 µl RIPA buffer for 30 min at 4°C, followed by centrifugation at 12,000 × g for 10 min to pellet debris. Protein concentration was assayed by BCA Protein Assay Kit (Pierce) and equal amounts of protein extract were separated by 10% SDS-PAGE and transferred to PVDF or nitrocellulose membranes. Samples were analyzed by Western blotting using mouse anti-rpS6 (Cell Signaling Technology), rabbit anti-phospho-rpS6 (Cell Signaling Technology), rabbit anti-Bcl-xL (Cell Signaling Technology), anti-XIAP (rabbit anti-GST-XIAP (AEgera) or rabbit anti-RIAP3 (22)), mouse anti-Nucleolin (Santa Cruz Biotechnology), mouse anti-GAPDH (Advanced
Immunochemical Inc), rabbit anti-PDCD4 (Rockland), rabbit anti-PDCD4(S67-P) (Millipore), rabbit anti-GST (Santa Cruz Biotechnology), or HRP-conjugated anti-HA (Roche) antibodies followed by species-specific horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Antibody complexes were detected using the ECL or ECL Plus systems (GE Biosciences) and were quantified using Odyssey densitometry software (Li-COR Biosciences).

2.4.13 RNA extraction and quantitative RT-PCR analysis
Total RNA was isolated from transfected cells using the Absolutely RNA miniprep kit according to the manufacturer’s instructions (Stratagene). cDNA was generated using an oligo dT$_{18}$ primer and the Bulk 1st-Strand Synthesis kit according to the protocol provided by the manufacturer (GE Biosciences). The synthesized cDNA was used as the template for quantitative PCR using the PerfeCTa SYBR Green FastMix (Quanta Biosciences) along with gene specific primers for XIAP coding region (QuantiTect Primer Assay, Qiagen), XIAP IRES- or non-IRES containing mRNA variants (38), Bcl-xL (QuantiTect Primer Assay, Qiagen), Apaf-1 (5′-CTTGAGCCCTGGAGTTTGAG, 5′-TGCATGAACTGCCATGAAAT), VEGF-A (5′-CGCGAGGCTTGGGGCA, 5′-GGTTTCGGAGGCCCGACC), cIAP1 (15), or GAPDH (11), and analyzed on a Mastercycler-realplex (Eppendorf) real-time thermocycler using the associated realplex software. Relative expression levels were determined using the standard curve method. Controls lacking RT demonstrated no significant genomic DNA amplification (>10 cycle difference).

2.4.14 Polysome profiling
HEK293T cells from three 15 cm plates per condition were lysed in cold polysome lysis buffer (15 mM Tris-HCl, pH 7.4, 15 mM MgCl₂, 300 mM NaCl, 1% (v/v) Triton X-100, 0.1 mg/ml cycloheximide, 100 U/ml RNasin). Equal OD₂₅₄ units were loaded onto 10-50% linear sucrose gradients and centrifuged at 39,000 rpm for 90 min at 4°C. Gradients were fractionated from the top (Auto Densi-Flow, Labconco) and RNA was monitored at 254 nm using a HPLC system (Äkta Explorer, GE Biosciences). 1 ml fractions were collected and frozen. RNA was isolated from individual fractions by proteinase K digestion followed by phenol:chloroform extraction and ethanol precipitation. Equal volumes of RNA from each fraction were used to generate cDNA using oligo-dT primers and a reverse transcription kit (First-Strand cDNA synthesis kit, GE Biosciences). PCR primers specific for XIAP isoforms, Bcl-xL, Apaf-1, VEGF-A, cIAP1 or GAPDH were used to amplify messages using quantitative PCR as described above. Weighted averages (Fₖ) for the distribution of each mRNA were calculated as described (8). ΔFₓ represents the difference in mRNA polysome distribution between siControl and siPDCD4 treated cells.

### 2.4.15 Toeprinting assay

Toeprinting was performed as described (42). Briefly, rabbit reticulocyte lysate (RRL; Green Hectares) was supplemented with recombinant His-PDCD4 or GST, as indicated, at 37°C for 15 min. Subsequently, RRL was treated with RNAsin (Promega) and GMP-PNP (1.7 mM) for 5 min at 37°C. Uncapped, poly A-tailed XIAP IRES RNA (800 ng) and ATP (1.82 mM) were added and the reactions were incubated at 37°C for 5 min. The reaction volume was brought to 40 µl by the addition of toeprinting buffer (20 mM Tris-HCl, pH 7.6, 100 mM KOAc, 2.5 mM MG(OAc)₂, 5% (w/v) sucrose, 2 mM DTT and 0.5 mM spermidine) and
incubated at 37°C for 3 min. Subsequently, 5 pmol of toeprinting primer (5′-CTCGATATGTGCATCTGTA; 5′ end labeled with IRDye™800) was added and reaction was incubated on ice for 10 min. 1 mM dNTPs, 5 mM Mg(OAc)$_2$ and 1 µl of avian myeloblastosis virus reverse transcriptase (Promega) were added to the reaction and the final volume was brought to 50 µl with toeprinting buffer. Primer extension occurred for 45 min at 37°C. The cDNA products were purified by phenol:chloroform extraction and analyzed on a standard 6% sequencing gel using a model 4200 IR2 sequence analyzer (LI-COR, Lincoln, Nebraska, USA).

### 2.4.16 Statistical analysis

All data are expressed as mean +/- standard deviation (SD). Unless otherwise stated, all results were obtained through a minimum of three independent experimental replications. For reporter assays, independent replicates consist of three biological triplicates. Unpaired $t$-test was performed to determine data significance using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego).

### 2.5 Results

#### 2.5.1 S6K2 directly interacts with and phosphorylates PDCD4

We have previously determined that upon FGF-2 stimulation a complex forms among S6K2, B-Raf and PKCε leading to the activation of S6K2. Importantly, this activation results in a translational upregulation of the anti-apoptotic XIAP and Bcl-xL proteins, leading to
enhanced cell survival (37). These findings prompted us to determine the mechanistic link between S6K2 and the upregulation of XIAP and Bcl-xL expression.

To identify novel interactors of S6K2 that may participate in XIAP and Bcl-xL translation, we performed tandem affinity purification (TAP) on cells expressing S6K2 as a bait, in the presence and absence of FGF-2 stimulation. The cell lysates were prepared and purified according to TAP published methodology (6), the eluates separated by SDS-PAGE, and proteins within each lanes identified by mass spectrometry. We were interested in binding partners that were enriched upon FGF-2 treatment and discovered several proteins that fulfilled this criteria (Table 2.1). Among these, we identified programmed cell death 4 (PDCD4), a known tumour suppressor, as an S6K2 binding partner. PDCD4 was previously implicated in translational control (9, 30, 47) and therefore we were interested in determining if PDCD4 had a specific effect on XIAP and Bcl-xL translation. In order to validate the TAP data, we overexpressed HA-tagged S6K2 in HEK293T cells and performed immunoprecipitation (IP) using anti-HA agarose beads. We found that endogenous PDCD4 does co-precipitate with S6K2, and this interaction is enhanced following FGF-2 treatment (Fig. 2.1A). To verify that this interaction is specific and indeed occurs in cells between native proteins, we also performed reciprocal co-IP experiments using endogenous S6K2 or PDCD4. Again, we identified an interaction between endogenous S6K2 and endogenous PDCD4 following FGF-2 stimulation (Fig. 2.1B).

To study this interaction further, we sought to determine if S6K2 can directly phosphorylate PDCD4. HA-tagged S6K2 was overexpressed in HEK293T cells, immunoprecipitated 24 hours later, and used in an in vitro kinase assay with purified recombinant GST-PDCD4, or ribosomal protein S6 peptide, a known S6K2 target. We found that S6K2 is in fact able to phosphorylate PDCD4 (Fig. 2.1C). In contrast, a mutant version
Table 2.1. Identification of S6K2 interacting proteins in FGF-2 treated and untreated cells. HEK293 cells were transiently transfected with TAP-S6K2 plasmid and the identification of binding partners was performed as described in Material and Methods. LC/MS/MS data were searched against a non-redundant protein database (UniProt 12.4) using the Mascot search engine programme (Matrix Science, UK) to determine the protein identity (probability-based protein identification). Only proteins differentially identified in FGF-2 treated cells are shown.
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<th>MW</th>
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<th>Probability FGF2-</th>
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Fig. 2.1: PDCD4 associates with and is phosphorylated by S6K2. (A) HEK293T cells were transiently transfected with HA-S6K2 expressing plasmid, treated or not with FGF-2, and the lysates were immunoprecipitated with anti-HA agarose beads; PDCD4 and S6K2 protein levels were monitored by anti-PDCD4 or anti-HA antibodies, respectively. (B) HEK293T cells were treated with FGF-2 and endogenous PDCD4 or S6K2 was immunoprecipitated using anti-PDCD4 or anti-S6K2 antibodies. The levels of co-precipitated PDCD4 or S6K2 were monitored by Western blot analysis. (C) Immunoprecipitated HA-S6K2 was incubated with purified recombinant GST, GST-S6 peptide, GST-PDCD4 WT or GST-PDCD4 S67/71/76A mutant proteins in the presence of $^{32}$P-gamma ATP, separated by SDS-PAGE, and transferred to a PVDF membrane. Phosphorylation was detected by autoradiography and protein levels were analysed by Western blot analysis with a mixture of anti-GST and anti-HA antibodies on the same membrane. (D) Recombinant GST-S6K2 was incubated with GST-PDCD4 WT or GST-PDCD4 S67/71/76A mutant and processed as described in (C) (E) HEK293T cells were transfected with control siRNA (siCTRL) or S6K2 targeting siRNA (siS6K2) for 48h, treated with FGF-2 for the indicated times, and cells were harvested and analysed by Western blot. siPDCD4 was used as a control for the phospho-specific antibody.
of PDCD4, in which serines (S67, S71, S76) within the putative S6K-consensus site were mutated to alanines, was not phosphorylated. In order to eliminate the possibility that another kinase co-precipitated with S6K2 and was thus responsible for PDCD4 phosphorylation, we performed the in vitro kinase assay using purified recombinant GST-S6K2 (Fig. 2.1D). We observed that S6K2 robustly phosphorylates wildtype and not mutant PDCD4, thus identifying PDCD4 as a novel substrate of S6K2. Furthermore, we investigated the effect of FGF2 stimulation on PDCD4 phosphorylation in vivo. We treated HEK293T cells with FGF2 after S6K2 knockdown and measured phosphorylation of PDCD4 at serine 67 using a phospho-specific antibody. We observed that PDCD4 is rapidly phosphorylated after FGF2 treatment, but this phosphorylation is lost when levels of S6K2 are reduced by siRNA (Fig. 2.1E). Together, our data demonstrate that S6K2 is required for the phosphorylation of PDCD4 in response to FGF-2 stimulation.

2.5.2 S6K2 activation results in degradation of PDCD4 and a concomitant increase in XIAP and Bcl-xL proteins

We have shown that S6K2 interacts with and phosphorylates PDCD4. We next investigated the consequences of PDCD4 phosphorylation by S6K2 in cells. In order to do so, we utilized a tetracycline-inducible system where kinase active S6K2 (KAS6K2) is expressed upon stimulation with doxycycline (37). We observed that stimulation of cells with 1 µg/ml of doxycycline for 24 hours resulted in a marked loss of PDCD4 (Fig. 2.2A), which correlated with an increase in XIAP and Bcl-xL levels as previously reported ((35, 36); Fig. 2.2A). In contrast, addition of doxycycline had no effect on PDCD4, XIAP or Bcl-xL levels in control pTripz-Kate transfected cells (Fig. 2.2A). The loss of PDCD4 preceded the increase in XIAP and Bcl-xL (Fig. 2.2B), and was mediated by the proteasome, since treatment of the cells
Fig. 2.2: Activation of S6K2 leads to PDCD4 degradation with a concomitant increase in XIAP and Bcl-xL protein levels. (A) TOKAS6K2 stable or control cells (transiently transfected with an inducible pTripz-Kate plasmid) were treated with doxycycline (1 µg/ml) or DMSO for 24 hr and cell lysates were analysed by Western blot analysis. KAS6K2 induction is indicated by an increase in phosphorylated rpS6. (B) TOKAS6K2 cells were treated with doxycycline as in (A), harvested after the indicated time points, and analyzed by Western blot. (C) TOKAS6K2 cells were treated with doxycycline (1 µg/mL) in the presence of the proteasome inhibitor MG132 (2 µM) for 18 hr; PDCD4 and nucleolin levels were monitored by Western blot. (D) TOKAS6K2 cells were transfected with a control (pcDNA3) or a PDCD4-expressing plasmid for 24 hr followed by doxycycline treatment for 24 hr. Protein levels were monitored by Western blot analysis. (E) The protein levels of XIAP and Bcl-xL from (D) were quantified relative to nucleolin expression (**p<0.01).
A. TOKAS6K2  pTripz-Kate
Dox (μg/mL)

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B. Dox (1 μg/mL)

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C. MG132 (μM): Dox (μg/mL):

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D. pcDNA3  PDCD4
Dox (μg/mL)

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E. Relative protein levels

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<tr>
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** p < 0.01
with the proteasomal inhibitor MG132 completely prevented PDCD4 loss upon doxycyline treatment (Fig. 2.2C). Furthermore, when PDCD4 expression was rescued in TOKAS6K2 cells by transient transfection, the enhanced expression of XIAP and Bcl-xL was blunted (Fig. 2.2D,E), further supporting the notion that the degradation of PDCD4 and the enhanced expression of both XIAP and Bcl-xL are mechanistically linked.

2.5.3 PDCD4 is a repressor of XIAP and Bcl-xL IRES-mediated translation

XIAP and Bcl-xL belong to the small subset of cellular mRNAs whose stress-induced translation is regulated by an IRES element found within their respective 5’ UTRs (21, 49). In addition, XIAP is encoded by two distinct mRNAs that differ in their 5’ UTRs; the major, shorter 5’ UTR promotes a basal level of XIAP expression under normal growth conditions, while the less abundant, longer 5’ UTR contains an IRES element and supports cap-independent translation during stress (38). We were therefore interested in determining if PDCD4 is a specific regulator of the IRES-mediated translation of XIAP and Bcl-xL in response to S6K2 activation. HEK293T cells were transfected with either PDCD4-targeting or non-silencing control siRNA and the levels of XIAP and Bcl-xL proteins were determined 48 hours later by Western blot analysis. We observed that reducing the levels of PDCD4 by siRNA resulted in a marked increase in XIAP and Bcl-xL protein levels compared with a non-silencing control (Fig. 2.3A), while having no effect on XIAP and Bcl-xL total mRNA levels (Fig. 2.3B). To demonstrate that PDCD4 regulates the translation of endogenous XIAP and Bcl-xL mRNAs we performed polysome profiling to examine the association of XIAP and Bcl-xL mRNAs with translating ribosomes in cells with reduced levels of PDCD4. We observed that reduction of PDCD4 by siRNA did not change the overall polysome profile (Fig. 2.3C top panel) indicating that PDCD4 is not essential for general protein synthesis.
Fig. 2.3: Loss of PDCD4 correlates with an increase in XIAP and Bcl-xL translation through their respective IRES elements. (A) HEK293T cells were treated with PDCD4 siRNA or control (CTRL), non-targeting siRNA. Cell lysates were harvested and subjected to Western blot analysis. (B) Steady-state mRNA levels were measured by qRT-PCR in PDCD4 siRNA or control (CTRL) siRNA treated cells. (C) PDCD4 was knocked down as in (A) and cell lysates were subjected to polysome profiling. The polysome profile is shown in the top panel; distribution of the XIAP IRES, XIAP non-IRES, and Bcl-xL mRNAs relative to that of GAPDH is shown as the percent of total mRNA (% mRNA) in each fraction. Representative experiment is shown. (D) The distribution of mRNA in each polysome fraction from (C) was calculated as a ΔFW (8). (E) Bicistronic DNA constructs containing XIAP or Bcl-xL IRES elements were transfected into HEK293T cells after treatment with PDCD4 siRNA or control (CTRL) siRNA. IRES activity was measured as a ratio of CAT to β-gal expression. (F) In vitro-transcribed IRES reporter mRNAs were transfected into HEK293T cells after treatment with PDCD4 siRNA or control, non-targeting siRNA. IRES activity was quantified as a ratio of CAT protein to CAT mRNA (**p<0.01).
This observation was somewhat surprising since PDCD4 has been reported to affect global rates of protein synthesis (46). However, monitoring the relative distribution of XIAP IRES or Bcl-xL mRNA within the polysome by qRT-PCR showed that both mRNAs were shifted into the heavier polysomes in cells with reduced PDCD4 levels, confirming that the translation of XIAP IRES and Bcl-xL mRNAs is enhanced when PDCD4 levels are reduced. Importantly, the relative distribution of the XIAP mRNA splice variant that does not contain an IRES (XIAP non-IRES) remained unchanged in cells with reduced levels of PDCD4 (Fig. 2.3C). These data indicate that PDCD4 specifically represses translation of cellular IRES-containing mRNAs, suggesting that PDCD4 is a novel IRES trans-acting factor (ITAF). Additionally, we investigated the translational patterns of three other mRNAs, Apaf-1, cIAP1, and VEGF, which are all known to contain IRES elements in their 5′ UTRs (1, 43, 45), to determine if PDCD4 has a global effect on IRES-mediated translation or if this effect is specific to a subset of IRES mRNAs. After analyzing the polysome distribution of these mRNAs by qRT-PCR, we observed that their translation is not affected by reduced levels of PDCD4, suggesting that PDCD4 has specific targets and is not a general inhibitor of IRES-mediated translation (Fig. 2.3D).

To further study the effect of PDCD4 on IRES-mediated translation of XIAP and Bcl-xL, we utilized a previously characterized bicistronic reporter system that contains the XIAP (21) or Bcl-xL (49) IRES element. In this system, translation of the first cistron (β-galactosidase; βgal), is cap-dependent, whereas translation of the second cistron (chloramphenicol acetyl transferase; CAT), is driven by the IRES. By calculating the ratio of CAT expression to βgal expression, the relative IRES activity can be determined. HEK293T cells were initially transfected with PDCD4-targeting siRNA or a non-silencing control siRNA. 48 hours later, the cells were transfected with the bicistronic reporter plasmids and
reporter protein levels were assayed after 24 hours. We found that the activity of both the XIAP and Bcl-xL IRES elements was enhanced approximately 2-fold when PDCD4 levels were reduced by siRNA (Fig. 2.3E). In contrast, reduction in PDCD4 levels did not increase CAT expression from control plasmids (XIAP non-IRES 5’ UTR and a short unrelated sequence). Since the validity of data generated with bicistronic DNA constructs has been questioned recently (29, 44), we also used direct RNA transfection of *in vitro* transcribed XIAP and Bcl-xL reporter RNAs. These capped and polyadenylated reporter RNAs incorporate a strong hairpin structure at the 5’ end to restrict their capacity for cap-dependent translation, and thus the expression of CAT is driven solely by the IRES (38). Following transfection of these synthetic transcripts into HEK293T cells in which the PDCD4 levels were reduced by siRNA, CAT protein levels were quantified. Similar to the data obtained with the bicistronic DNA constructs, we observed an increase in IRES activity for both XIAP and Bcl-xL when PDCD4 levels were reduced (Fig. 2.3F). These observations confirm that PDCD4 is a specific ITAF for the XIAP and Bcl-xL IRESes and under normal conditions PDCD4 acts as a repressor of their activity.

### 2.5.4 PDCD4 binds directly to IRES-containing RNA

Our observations that PDCD4 represses IRES-mediated translation of XIAP and Bcl-xL mRNAs suggest that PDCD4 associates with these mRNAs by binding to their IRES sequences. To determine if PDCD4 binds directly to IRES RNA we performed a UV-crosslinking experiment using purified, recombinant His-PDCD4 and *in vitro* transcribed \(^{32}\text{P}\)-labelled RNAs. We found that both XIAP and Bcl-xL IRES RNA crosslinked to His-PDCD4 in a dose-dependent manner, whereas a non-specific, cIAP1 3’ UTR RNA (50) did not bind under the same conditions (Fig. 2.4A). Furthermore, we performed nitrocellulose
binding assays to determine the relative binding affinity of PDCD4 for the individual IRES RNAs. We observed that PDCD4 binds the XIAP and Bcl-xL IRESes with a similar apparent Kd (253 nM and 183 nM, respectively); however, there was no binding detected with the cIAP1 3′ UTR RNA (Fig. 2B). These data confirm that PDCD4 does indeed bind directly and specifically to IRES RNA.

We were further interested in identifying the specific domain(s) of PDCD4 required for binding to the IRES, as well as the sequence or structure of the IRES RNA that PDCD4 binds to. PDCD4 contains a putative N-terminal RNA binding domain (RBD) followed by two MA-3 domains involved in protein-protein interactions (47). We therefore generated HIS-tagged mutants of PDCD4 where the RBD domain was deleted leaving only the MA-3 domains (PDCD4ΔRBD; amino acids 157-469), or by deleting both MA-3 domains (PDCD4RBD, amino acids 1-156). We measured the binding of these mutants with the XIAP IRES as compared to wildtype PDCD4 by nitrocellulose filter binding assays. We observed that the RBD domain of PDCD4 is both necessary and sufficient for binding to the XIAP IRES since the PDCD4RBD mutant exhibits binding similar to full length PDCD4, whereas the PDCD4ΔRBD mutant shows no binding (Fig. 2C). Additionally, we were interested in determining more specifically where PDCD4 binds on the XIAP IRES. The structure of the XIAP IRES was previously determined to contain two stem loops joined by a flexible linker region (Fig. 2D)(2); interestingly, two known negative regulators of XIAP IRES, PTB and hnRNP A1 were both shown to bind domain II of the IRES, suggesting that repression of the IRES function is accomplished by repressor protein(s) binding to the 3′ end of the IRES, in the vicinity of the initiation AUG codon (2, 33). We therefore hypothesized that PDCD4 will
Fig. 2.4: PDCD4 specifically binds to XIAP and Bcl-xL IRES RNA both \textit{in vitro} and \textit{in vivo}. (A) Recombinant His-PDCD4 was incubated in the presence of $^{32}$P-labelled, \textit{in vitro}-transcribed RNA and UV-crosslinked. RNA-protein complexes were separated by SDS-PAGE and analysed by autoradiography. GST was used as a negative control. (B) Increasing concentrations of His-PDCD4 was incubated with $^{32}$P-labelled, \textit{in vitro}-transcribed RNA and nitrocellulose filter binding assays were performed and analyzed as described in Materials and Methods. Filter-bound RNA is plotted as a function of protein concentration. (C) Increasing concentrations of PDCD4 wildtype or mutant proteins, PDCD4$^{\text{RBD}}$ or PDCD4$^{\Delta \text{RBD}}$, were incubated with $^{32}$P-labelled, \textit{in vitro}-transcribed XIAP IRES RNA and analyzed as in (B). Filter-bound RNA is plotted as a function of protein concentration. (D) The structure of the minimal XIAP IRES (2) is shown on the left. The XIAP IRES 3'(-47) deletion is indicated by an arrow. Wildtype His-PDCD4 was incubated with $^{32}$P-labelled, \textit{in vitro}-transcribed XIAP RNA or mutant XIAP IRES 3'(-47) RNA, and protein-RNA complexes were analyzed as in (B). (E) Flag-PDCD4, Flag-hnRNPA1(F1) (positive control), and pCDNA3-Flag (negative control) were transfected into HEK293T cells for 24 hr and immunoprecipitated with anti-Flag agarose beads. RNA was isolated and detected by RT-PCR. GAPDH RNA was used as a negative control. RNA only samples show no genomic DNA contamination.
A. 

His-PDCD4 GST 

- PDCD4:RNA complex

XIAP IRES  Bcl-xL IRES  cIAP1 3' UTR

B. 

C. 

D. 

E. 

- RNA (%) bound

His-PDC4 (nM) 

protein concentration (nM) 

- RNA (%) bound

His-PDCD4 (nM)

- RNA (%) bound

1% input  Flag IP

<table>
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<tr>
<th>cDNA</th>
<th>XIAP</th>
<th>GAPDH</th>
<th>Bcl-xL</th>
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<td>RNA</td>
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- pDNA1-Flag
- Flag
- pDNA3-Flag
- Flag
- pDNA3-Flag
- Flag
also bind to this region. We have previously generated a mutant XIAP IRES RNA deleted of stem loop II (XIAP 3′(-47) (21)) and used the RNA derived from this mutant to measure the binding of PDCD4 in nitrocellulose filter binding assays. We observed that when compared to the full length XIAP IRES, the binding of PDCD4 is abolished when stem loop II is deleted (Fig. 2.4D).

We further to determine if the association between PDCD4 and XIAP and Bcl-xL IRES RNAs occurs *in vivo*. HEK293T cells were thus transiently transfected with Flag-PDCD4, pCDNA3-Flag (negative control), or Flag-hnRNP A1 (a known XIAP and Bcl-xL ITAF; (4, 33)) and the RNA-protein complexes were immunoprecipitated using anti-Flag agarose beads in conditions that preserve RNA-protein complexes. Following isolation of the RNA from these immunoprecipitates, cDNA was generated by reverse transcription and PCR amplified using gene-specific primers (Fig. 2.4E). We were unable to amplify significant amounts of XIAP or Bcl-xL mRNA in immunoprecipitates from Flag-transfected cells. However, we identified both XIAP and Bcl-xL RNAs in lysates from either Flag-hnRNP A1 or Flag-PDCD4 transfected cells. We were unable to amplify the high-abundance GAPDH transcript from any lysates, indicating that the immunoprecipitation of endogenous XIAP and Bcl-xL mRNAs is specific. These data support the *in vitro* binding assays and confirm that PDCD4 associates with both endogenous XIAP and Bcl-xL mRNAs *in vivo* to repress IRES-mediated translation.

### 2.5.5 PDCD4 prevents formation of the 48S initiation complex on IRES

We have recently established an *in vitro* toe-printing assay to characterize the formation of the translation-competent 48S initiation complex on XIAP IRES RNA (42). We therefore used this technique to investigate the mechanism by which PDCD4 represses IRES-mediated
translation of XIAP. We first examined the levels of PDCD4 in rabbit reticulocyte lysates (RRL) and noted that in contrast to HEK293T the levels of PDCD4 are substantially reduced in RRL (Fig. 2.5A). After XIAP IRES initiation complexes were allowed to form in RRL supplemented with increasing amounts of purified His-PDCD4, or GST as a negative control, we observed that the ability of the XIAP IRES to recruit ribosomes (as determined by a toeprint +17 to +19 nt downstream of AUG) was severely impaired in the presence of His-PDCD4 (Fig. 2.5B, compare lanes 1, 2 and 4, 5). In contrast, addition of GST had no impact on the formation of the XIAP 48S complex (lanes 3, and 4, 5).

Taken together our data strongly suggest that the mechanism of PDCD4-mediated inhibition of IRES-dependent translation involves direct binding of PDCD4 to its target RNA and subsequent interference with the formation of a translation-competent 48S initiation complex.

2.6 Discussion

Several cellular mRNAs have been identified as containing IRES elements, which allow them to be translated in times of cellular stress when global translation is attenuated. The mechanism of IRES-mediated translation is still poorly understood and the many proteins involved in regulating the process are still unknown.

The ribosomal S6 kinases (S6K) 1 and 2 have been identified as important kinases in the response to mitogen signalling that regulate a variety of cellular functions, including cell growth and proliferation. We have identified a novel signalling pathway wherein FGF-2 stimulation causes formation of a complex consisting of B-Raf, PKCe, and S6K2, leading to S6K2 phosphorylation and activation (35-37). In the present work we extend these
Fig. 2.5: PDCD4 inhibits recruitment of the ribosome on the XIAP IRES. (A) The lack of PDCD4 in rabbit reticulocyte lysate (RRL) was revealed by Western blot analysis. (B) XIAP IRES initiation complexes were formed on the \textit{in vitro}-transcribed RNA in untreated RRL, and RRL pre-incubated with His-PDCD4, GST, or toeprinting buffer. Subsequently, initiation complexes were analyzed by toeprinting and are indicated by the ‘17 to 19 nt from AUG’ line.
A.

HEK293T RRL

PDCD4 rpS6

B.

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<th>RRL (μl)</th>
<th>Buffer (μl)</th>
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<th>GST (μM)</th>
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17 to 19 nt from AUG

Full length
observations and identify tumour suppressor PDCD4 as a target of S6K2. We show, both in vitro and in vivo, that activated S6K2 phosphorylates PDCD4 leading to its proteasomal degradation. We further demonstrate that this reduction in PDCD4 levels relieves translational repression on XIAP and Bcl-xL mRNAs, thus allowing for their increased expression. FGF-2 signalling is an important factor in tumour formation since cancer cells frequently acquire the ability to produce growth factors and increase the number of membrane receptors for such factors, thus resulting in a stimulation in proliferation through autocrine signalling (reviewed in (17)). Our data suggest that FGF-2 signalling and subsequent PDCD4 degradation are key factors that cause an increase in the levels of the anti-apoptotic proteins XIAP and Bcl-xL, which likely play a role in the chemoresistance of cancer cells.

PDCD4 has been shown to be phosphorylated by S6K1, leading to its proteosomal degradation (9). The phosphorylation sites on PDCD4 appear to be same for S6K1 and S6K2, since mutations within the reported S6K1 phosphorylation sites also prevented phosphorylation of PDCD4 by S6K2. This suggests that PDCD4 acts as an effector molecule in either the S6K1- or S6K2-mediated signal transduction pathways, broadening the range of physiological events that regulate cellular translation via PDCD4. In addition, we were able to recapitulate the de-repression of XIAP and Bcl-xL protein synthesis by either overexpression of a kinase active S6K2 or by targeting PDCD4 with siRNA in the absence of any additional stimuli. These data indicate that lowering the abundance of PDCD4 is sufficient for translational de-repression of XIAP and Bcl-xL mRNAs, and no additional modification of PDCD4 by S6K2 is required for this activity. Our results highlight the importance of S6K2 for the apoptotic resistance of tumour cells; targeting S6K2 to reduce its activity could be used to inhibit the pro-survival effect of FGF-2 on cancer cells, since the
levels of PDCD4 will remain high and thus the levels of anti-apoptotic proteins, particularly XIAP and Bcl-xL, would be reduced. This decrease in XIAP and Bcl-xL proteins is expected to increase the resistance of cancer cells to death, as we have shown previously (37).

PDCD4 is a known tumour suppressor whose levels are decreased in a variety of cancers, and a link has been identified between decreased levels of PDCD4 and increased invasiveness and more aggressive tumours (7, 27, 48). Recent studies have been aimed at elucidating the mechanism of PDCD4 action. It has been shown that PDCD4 is able to bind to eIF4A and eIF4G (41, 46), thus inhibiting the helicase activity of eIF4A and preventing cap-dependent translation, which suggests that PDCD4 acts as a general repressor of translation. However, some studies have shown that mRNAs containing a highly structured 5′ UTR are preferentially repressed by PDCD4, suggesting an alternative model whereby PDCD4 has specific targets and is not a general inhibitor of translation (46, 47). Further support for this model was provided recently when it was reported that PDCD4 contains a putative RNA-binding domain within its N-terminus capable of binding to RNA (5). In addition, Singh et al. identified the c-myb coding region as an RNA target of PDCD4 and demonstrated that increased protein levels of PDCD4 repressed translation of c-myb; however no molecular mechanism of this inhibition was identified (40).

Our data demonstrate that PDCD4 specifically interacts with distinct cellular mRNAs and mediates repression of their translation by interfering with the assembly of the 48S initiation complex. We have identified both XIAP and Bcl-xL mRNAs as novel targets of PDCD4-mediated translation repression. Binding of PDCD4 to in vitro-transcribed XIAP and Bcl-xL 5′ UTR RNAs, but not cIAP1 3′ UTR RNA, supports the notion that PDCD4 exhibits binding specificity for a particular RNA sequence or structure. To further support this observation, we identified the stem loop II of the XIAP IRES as the structure that is
bound by PDCD4 since deletion of this stem loop causes a loss in binding as measured by nitrocellulose filter binding assays. Moreover, we were able to define the N-terminal putative RNA binding domain of PDCD4 as being both necessary and sufficient for binding to RNA. We further demonstrate that PDCD4 interacts with both the XIAP and Bcl-xL 5’ UTRs in vivo by using RNA-immunoprecipitation assays, indicating that the binding occurs in a cellular context. Most importantly, we show that PDCD4 translationally regulates both XIAP and Bcl-xL through the IRES elements located within their respective 5’ UTRs. Using reporter constructs we demonstrated that IRES activity of both XIAP and Bcl-xL increases upon the loss of PDCD4, which correlates well with increasing protein levels. It has been suggested that PDCD4 does not inhibit IRES-mediated translation (46). It should be noted, however, that only one viral IRES element, EMCV, was tested to support that conclusion. Viral and cellular IRES elements do not share sequence or structural homology (2, 3), and the mechanism of viral and cellular IRES-mediated translation is thus suggested to proceed through differing mechanisms. For example, while the canonical initiation factors eIF4GI and p97/DAP5 were shown to stimulate translation mediated by some cellular IRES (ie. c-myc, XIAP, cIAP1, p97) (25, 31), several viral IRES function independently of these factors (ie. HCV, CrPV (10). Notably, we confirmed an increase in both XIAP and Bcl-xL translation using polysome profiling after knockdown of PDCD4 expression, which is represented as a shift of XIAP and Bcl-xL mRNA from monosomes or light polysomes into the heavier polysomes. We did not observe any change in the overall polysome profile of PDCD4 deficient cells, suggesting that PDCD4 knockdown does not significantly affect global translation, but rather a subset of mRNAs. Our data thus corroborates published reports that challenge the notion that PDCD4 is ‘only’ a general inhibitor of translation. Instead, the sequence and structural features of the 5’ UTR are important determinants of
whether or not the mRNA is regulated by PDCD4 (12, 46, 47). Finally, our toeprinting data provide a molecular mechanism for PDCD4’s mode of action. We show that a functional translation-competent 48S initiation complex is unable to form on cellular IRES in the presence of increasing concentrations of PDCD4. This suggests that direct binding of PDCD4 to domain II of the IRES interferes with ribosome recruitment, thus preventing translation initiation. It is possible that other proteins may be involved in this repression and it will be interesting to determine if binding of PDCD4 to its target mRNAs is altered in the presence of other binding partners. We have identified PDCD4 as a novel ITAF of both XIAP and Bcl-xL mRNAs. However, as observed for other ITAFs, it is likely that other mRNA exist that are translationally regulated by PDCD4 through their IRES elements. Given the tumour suppressing properties of PDCD4, we predict that this ‘PDCD4 operon’ will be comprised of mRNAs that encode cell growth and cell survival/death regulating proteins. Indeed, our previously reported data support this notion - we have observed that the expression of Bcl-2, an anti-apoptotic protein whose translation is also mediated by an IRES, was increased upon FGF-2 stimulation (35, 36). In a preliminary analysis we found, however, that mRNAs of VEGF, Apaf-1, and cIAP1 that are known to harbour an IRES are not targets of PDCD4. It will be interesting to further examine the effect of PDCD4 on additional mRNAs through a genome-wide analysis.

In summary, we have identified PDCD4 as a novel target of S6K2 upon FGF-2 stimulation, providing a novel role for S6K2 within the cell. We have also identified PDCD4 as an ITAF that represses both XIAP and Bcl-xL IRES-mediated translation by interfering with the formation of the 48S ribosome initiation complex. Our work is the first to identify a mechanism of action for PDCD4’s specific regulation of cellular IRES activity, and thus increases our understanding of the regulation of IRES-mediated translation in general.
mediated translation of cellular mRNAs has emerged as a key mechanism that contributes to
the survival and enhanced apoptotic resistance of cancer cells; targeting multiple steps of the
FGF-2 pathway (such as regulation of IRES activity by PDCD4) may thus provide
therapeutic options for a variety of cancers.

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2.8 Author Contributions

UL, OP, MS and MH conceived the experiments; UL, NT, LEJ, SML and RR conducted the
experiments; UL and MH wrote the paper.

2.9 Conflict of interest

The authors have no competing financial interests in relation to the work described in this
manuscript.
Supplementary Table 2.2. Identification of S6K2 interacting proteins in FGF-2 treated and untreated cells. Proteins differentially identified in FGF-2 treated cells are shaded in grey.
2.10 References


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CHAPTER 3

Loss of PDCD4 contributes to enhanced chemoresistance in Glioblastoma Multiforme through de-repression of Bcl-xL translation
3.1 Preamble

"Loss of PDCD4 contributes to enhanced chemoresistance in Glioblastoma Multiforme through de-repression of Bcl-xL translation" is published in the journal Oncotarget (Volume 4, September 2013). This article describes a strong correlation between low PDCD4 protein levels and high expression of Bcl-xL protein in adult de novo GBM, GBM tumor initiating cells, and established GBM cell lines. These levels correlate significantly with poor progression and patient survival. We further demonstrate that re-expression of PDCD4 in GBM cells down-regulates Bcl-xL expression and decreases cell viability. Most importantly, inhibition of Bcl-xL with ABT-737 sensitizes GBM cells to the effects of the chemotherapeutic doxorubicin. These results identify Bcl-xL as a novel marker of GBM chemoresistance and provide treatment options for this aggressive tumor.

Author List

Urszula Liwak, Lindsay E. Jordan, Sally Davidson Von-Holt, Poonam Singh, Jennifer E.L. Hanson, Ian A. Lorimer, Federico Roncaroli, and Martin Holcik

Author Contributions

UL and MH wrote the paper; U.L., L.E.J., I.A.L., F.R., and M.H. conceived the experiments; U.L., L.E.J., and F.R. conducted the experiments; UL designed and performed experiments presented in Figures 3.2C, 3.3, 3.4.
3.2 Abstract

Glioblastoma multiforme (GBM) is the most common and aggressive form of tumor of the central nervous system. Despite significant efforts to improve treatments, patient survival rarely exceeds 18 months largely due to the highly chemoresistant nature of these tumors. Importantly, misregulation of the apoptotic machinery plays a key role in the development of drug resistance. We previously demonstrated that Bcl-xL, an important anti-apoptotic protein, is regulated at the level of translation by the tumor suppressor programmed cell death 4 (PDCD4). We report here a strong correlation between low expression of PDCD4 and high expression of Bcl-xL in adult de novo GBM, GBM tumor initiating cells, and established GBM cell lines. Importantly, high Bcl-xL expression correlated significantly with poor progression and patient survival. We demonstrate that re-expression of PDCD4 in GBM cells down-regulated Bcl-xL expression and decreased cell viability. Finally, we show that direct inhibition of Bcl-xL by small molecule antagonist ABT-737 sensitizes GBM cells to doxorubicin. Our results identify Bcl-xL as a novel marker of GBM chemoresistance and advocate for the combined use of Bcl-xL antagonists and existing chemotherapeutics as a treatment option for this aggressive tumor.

3.3 Introduction

Glioblastoma multiforme (GBM) is the most common primary intrinsic tumor of the central nervous system in adults and one of the most aggressive types of cancer [1, 2]. Despite significant efforts to improve the outcome of patients with GBM, their median
survival remains approximately 18 months, with a five-year survival of 5%. Both extensive infiltration of the adjacent brain that prevents radical surgical removal, and resistance to chemotherapy are the key factors of poor outcome. About 90% of GBM occurs de novo, while the remaining 10% result from the progression of a lower grade astrocytoma. Defined as secondary GBMs, these latter tumors show molecular and genetic changes that are different from de novo GBMs, including frequent mutation of p53 and mutation of IDH1[1, 2].

Low expression levels of the tumor suppressor programmed cell death 4 (PDCD4) have been correlated with poor outcome in patients with GBM. The frequent loss of PDCD4 in GBM is partly due to epigenetic silencing secondary to 5’CpG island methylation [3] as well as over-expression of microRNA 21 (miR-21) which targets PDCD4 mRNA for degradation [4]. Furthermore, frequent over-activation of kinases, in particular S6K1 and S6K2, observed in GBM leads to phosphorylation and subsequent degradation of PDCD4 [5-7]. PDCD4 plays key roles in a number of cellular processes including cell growth and invasion via inhibition of the AP-1 transcription factor as well as translation suppression through the eukaryotic initiation factor (eIF) 4A (reviewed in [8]). Since eIF4A is thought to be required for translation of virtually all cellular mRNAs, the PDCD4-dependent inhibition of eIF4A results in a decrease in global translation. Recently, however, several reports identified specific targets of PDCD4, thus pointing towards a novel role for this molecule in regulating selective translation of distinct mRNAs and not as a general inhibitor of translation [6, 9, 10]. Among the specific PDCD4 targets we identified the Bcl-2 family member Bcl-xL. Bcl-xL is an inhibitor of mitochondrial outer membrane permeabilization
thus is a strong anti-apoptotic protein [6]. In addition it plays a role in p53 signaling [11] and cell cycle progression and checkpoints [12]. We demonstrated that PDCD4 specifically binds to and represses translation from the internal ribosome entry site (IRES) of Bcl-xL and that loss of PDCD4 removes the repression on the Bcl-xL IRES and results in an increase in Bcl-xL protein levels [6].

Given the known roles of Bcl-xL in regulation of apoptosis and chemoresistance, we sought to determine if the loss of PDCD4 expression observed in GBM causes elevated Bcl-xL expression, which could explain the high chemoresistance of GBM cells. Indeed, we find that low levels of PDCD4 correlate with high levels of Bcl-xL in both de novo GBM patient tumors and in established GBM cell lines and that high Bcl-xL correlates with poor progression and patient survival. Furthermore, we demonstrate that re-expression of PDCD4 in GBM cells results in a repression of Bcl-xL protein expression and a decrease in cell viability. Finally, we demonstrate that direct inhibition of Bcl-xL by the small molecule inhibitor ABT-737 results in a sensitization of GBM cells to doxorubicin. Our data identify Bcl-xL as a target of PDCD4 whose elevated levels contribute to high chemoresistance in GBM, thus providing a novel treatment option for this aggressive tumor.

3.4 Results

3.4.1 Loss of PDCD4 correlates with increased Bcl-xL in human GBM samples

Our previous work demonstrated the role of PDCD4 in regulating Bcl-xL, an inhibitor of apoptosis, through its IRES-mediated translation. Under normal proliferative
conditions, we demonstrated that PDCD4 specifically and directly binds to and inhibits Bcl-xL IRES translation. However, when expression of PDCD4 is down-regulated, the repression on Bcl-xL is relieved thus resulting in an increase in Bcl-xL protein levels. These findings prompted us to investigate the link between PDCD4 and Bcl-xL in GBM.

In order to study the relationship between Bcl-xL and PDCD4 expression in a clinical setting, we investigated with immunohistochemistry a cohort of 50 human de novo GBMs. Twenty-six GBMs were positive for Bcl-xL, where 15 of them showed expression in more than 50% of neoplastic cells (score 2) (Figure 3.1A, 1B) and 11 showed focal expression (score 1). Thirty cases did not show any detectable PDCD4. Interestingly, 18 cases with no PDCD4 showed Bcl-xL positive cells and 12 PDCD4 positive cases had no Bcl-xL. Immunopositivity for Bcl-xL was cytoplasmic and granular in keeping with its mitochondrial localization (Figure 3.1A). Bcl-xL immunolabelling was also found in reactive astrocytes, a few microglial cells and some neurons. Sixteen tumors showed PDCD4 nuclear and/or cytoplasmic expression but it was only limited to a minority of tumor cells (score 1). In all 50 lesions, PDCD4 was present in endothelial and inflammatory cells, including perinecrotic macrophages (Figure 3.1A). Six out of the seven recurrent cases demonstrated diffuse Bcl-xL expression that was more intense than in the primary tumor. No change in PDCD4 was seen in recurrent tumors compared to the primary lesion. Chi square test for trend (p=0.0469) of this cohort suggested that PDCD4 likely regulates Bcl-xL protein in human samples, therefore confirming our previous in vitro data. The distribution of Bcl-xL and PDCD4 positive cases is represented in Figure 3.1B. To understand if Bcl-xL and PDCD4 have any impact on patient outcomes, we correlated their expression with progression and survival.
**Figure 3.1:** Patient GBM tumors show a correlation between low PDCD4 and high Bcl-xL levels (A) I – Hematoxylin-eosin stain of a representative case of glioblastoma multiforme (x10); II – diffuse Bcl-xL expression in neoplastic cells (immunoperoxidase x10); III – Granular cytoplasmic immunolabeling showing mitochondrial localization of Bcl-xL (immunoperoxidase x20); IV – PDCD4 is positive in endothelial cell and intratumoral lymphocytes (immunoperoxidase x20); V – Colorectal carcinoma as positive control for Bcl-xL (immunoperoxidase x20); VI – immunoreactions with omission of the primary antibody, (immunoperoxidase x20). (B) Dot plot representation of 50 cases of adult de novo GBM with respect to PDCD4 and Bcl-xL expression (Score 0 – expression between 0-5% was considered negative, Score 1 – expression between 5-50%, Score 2 – expression over 50%). More than 70% of Bcl-xL positive tumors are PDCD4 negative (Chi square test for trend, p=0.0469). (C) Kaplan-Meier curve showing a significant correlation between high Bcl-xL expression (score 1, 2) and progression (p=0.0187, Geham-Breslow-Wilcoxon test) and (D) significant correlation between high Bcl-xL expression (score 1, 2) and poor survival (p=0.0476, Geham-Breslow-Wilcoxon test).
Patients with Bcl-xL positive GBM (score 1 and 2) showed a median progression of 136 days and median survival of 280 days against median progression of 284.5 days and survival of 611 days of patients with Bcl-xL negative lesions. Gehan-Breslow-Wilcoxon test showed a significant difference between the Bcl-xL positive and Bcl-xL negative cases for both progression (p=0.0187) and survival (p=0.0476). The effect on MGMT status did not correlate with either progression or survival suggesting that Bcl-xL represents an important biomarker of chemoresistance of GBM.

In order to further explore the link between PDCD4 and Bcl-xL we examined a panel of established GBM cells lines and patient-derived tumor initiating cells (TICs). We measured the relative ratio of Bcl-xL and PDCD4 using HEK293 cells as a reference. In concordance with patient samples, we find that GBM cells exhibit low levels of PDCD4, which correlate with a robust expression of the Bcl-xL protein (Figure 3.2A, 2C). We have previously described the mechanism by which PDCD4 regulates expression of Bcl-xL through translational repression of the Bcl-xL IRES [6]. In accordance with this, other than U343 cells, we find that the levels of Bcl-xL mRNA do not differ significantly between HEK293 and GBM cells (Figure 3.2B), lending further support to the notion that PDCD4 is an inhibitor of Bcl-xL translation and that the loss of PDCD4 in GBM results in derepression of Bcl-xL translation.

To further demonstrate the causal link between PDCD4 and Bcl-xL expression, U373 cells were transiently transfected with GFP- or PDCD4-expressing plasmids and the expression of Bcl-xL was determined by Western blot analysis. We find that restoring the expression of PDCD4 resulted in a significant decrease in Bcl-xL (Figure 3.3A).
Figure 3.2: GBM cell lines and tumor initiating cells have low levels of PDCD4 and high levels of Bcl-xL protein. (A) Western blot analysis of a panel of GBM cell lines indicating the correlation between low PDCD4 levels and high Bcl-xL levels. (B) qPCR analysis of GBM cell lines showing no increase in Bcl-xL versus GAPDH mRNA as compared to HEK293 reference. (C) Western blot analysis of GBM tumor initiating cells (TICs) showing correlation between low PDCD4 and high Bcl-xL levels.
Figure 3.3: Re-introduction of PDCD4 into GBM causes reduction in Bcl-xL and cell viability. (A) PDCD4 was overexpressed in U373 for 24 h followed by western blot analysis showing decrease in Bcl-xL. (B) Alamar blue analysis of cells overexpressing PDCD4 for 24 h to measure cell viability (* p<0.05).
Furthermore, since Bcl-xL is a known inhibitor of apoptosis, we were interested in determining the effect that the PDCD4-mediated decrease in Bcl-xL expression would have on cell viability. We thus measured cell viability in GFP- or PDCD4-transfected cells by Alamar blue analysis. We observed that restoring PDCD4 levels in U373 cells resulted in a significant reduction in cell viability (Figure 3.3B). Taken together, this data suggests that the apoptotic resistance typically observed in GBM cells is due in part by the large increase in the expression of Bcl-xL, which is a result of the loss of PDCD4. More importantly, our data suggest that by manipulating the levels of Bcl-xL, GBM cells could be sensitized to chemotherapeutics.

3.4.2 Inhibition of Bcl-xL sensitizes GBM cells to Doxorubicin

Since re-introduction of PDCD4 protein into tumor cells is likely not a feasible therapeutic option, we sought to explore if targeting downstream of PDCD4 would offer an effective strategy. We therefore chose to inhibit Bcl-xL using the small molecule inhibitor ABT-737 in combination with the chemotherapeutic drug doxorubicin. We expected that inhibition of Bcl-xL would sensitize cells to doxorubicin since high Bcl-xL levels would no longer inhibit the apoptotic machinery. Four different GBM cell lines (U87, U373, SNB19, SNB75) were treated with ABT-737 alone or in combination with doxorubicin for 24 hours and cell viability was determined (Figure 3.4). We observed that treatment of three out of four GBM cell lines with ABT-737 alone resulted in minimal cell death even at highest concentrations of the inhibitor (Figure 3.4A). In contrast, one cell line, U87, was sensitive to ABT-737 at high concentrations. Similarly, with the exception of U87 cells, the treatment of
Figure 3.4: ABT-737 sensitizes GBM cells to doxorubicin. (A) Cytotoxicity was measured by incorporation of Yoyo-1 dye after treatment of indicated cell lines with increasing concentrations of doxorubicin for 24 h. (B) Cytotoxicity was measured after treating indicated cell lines with increasing concentrations of ABT-737 in the presence of Yoyo-1. (C) Cytotoxicity was measured after treating indicated cells with ABT-737 (1uM), doxorubicin (dox, 500nM), or a combination of both in the presence of Yoyo-1 (* p<0.05, ** p<0.01).
these cells with doxorubicin alone resulted in minimal cell death (Figure 3.4B). However, combined treatment with sublethal doses of doxorubicin (500nM) and ABT-737 (1 uM) significantly increased cytotoxicity in all four cell lines tested (Figure 3.4C).

3.5 Discussion

Despite the recent advances in surgical resection techniques, radiotherapy planning and dose delivery, and stratified targeted medical treatment, more than 90% of patients with GBM succumb to disease within 18 months from onset and show an overall median progression-free survival time of approximately 7 months [2]. Recent clinical trials have achieved better outcomes with median overall survival of up to 22 months but such an improvement is likely influenced by better supportive care, more aggressive salvage therapy and a more stringent selection of patients enrolled. In fact, good preoperative and postoperative performance status and extensive neurosurgical tumor resection are important factors influencing the outcome. Extensive infiltration of normal brain, intra and inter-tumoral molecular and genetic heterogeneity, and chemoresistance of neoplastic cells are the key determinants of poor treatment response.

Chemotherapy represents the mainstream treatment modality in GBM but glioma cells characteristically show simultaneous resistance towards cytotoxic drugs. Such multidrug resistance can occur at the beginning of treatment or be acquired during chemotherapy. The mechanisms causing chemoresistance are complex and multifactorial [13]. In addition, defects in the apoptotic pathway are also important in tumor drug resistance, which can be secondary to either over-activation of anti-apoptotic proteins or to the loss of expression or loss of function of pro-apoptotic molecules [14]. In our previous
studies, we demonstrated that the tumor suppressor protein PDCD4 plays a critical role in regulating IRES mediated translation of key anti-apoptotic factors including Bcl-xL [6]. We showed that under normal conditions, PDCD4 directly binds to and inhibits translation of Bcl-xL mRNA. However, upon loss of PDCD4, the repression is relieved thus resulting in IRES mediated translation and an increase in protein production. High levels of Bcl-xL have been associated with chemoresistance by inhibiting the cell from undergoing apoptosis upon the addition of stressors [15]. This chemoresistance is through Bcl-xL’s ability to prevent mitochondrial outer membrane permeabilization and cytochrome c release. Since PDCD4 is a known tumor suppressor, and is lost in a variety of tumors including GBM, we were interested in determining if this loss also corresponds with an increase in Bcl-xL, which would contribute to the highly chemoresistant nature of GBM tumors. Indeed, we identified a correlation between low levels of PDCD4 and high levels of Bcl-xL in a cohort of 50 primary GBM tumors. Furthermore, we observed a significant correlation between Bcl-xL and tumor progression and patient survival. This data suggests that Bcl-xL may be a good prognostic tool to identify patient outcome and response to chemotherapy, independent of MGMT status. Our results differ from the study published by Cartron et al [16] where the authors examined 13 members of the Bcl-2 family, including Bcl-xL, and found no correlation with overall survival. Their analysis was based on Western blot and Elisa data and the results were evaluated semi-quantitatively attributing score 0 to tumors with low expression and 1 to tumors showing high expression. Given the heterogeneity of the microenvironment of GBMs that contains several non-neoplastic cell populations [17], we decided to assess Bcl-xL by immunoperoxidase immunohistochemistry on tissue sections.
This allowed us to count the expression in tumor cells only. With this approach entrapped neurons, microglia and reactive astrocytes that were seen to express the protein were excluded from our assessment. In contrast, expression of Bcl-xL in non-neoplastic cells may have affected Cartron et al. results that were obtained using tumor homogenates. Given the retrospective nature of our study, semi-quantitative analysis of Bcl-xL was based on number of positive cells rather than intensity of expression accounting for another difference between the two studies. Correlation with outcome was obtained considering positive cases with those with more than 5% of expression irrespective of intensity. With a similar semi-quantitative approach Yoshimine et al [18] observed a correlation between lower survival and increased expression of Bcl-xL in urothelial carcinoma, further suggesting a role for this molecule in cancer progression.

Furthermore, we also observed this correlation in tumor initiating cells and in a panel of established GBM cell lines, which we utilized for further analysis. Importantly, we did not see a significant change in Bcl-xL mRNA levels that would account for the drastic increase in protein levels. This finding suggests that transcription and/or stability of the mRNA is not a contributing factor; however, translation of the Bcl-xL mRNA is critical for the increase in protein. This further supports our previous findings that PDCD4 can negatively regulate Bcl-xL IRES mediated translation. To verify that PDCD4 directly affects Bcl-xL in GBM cells, we rescued PDCD4 expression, which resulted in reduction in Bcl-xL levels and importantly, a reduction in cell viability. In order to use this therapeutically to our advantage, we decided to target downstream of PDCD4 since overexpressing a protein in patient tumor samples is likely not a feasible option. We treated four different GBM cell lines with the chemotherapeutic doxorubicin, which resulted in minimal cell death at low concentrations
likely due to the high levels of Bcl-xL. Increase in cell death was observed at high concentrations of the drug in accordance with previous reports [19]. Importantly, by directly inhibiting Bcl-xL with the chemical inhibitor ABT-737, we were able to sensitize these cells to the effects of low concentrations of doxorubicin. These data clearly indicate the importance of monitoring the levels of Bcl-xL in patient tumors as well as the possibility of using ABT-737 as an adjuvant to existing chemotherapeutics in treating GBM tumors.

3.6 Materials and Methods

3.6.1 Patient selection: From the Brain Tumor Registry at Imperial College, London UK, we have selected 50 adult patients operated de novo GBM whom the clinical history, pre-operative and post-operative MRI scan, and data on post-operative radio-chemotherapy and follow-up were available for review (Supplementary Table) and examined their tumors for the expression of PDCD4 and Bcl-xL. Given the heterogeneous nature of GBM, we chose samples obtained from maximally safe surgical debulking rather than stereotactic biopsies because they allowed us to examine as much tumor tissue as possible and investigate the full extent and distribution of the two proteins. We chose supratentorial, hemispheric GBMs because gross total excision is not achievable in tumors occurring in the subcortical grey matter, cerebellum, brainstem and spinal cord. None of the patients had previous evidence of a lower grade astrocytoma. In order to correlate PDCD4 and Bcl-xL expression with patients’ outcome, we selected patients that had Karnofsky’s performance status at onset of 70 or higher. Evidence of disease progression documented at follow-up
neuroimaging and survival time were used as measures of outcome and were calculated in
days from the day of operation.

Of the 50 patients, 19 were female; the mean age at the time was 57 years and 2
months (range between 31yrs 5mo–78yrs 7mo; median 58yrs). Progression time ranged
between 23-1154 days (average 316 days; median 207 days). Survival ranged 30-1611 days
– average 492 days; median 355 days). Five patients were alive at the time of the last follow-
up and four of them were clinically stable with no evidence of progression at neuroimaging.
Ten patients had a second operation following progression after chemoradiation but
diagnostic tissue for further immunostains was only available in 7 cases. Two of these 10
patients had progression after the second operation. All patients had consented for their
tissue to be used for research.

3.6.2 Pathological assessment: Tissue was routinely fixed in formalin and embedded
in paraffin (FFPE). The original HE-stained sections of each case were re-examined to
confirm the diagnosis and the most representative sample from each case was used for
immunohistochemical stains. Five micron sections were cut from each block for
immunohistochemistry. All lesions were routinely assessed for MGMT gene promoter
methylation and expression of the mutant IDH1R132H. Peroxidase immunohistochemistry was
performed on FFPE tissue sections with antibodies directed against PDCD4 (Rabbit
polyclonal, Rockland at the dilution of 1:350 following antigen retrieval 20 in steamer in
citrate buffer at pH 6) and anti-Bcl-xL (Rabbit polyclonal Cell Signalling at the dilution of
1:200, following antigen retrieval 20 in steamer in citrate buffer at pH 6). Briefly, sections
were dewaxed in xylene and dehydrated in decreasing alcohols to distilled water.
Endogenous peroxidase quenching was obtained by incubating the sections in 0.3% hydrogen peroxide for 30 min. Immunostains were then performed with an automated immunostainer (Leica bond III, Leica Microsystems Ltd, Milton Keynes, UK). Sections were counterstained in Harris’ hematoxylin, dehydrated in progressive alcohols and xylene and coverslipped. Sections of pharyngeal tonsils were used as a positive control for PDCD4 and sections of colon cancer were used as positive control for Bcl-xL. Immunostains following similar protocol but with omission of the primary antibody were performed as negative controls. The extent of expression of the two proteins was assessed semi-quantitatively on tissue sections. Expression between 0-5% was considered negative (score 0), while tumors featuring more than 50% of positive cells were scored 2. Any tumor showing between 5 and 50% positivity was scored 1.

3.6.3 Cell Culture. Human embryonic kidney (HEK293), and human glioblastoma (SNB19, SNB75, U87, SF295, U343, SF268, U373) cells were maintained in standard conditions in Dulbecco’s modified Eagle’s medium supplemented with heat-inactivated 10% fetal calf serum, 2 mM L-glutamine, and 1% antibiotics (100 units/ml penicillin-streptomycin). Cells were transfected using Fugene (Roche) as per the manufacturer’s protocol. For the isolation of tumor initiating cells, consent was obtained from patients in accordance with a protocol approved by the Ottawa Hospital Research Ethics Board. Surgical samples were digested with Accutase (Sigma-Aldrich, Oakville, ON, Canada), filtered through 100 µM and 40 µM nylon mesh filters, and plated on laminin-coated plates in Neurobasal A medium supplemented with B27, N2 (all from Life Technologies,
Burlington, ON, Canada), EGF and FGF (Peprotech, Rocky Hill, NJ, USA) as described by Pollard et al [20]. Cells were cultured in 5% O₂/CO₂ at 37°C. Debris and other cell types were removed by media changes once tumor initiating cells had adhered. Cells were characterized for chromosomal abnormalities typical of glioblastoma using chromogenic in situ hybridization, positive expression of nestin by immunofluorescence, the ability to form neurospheres in the absence of laminin, the ability to differentiate in response to serum exposure and/or growth factor withdrawal, and the ability to form invasive glioblastoma in immunocompromised mice as described by Gont et al [21].

3.6.4 Western blot. Cells were lysed in RIPA buffer for 15 min on ice followed by centrifugation at 12,000 x g for 15 min to pellet cell debris. Protein concentrations were measured by a Bradford Assay (Bio-Rad) and equal amounts of proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Western blotting was performed using rabbit anti-PDCD4 (Rockland), rabbit anti-Bcl-xL (Cell Signaling Technology), or mouse anti-GAPDH (Advanced ImmunoChemical Inc), followed by species-specific horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Antibody complexes were detected using ECL (GE Biosciences) and X-ray film (Kodak).

3.6.5 RNA extraction, qRT-PCR. Total RNA was isolated using the Absolutely RNA Miniprep kit (Stratagene). Reverse transcription was done using the First-strand cDNA synthesis kit (GE Biosciences) and quantitative PCR was performed using the QuantiTect
SYBR green PCR kit (Qiagen) with gene specific primers for Bcl-xL (QuantiTect Primer Assay; Qiagen) and GAPDH [6].

3.6.6 Alamar Blue Analysis. U373 cells were transfected with GFP or GFP-PDCD4 for 24 h followed by analysis of cell viability by Alamar Blue (Invitrogen) as per manufacturer’s protocol.

3.6.7 Cytotoxicity Assays. Cells were seeded in a 96-well plate at 5000 cells/well for 24 h. Cells were treated with ABT-737, Doxorubicin, or their combinations for 24 h in the presence of Yoyo-1 (Molecular Probes) and the percentage of Yoyo-1 positive cells versus total cells as indicated by Vybrant DyeCycle Green (Molecular Probes) was measured using the IncuCyte Live-Cell Imaging System (Essen Bioscience).

3.6.8 Statistical analysis. With the exception of patient data all data are expressed as means +/- standard deviation (s.d), with a minimum of three independent experimental replicates unless otherwise noted. Chi square test for trend, Geham-Breslow-Wilcoxon test, and student’s t-test were performed to determine data significance using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA).

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Chapter 4

The RNA-binding protein HuR protects PDCD4 mRNA from microRNA-21 induced silencing
4.1 Preamble

“The RNA-binding protein HuR protects PDCD4 mRNA from microRNA-21 induced silencing” is an unpublished manuscript. This article identifies HuR as a regulator of PDCD4 expression through its ability to bind to the PDCD4 3’UTR. Furthermore, it demonstrates that HuR binding prevents miR-21 association and subsequent mRNA degradation. Finally, we show that H₂O₂ treatment correlates with reduced PDCD4 mRNA and protein expression and that this effect is mediated by miR-21.

Author list

Urszula Liwak, Thet Naing, and Martin Holcik

Author contributions

UL and MH wrote the article. UL designed and performed the experiments. TN performed repeats of Figure 4.4A.
4.2 Abstract

Programmed cell death 4 (PDCD4) is a tumour suppressor implicated in the development and progression of many cancers. Recently, it has been identified as a negative regulator of alternative translation of the anti-apoptotic genes XIAP and Bcl-xL, where a loss of PDCD4 correlates with an increase in protein expression. This increase contributes to the cancer cells enhanced chemoresistance; therefore, understanding how PDCD4 is regulated is important for elucidating its mis-regulation in diseased states. We now show that the RNA-binding protein HuR binds to the PDCD4 3'UTR to stabilize it. We further demonstrate that HuR binding protects PDCD4 from miR-21 induced silencing. Finally, we show that PDCD4 expression is lost following H₂O₂ treatment, which is mediated by miR-21 binding. Our results identify HuR as a regulator of PDCD4 stability by preventing the interaction with miR-21.

4.3 Introduction

Programmed cell death 4 (PDCD4) is a tumour suppressor that was initially discovered as a protein whose expression increases during apoptosis (1), and has since been implicated in the development of many cancers including lung, colon, liver, breast, and brain (2-6). PDCD4’s ability to bind to and inhibit the eukaryotic initiation factor (eIF) 4A, the main helicase required for cap-dependent translation, points towards a role as a general inhibitor of translation (7, 8). Recently, however, a role for PDCD4 in inhibiting translation of a subset of specific targets has emerged. For example, Wedeken et al (9) demonstrated that PDCD4 binds to the 5'UTR of p53 and inhibits its translation. Furthermore, we have demonstrated that PDCD4 can specifically bind to the internal ribosome entry site (IRES)
elements of both the X-linked inhibitor of apoptosis (XIAP) and Bcl-xL messenger RNAs (mRNAs) and repress their cap-independent translation (10). These observations demonstrate that PDCD4 is not a general translational inhibitor, but in fact, is a regulator of specific targets. We demonstrated the importance of this regulation recently, where the loss of PDCD4 in Glioblastoma multiforme (GBM) tumours correlates with an increase in the anti-apoptotic Bcl-xL protein. We further demonstrated that re-expression of PDCD4 in GBMs down-regulates Bcl-xL expression thus sensitizing these cells to chemotherapeutics. Given PDCD4’s novel role in regulating alternative translation of anti-apoptotic proteins and the effect this has on tumourigenesis; understanding how PDCD4 expression itself is regulated is crucial for understanding tumour development. At the protein level, PDCD4 can be phosphorylated by S6 kinase 1 (S6K1) in response to mitogens (11) or S6K2 in response to fibroblast growth factor -2 (FGF-2) (10, 12), leading to its degradation. Additionally, PDCD4 is also regulated at the mRNA level by microRNA (miR)-21 whose expression is upregulated in a variety of cancers (13-15). Interestingly, recent evidence shows that miRNAs ability to regulate transcripts can be controlled through several distinct RNA binding proteins including Human antigen R (HuR).

HuR is a ubiquitously expressed, RNA binding protein that belongs to the Hu/embryonic lethal abnormal vision (ELAV) family of proteins (16). HuR localizes primarily to the nucleus where it is involved in regulating mRNA splicing (17), export (18), and polyadenylation (19) via its three RNA recognition motifs (RRMs). Notably, a hinge region between RRM2 and RRM3 contains a nucleocytoplasmic shuttling domain responsible for shuttling HuR into the cytoplasm in response to a variety of stressors such as UV, arsenite, and hydrogen peroxide, H₂O₂, treatment (20, 21). It is this cytoplasmic
accumulation that allows HuR to modulate mRNA stability and translation (22-24). Many functions of HuR are mediated through its binding to AU-rich elements (AREs) of target mRNAs found in their 3' untranslated regions (UTRs). However, HuR can also bind the 5'UTR where it has been shown to either positively or negatively regulate translation. For example, HuR binds to the 5'UTR of IGF-IR to downregulate its translation (25). Moreover, similarly to PDCD4, we demonstrated that HuR binds to and regulates IRES mediated translation of XIAP (23) and Bcl-xL (24). Aside from this, HuR has also been implicated in regulating translation through its ability to influence microRNA regulation. Generally, in a competitive role, the binding of HuR to the mRNA prevents miR/RISC (RNA induced silencing complex) binding thus resulting in stabilization of the mRNA and an increase in translation (26). Conversely, other studies suggest that HuR binding can result in conformational changes in the mRNA that promote miR/RISC binding leading to mRNA degradation or translation inhibition (26). Given the diverse functions of HuR, it is no surprise that it plays a major role in the development and progression of cancer. Mainly, this is through its ability to regulate the stability or translation of target mRNAs important for tumour growth, angiogenesis, invasion, and metastasis (27).

Since both HuR and PDCD4 mediate IRES translation of the same targets, which is important for cancer development, and since HuR can alter miRNAs ability to regulate transcripts, we were interested in determining if HuR can regulate PDCD4. Indeed, we discovered that HuR is able to regulate PDCD4 mRNA levels, in turn regulating PDCD4 protein expression. We show that knockdown of HuR results in a loss of PDCD4 that is mediated through miR-21. We further demonstrate that treatment of cells with H$_2$O$_2$ correlates with a loss of PDCD4 that is also mediated through miR-21. This leads us to
propose that HuR and miR-21 are involved in co-regulating PDCD4 expression in response to stress, thus affecting its ability to repress anti-apoptotic genes involved in the cellular stress response.

4.4 Materials and Methods

4.4.1 Cell culture, expression constructs, and transfection

HeLa cells were maintained in standard conditions in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal calf serum, 2 mM L-glutamine, and 1% antibiotics (100 units/ml penicillin-streptomycin). HuR cDNA was cloned after the C-terminus of GST in the pGEX-KG plasmid for recombinant protein purification. Transfections of siRNA, or miRVana microRNA mimics and inhibitors were performed using Lipofectamine RNAiMax (Life Technologies). Briefly, 2.5 x 10⁴ HeLa cells were seeded in 12-well plates for 24 hours. Transfections were performed at a final concentration of 20 nM HuR siRNA (AAGUCUGUUCAGCAGCAUUGGUdTdT, Dharmacon), nonsilencing control (Qiagen, Cat. # 1022076), miR-21 mimic (Ambion, Cat. # 4464066), miR negative Control mimic (Ambion, Cat. # 4464058), anti-miR-21 (Ambion, Cat. # 4464084), anti-miR control (Ambion, Cat. # 4464076). Cells were treated in the presence of 0.5 mM H₂O₂ for 4 hours. Cells were harvested for analysis after the indicated time points.

4.4.2 Western Blot Analysis

Cells were washed with PBS, scraped, and transferred to an eppendorf tube. Cells were pelleted and resuspended in RIPA buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% SDS, 1 mM PMSF) for 15 minutes on ice. Lysates were centrifuged
at 12,000 x g for 15 minutes to pellet cell debris. Bradford Assay (Bio-Rad) was used to quantify protein concentration and equal concentrations were loaded on SDS-PAGE gels. Proteins were transferred to a PVDF membrane and analysed by rabbit anti-PDCD4 (Rockland), mouse anti-HuR (Santa Cruz Biotechnology), mouse anti-Tubulin (Abcam), and rabbit anti-GST (Santa Cruz Biotechnology) antibodies followed by species-specific HRP-conjugated secondary antibodies (Cell Signaling Technology). Antibody complexes were detected using an ECL or ECL Plus system (GE Biosciences) and were quantified using Odyssey densitometry software (Li-COR Biosciences).

4.4.3 RNA extraction and quantitative RT-PCR (qRT-PCR) analysis

Total RNA was isolated from cells using RNAzol (Molecular Research Center, Inc.) as per manufacturer's protocol. cDNA was generated using the First-strand cDNA synthesis kit (GE Biosciences). Quantitative PCR was performed using the QuantiTect SYBR green PCR kit (Qiagen) with gene specific primers for PDCD4 (QuantiTect Primer Assay; Qiagen) and GAPDH (10).

4.4.4 In vitro synthesis of $^{32}$P-labeled RNA and UV-crosslinking

The first 610 nt of the PDCD4 3'UTR containing the miR-21 binding site (228-249 nt; (13)) was cloned after the chloramphenicol acetyl transferase (CAT) coding sequence in the pMC.pa plasmid described in (28) using the forward 5'-

CAGGATCCATATAAGAAGCTTTGCGTC and the reverse 5'-

CTTCTAGAACCAGTTCATTTTTCC primers. DNA templates containing the T7 promoter were generated from this pMC.PDCD4_3'UTR.pa plasmid by PCR (S1 fragment: forward primer 5'-'CAGGATCCATATAAGAAGCTTTGCGTC, reverse primer 5'-'
CTTCTAGACTTGCCCCCTCGAAAAAC; S2 fragment: forward primer 5’- CAGGATCCGAGGGACAGAAAAAGTAAC, reverse primer 5’- CTTCTAGATTTTAGCAGCTTAACTTT; S3 fragment: forward primer 5’- CAGGATCCCCCATGTTGGCTGCTGC, reverse primer 5’- GGAAAAATGAACCTGGTTCTAGAAG). RNA was generated using [α-32P]UTP and a MAXIscript T7 kit (Ambion) as per the manufacturer's protocol. The RNA was run on a 5% acrylamide–8 M urea denaturing gel and purified. The RNA was then incubated with purified GST or GST-HuR in RNA binding buffer (10 mM Tris-HCl [pH 7.4], 3 mM MgCl2, 300 mM KCl, 1 mM dithiothreitol [DTT], 0.2 mM PMSF, leupeptin [20 µg/ml]) for 30 minutes at room temperature then cross-linked at 250 mJ/µm² in a Stratalinker. The complexes were treated with RNase T1 (1 U/µl), RNase A (10 µg/ml), and heparin (5 mg/ml) for 10 minutes. The samples were separated by SDS-PAGE gel, and exposed to X-ray film at −80°C overnight.

**4.4.5 GST-tag protein purification**

E. coli was transformed with the pGEX or pGEX-KG_HuR plasmid and grown overnight in 4 mL Luria-Bertani (LB) media containing ampicillin (100 mg/mL). The culture was added to 100 mL of Luria-Bertani (LB) media containing 100 mg/mL of ampicillin and grown to an OD of 0.7. Isopropylthio-β-galactoside (IPTG) was added to a final concentration of 1 mM and grown for 4 hours longer. The cultures were centrifuged at 5000 x g for 10 minutes at 4°C and supernatant discarded. The pellet was resuspended in 10 mL of ice-cold PBS and the samples were centrifuged again at 5000 x g for 10 minutes at 4°C. Samples were then lysed with 10 mL lysis buffer (50 mM Tris-HCl [pH 8.0], 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 mM PMSF). Lysates were sonicated twice, 1% Triton X-100 was added, and
samples were sonicated again. Samples were centrifuged at 13000 \times g for 10 minutes at 4°C. Glutathione sepharose beads (200 μL) were added to the supernatant and rotated at 4°C for 2 hours. Samples were washed 5 times with cold PBS and proteins were eluted using 20 mM L-glutathione, pH 8.0 in PBS rotating at 4°C for 1 hour.

**4.4.6 RNA-protein complex immunoprecipitation**

HeLa cells were cross-linked *in vivo* with 1% formaldehyde for 30 min at room temperature followed by 0.2 M glycine for 5 minutes to stop cross-linking. Cells were washed in PBS and lysed in 1 mL/10 cm plate of NP40 buffer (50 mM HEPES [pH7.4], 120 mM NaCl, 0.5% NP40, 2 mM EDTA, 5% glycerol, PMSF, 40 U/mL RNase inhibitor) at 4°C for 30 minutes. Lysates were treated with 12 μL DNaseI (20 mg/mL), 40 U RNAse inhibitor, 25 mM MgCl$_2$, 5 mM CaCl$_2$ and incubated at 37°C for 30 minutes. The reaction was stopped by adding EDTA to a final concentration of 20 mM followed by centrifugation at 14 000 xg for 15 minutes at 4°C. Lysates were incubated with mouse anti-HuR (Santa Cruz Biotechnology) or anti-Rabbit IgG for 2 hours at 4°C while rotating. Anitbody-supernatant complexes were mixed with Dynabeads (Life Technologies) and rotated for 1 hour at 4°C, washed 3 times in PBS, and RNA was eluted using RNAzol as per manufacturer’s protocol. RNA was quantified by qRT-PCR.

**4.4.7 Immunofluorescence**

Cells were grown on coverslips and treated with H$_2$O$_2$ or PBS and fixed with 3% formaldehyde in PBS for 15 minutes at room temperature. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 minutes at room temperature on shaker, rinsed twice with
PBS, and blocked with 1% fetal bovine serum in Tx-100/BSA (0.2% BSA, 0.004% Triton X-100 in PBS) for 15 minutes. Primary antibody was added (mouse anti-HuR (Santa Cruz Biotechnology; 1:500 dilution in Tx-100/BSA buffer) and incubated with cells for 45 minutes, followed by 3 washes with PBS for 5 minutes each. Secondary antibody (alexa fluor 488 goat anti-mouse (Life Technologies; 1:1000 dilution in PBS) was added for 1 hour followed by three 5 minute washes. Nuclei were stained with Hoechst 33342 (Pierce) for 5 minutes and washed with PBS twice. Coverslips were mounted on slides using Fluoromount (Sigma Aldrich). Confocal microscopy was performed using the 60X objective with water (Olympus Fluoview FV1000, Richmond Hill, Ontario Canada).

4.5 Results

4.5.1 HuR controls PDCD4 protein expression by regulating mRNA stability

HuR has recently been implicated in regulating mRNA stability by affecting miRNA interactions with their target mRNAs. Since HuR and PDCD4 have both been shown to regulate IRES-mediated translation of XIAP and Bcl-xL, we were interested in determining if HuR can modulate PDCD4 levels in order to control its functions. We began by transiently transfecting HeLa cells with small interfering (si) RNA to HuR and observed a marked reduction in PDCD4 protein levels (Figure 4.1A). Since HuR is known to bind to AU-rich elements (ARE) in the 3'UTR regions of many mRNAs to regulate their stability, we measured the mRNA levels of PDCD4 after HuR knockdown. Indeed, we observed a ~50% decrease in PDCD4 mRNA (Figure 4.1B) as compared to control, which suggests that a loss of HuR results in the loss of PDCD4 mRNA stability.

4.5.2 HuR directly and specifically binds to the PDCD4 3'UTR
Figure 4.1: HuR directly binds to PDCD4 3'UTR mRNA to regulate its mRNA stability. 
(A) Left panel: Western blot analysis of PDCD4 protein levels after HuR knockdown. HeLa cells were treated with siHuR or siCtrl (nonsilencing control) for 72 h and harvested for western blot analysis. Tubulin was used as a loading control. Right panel: PDCD4 protein levels are quantified relative to Tubulin. (B) HeLa cells were treated with siHuR or siControl for 72 h, harvested, and total RNA was isolated. PDCD4 mRNA levels versus GAPDH mRNA levels were quantified by qRT-PCR. (C) Left panel: HeLa cells were crosslinked with formaldehyde and endogenous HuR was immoprecipitated with mouse anti-HuR antibody and IgG was used as a control. HuR bound RNA was isolated and quantified by qRT-PCR. Right panel: Western blot showing the level of immunoprecipitated HuR. (D) PDCD4 3'UTR RNA was in vitro transcribed and $^{32}$P labeled. UV crosslinking was performed with recombinant GST (control) or GST-HuR, separated by SDS-PAGE, and exposed to X-Ray film.
HuR binds to the 3'UTR of many target mRNAs to regulate stability. Therefore, in order to identify if PDCD4 is a target mRNA that HuR binds to, we immunoprecipitated endogenous HuR from HeLa cells and analyzed bound RNAs by qRT-PCR. We were able to successfully immunoprecipitate HuR (Figure 4.1C right panel) and isolation of bound RNAs followed by qRT-PCR identified that PDCD4 mRNA associates with HuR as compared to IgG control (Figure 4.1C left panel). It is possible that this observed interaction is indirect; therefore, we performed in vitro binding experiments with purified recombinant GST-tagged HuR and in vitro transcribed ³²P-labeled PDCD4 3'UTR to determine if HuR can directly bind to the PDCD4 UTR. Indeed, we observed dose-dependent binding of HuR to the PDCD4 3'UTR (Figure 4.1D). These observations demonstrate that HuR affects PDCD4 stability through direct binding to its 3'UTR.

4.5.3 HuR regulates PDCD4 mRNA stability via miR-21

Recently, HuR has been implicated in regulating some mRNAs through their miR binding sites (26). Since PDCD4 is a known target of miR-21 (13), we decided to investigate the potential of HuR to regulate PDCD4 through miR-21. We confirmed that PDCD4 is a target of miR-21 by transiently transfecting a miR-21 mimic and observing a reduction in PDCD4 protein (Figure 4.2A) and mRNA (Figure 4.2B). To determine if the effect of HuR knockdown on PDCD4 expression is mediated through miR-21, we overexpressed an anti-miR that binds to endogenous miR-21 to inhibit its activity. HuR knockdown followed by a non-targeting antimiR-control showed a decrease in PDCD4 expression, however this reduction was rescued when cells were treated with the antimiR specific to miR-21 (Figure 4.2C). This data suggests that HuR prevents miR-21 from binding to the PDCD4 mRNA resulting in protection of the mRNA from degradation. One possibility
**Figure 4.2: HuR regulates PDCD4 stability via miR-21.** (A) HeLa cells were transiently transfected with a miR-21 mimic for 24 h and cells were harvested for western blot analysis. **(B)** HeLa cells were transiently transfected with a miR-21 mimic for 24 h and RNA was harvested. qRT-PCR analysis showing decrease of PDCD4 mRNA relative to GAPDH after miR-21 overexpression. **(C)** Left panel: AntimiR-21 or antimiR-Ctrl (control) was transiently transfected into HeLa cells for 24 h followed by siHuR transfection for 48 h. Cells were harvested and protein levels were analyzed by Western blot. Right panel: Quantification of PDCD4 protein levels relative to Tubulin. **(D)** Top panel: Schematic representation of a fragment of the PDCD4 3'UTR (nucleotides 1-610). S1: nucleotides 1-199, S2: nucleotides 200-401, S3: nucleotides 402-610. The grey box indicates the miR-21 binding site. Bottom panel: UV-crosslinking with GST or GST-HuR and the PDCD4 3'UTR fragments that were *in vitro* transcribed and $^{32}$P-labeled.
is that HuR can bind directly to the miR site on the 3'UTR thus blocking the miR from binding (26, 29). To determine if HuR binds the miR-21 site on PDCD4 we generated three ~200 nt fragments from the first 610 nucleotides of the PDCD4 3'UTR. Fragment S2 contains the miR-21 site highlighted in grey (Figure 4.2D top panel). We performed UV cross-linking experiments with purified GST-tagged HuR and $^{32}$P-labeled in vitro transcribed RNA probes. Interestingly, HuR did not bind to the miR-21 containing fragment S2 (Figure 4.2D bottom panel). Instead, HuR bound specifically to the first 200 nt S1 fragment. Moreover, HuR did not bind to the S3 probe, which further supports the specificity of the HuR-PDCD4 mRNA interaction. This data suggests that binding of HuR to the PDCD4 3'UTR prevents miR-21 binding, thus leading to increased stability of the transcript.

**4.5.4 Loss of PDCD4 expression after H$_2$O$_2$ treatment is mediated through miR-21**

HuR has an important role in cellular stress since elevated expression levels of HuR cause mis-regulation of many processes including cell survival, proliferation, and tumour invasion and metastasis (30). Many cellular stresses, such as oxidative stress, result in an accumulation of cytoplasmic HuR that is usually mediated by phosphorylation (26). This cytoplasmic accumulation is necessary for HuR’s ability to stabilize mRNAs and regulate translation. Therefore, we were interested in determining the effect of increased cytoplasmic HuR levels on PDCD4 expression. We were expecting that an increase in cytoplasmic HuR would provide a protective effect against miR-21 leading to increased PDCD4 expression. Surprisingly, treatment of cells with H$_2$O$_2$ correlated with a loss in PDCD4 expression both at the protein (Figure 4.3B) and mRNA (Figure 4.3C) levels, even
Figure 4.3: H$_2$O$_2$ causes cytoplasmic accumulation of HuR and correlates with a loss in PDCD4 expression. (A) HuR localization by immunofluorescence of HeLa cells treated with PBS (0 mM H$_2$O$_2$) or 0.5 mM H$_2$O$_2$ for 1 h. Nuclei are visualized by Hoechst staining. (B) Left panel: HeLa cells were treated with 0.5 mM H$_2$O$_2$ for the indicated times and harvested for Western blot analysis indicating a decrease in PDCD4 protein at 3 h as compared to Tubulin control. Right panel: PDCD4 protein levels are quantified relative to Tubulin. (B) Cells were treated with 0.5 mM H$_2$O$_2$ for the indicated time points and total RNA was isolated. qRT-PCR analysis indicates a loss of PDCD4 mRNA as compared to GAPDH control.
though HuR accumulated in the cytoplasm as monitored by immunofluorescence (Figure 4.3A). This data suggests that it is not the increase in cytoplasmic HuR that is important for PDCD4 regulation, but rather, the modifications of cytoplasmic HuR after H₂O₂ treatment affect its target binding ability. Nevertheless, we tested the requirement of miR-21 for PDCD4 loss after H₂O₂ treatment. We pre-treated the cells with antimiR-21 and then exposed the cells to H₂O₂. Inhibition of miR-21 rescued PDCD4 protein (Figure 4.4A) and mRNA (Figure 4.4B) expression after H₂O₂ treatment, thus suggesting that miR-21 binding is responsible for the reduced PDCD4 expression in response to H₂O₂ stress. These observations point toward a model where under normal conditions, cytoplasmic HuR binds to the PDCD4 3'UTR and protects it from miR-21 mediated degradation. However, under oxidative stress, this protective role of HuR is lost and miR-21 can bind to the PDCD4 3'UTR leading to degradation of the mRNA and loss of protein expression.

4.6 Discussion

PDCD4 is a tumour suppressor whose loss is correlated with the development and progression of many cancers including lung, colon, liver, breast, and brain (2-6). Previously, we identified PDCD4 as a regulator of IRES-mediated translation of XIAP and Bcl-xL (10). This regulation is particularly important in cancer development because loss of PDCD4 correlates with an increase in these anti-apoptotic proteins, thus contributing to the cell’s ability to evade apoptosis following chemotherapeutics. Importantly, we previously demonstrated that loss of PDCD4 in GBM tumours correlates with an increase in Bcl-xL and poor patient outcome. Furthermore, we showed that re-expression of PDCD4 down-regulates Bcl-xL expression and sensitizes cells to the chemotherapeutic doxorubicin (6). These important roles of PDCD4 highlight a need to elucidate the mechanism of PDCD4 protein
Figure 4.4: miR-21 mediates the decrease in PDCD4 in response to H$_2$O$_2$ treatment. (A) Left panel: HeLa cells were treated with antimiR-21 or a non-targeting antimiR-Ctrl (control) for 24 h followed by treatment with 0.5 mM H$_2$O$_2$ for 4 h. Cells were harvested and analysed by Western blot. Tubulin was used as a loading control. Right panel: Quantification of PDCD4 levels relative to Tubulin. (B) Cells were treated as in (A) and total RNA was isolated. PDCD4 mRNA relative to GAPDH mRNA was quantified by qRT-PCR.
A

antimiR-Ctrl
antimiR-Ctrl + H2O2
antimiR-21
antimiR-21 + H2O2

PDCD4
HuR
Tubulin

B

*P=0.0436

antimiR-Ctrl + 0mM H2O2
antimiR-21 + 0mM H2O2
antimiR-Ctrl + 0.5mM H2O2
antimiR-21 + 0.5mM H2O2

PDCD4/GAPDH
regulation. It is known that miR-21 regulates PDCD4 and targets it for degradation leading to a loss of protein expression. Moreover, an increase in miR-21 expression has been observed in many cancers, which contributes to the loss of PDCD4 that is frequently observed (13-15). Since HuR and PDCD4 regulate the same IRES-containing mRNAs, and recently HuR has been implicated in regulating miRNA-mediated degradation, we therefore wanted to determine if HuR is important for regulating PDCD4 expression. Additionally, we were interested if HuR regulation of PDCD4 was important in the cell’s response to stress. Indeed, we observed that HuR regulates PDCD4 protein expression via miR-21. Loss of HuR through knockdown renders PDCD4 mRNA readily available to miR-21 targeting, leading to its degradation and loss of protein levels. We demonstrated that HuR binds to the PDCD4 3'UTR but that it does not bind to the miR-21 binding site. Therefore, it is possible that HuR binding causes a conformational change in the mRNA, which prevents binding of miR-21. Alternatively, initial binding of HuR may cause further oligomerization of additional HuR proteins that would sterically inhibit miR-21 binding, which could be analysed by RNA electrophoretic mobility shift assays (REMSAs; (31)). It is also possible that HuR binding causes recruitment of the mRNA to a different part of the cell making it inaccessible to miR-21. This could be tested by RNA fluorescence in situ hybridization (FISH) experiments where PDCD4 mRNA localization is monitored after HuR knockdown or H2O2 treatment.

Frequently, HuR translocates from the nucleus and accumulates in the cytoplasm after cellular stress where it typically binds to and stabilizes target mRNAs. Therefore, we were expecting to see a rescue in PDCD4 expression after exposing cells to H2O2 stress. Unexpectedly, although we observed a cytoplasmic accumulation of HuR following H2O2 treatment, we did not observe a rescue in PDCD4 expression. Instead, H2O2 treatment
correlates with a loss in PDCD4 protein and mRNA levels. This observation is similar to the case described by Abdelmohsen and colleagues (30) where they demonstrated that H₂O₂ causes phosphorylation of HuR by Chk2 kinase thus leading to dissociation of HuR from SIRT1 mRNA and a consequent loss in SIRT1 protein. Additionally, Yoon and colleagues (32) demonstrated that tyrosine phosphorylation of HuR by JAK3 following arsenite treatment causes a dissociation of HuR from SIRT1 and VHL target mRNAs leading to their degradation. These findings point to a role for post-translational modifications of HuR in determining HuR’s ability to bind target mRNAs. This leads us to speculate that H₂O₂ treatment of cells not only causes a cytoplasmic accumulation of HuR, but also modifications that alter its ability to bind PDCD4 mRNA. It will be interesting to determine which kinase(s) is important for mediating this process and how this is regulated in cancer settings. Furthermore, increased expression of HuR has been linked to promoting cancer cell growth. For example, it was demonstrated that HuR expression is increased in primary glioblastoma cells (33, 34) and that a cytoplasmic accumulation of HuR occurs. However, the phosphorylation state of HuR in these cases has not been determined so it is possible that all or a subset of the HuR in the cytoplasm is modified, which may reduce its protective role in PDCD4 regulation. On the other hand, miR-21 levels are increased in glioblastoma cells (15, 35) and therefore it is possible that the relative ratios and accessibility of miR-21 versus HuR binding to PDCD4 will determine the fate of PDCD4 expression.

Finally, we have previously identified both HuR (23, 24) and PDCD4 (10) as important ITAFs of the XIAP and Bcl-xL IRES elements. Since both of these anti-apoptotic proteins are important in the cancer cell’s ability to evade apoptosis, it will be interesting to study how these ITAFs function together to modulate their expression.
Our findings demonstrate a novel role for HuR in regulating the tumour suppressor PDCD4 via miR-21. These observations provide insight into understanding the regulation of ITAFs, as well as how these ITAFs function together to mediate translation from IRES elements.

4.7 References


Chapter 5

General Discussion
5.1 Alternative mechanism of translation initiation

In response to cellular stress, modifications of the canonical translation initiation factors results in general inhibition of cap-dependent translation. This is thought to conserve cellular energy and prevent the synthesis of unwanted proteins (as discussed in General Introduction). In order for the cell to respond accurately to the insult, some cellular mRNAs must be translated. One alternative mechanism to cap-dependent translation is internal ribosome entry site (IRES)-mediated translation.

IRES-mediated translation was initially discovered in poliovirus and since then, IRES elements have been identified in many naturally uncapped viral RNAs. These viral IRESes can be grouped into four categories based on their structure, their need for protein factors, and the location of the start codon relative to the IRES. Structural similarity in viral IRESes allows for prediction and identification of new IRES elements (1, 2). On the other hand, cellular IRES elements vary widely and do not share sequence or structural homology, thus identifying cellular IRESes and elucidating their mechanism of translation has proven challenging. Nevertheless, many cellular IRESes have now been discovered through intensive experimental approaches and the growing list of IRESes can be found on the IRESite database (3). Frequently, mRNAs that harbour IRES elements encode growth factors, oncogenes, and genes involved in regulating apoptosis. Additionally, they are critical for the cell’s ability to respond to stress and to disease development thus, understanding the mechanism of IRES translation may provide potential targets for therapeutic strategies.

5.2 Key anti-apoptotic proteins are translated via IRES elements
Most cellular 5'UTR regions are approximately 300 nucleotides in length (2, 4). XIAP, on the other hand, has an extremely long 5'UTR of 1.7 kb length and is highly structured with many out-of-frame initiation codons and short open reading frames (5). These features provide difficulty for efficient ribosomal scanning involved in cap-dependent translation initiation. This led to the investigation of an alternative mode of translation initiation of XIAP and to the discovery of an IRES element located in a 162 nucleotide region upstream of the AUG start site (6). Furthermore, Yoon and colleagues (7) identified another important anti-apoptotic protein, Bcl-xL, as harbouring an IRES element in its mRNA. They mapped the IRES to 446 nucleotides upstream of the initiation codon. Although there are many other IRES-harbouring mRNAs, the co-regulation of XIAP and Bcl-xL in response to FGF-2 signaling (as discussed below) prompted us to focus on these two IRESes for our studies.

5.3 IRES containing transcripts are co-regulated

Not only is the discovery of cellular IRES elements important, but also the realization that these mRNAs may be co-regulated by similar environmental or physiological triggers. Yoon and colleagues (7) first suggested the possibility of IRES elements being co-regulated while investigating the mutation in the DKC1 gene in X-linked dyskeratosis congenital (X-DC) patients. This mutation results in the mis-regulation of ribosomal RNA (rRNA) modifications, which correlates with an impairment in IRES translation of XIAP, Bcl-xL, and the tumor suppressor p27kip1. Co-regulation of IRES containing transcripts was also demonstrated by Faye and colleagues (8), where the RNA-binding protein, NF45 was shown to activate IRES translation of XIAP and cellular inhibitor of apoptosis protein 1 (cIAP1). Furthermore, they showed that the requirement of NF45 by an IRES element could be
predicted based on the AU content of the IRES containing 5'UTR, where high AU content results in NF45 dependence. Moreover, Pardo and colleagues (9, 10) demonstrated that treatment of small cell lung cancer (SCLC) cells with FGF-2 results in an increase in Bcl-xL and XIAP expression and we further show that this increase is due to activation of IRES-mediated translation of these transcripts (11). This idea of co-regulation is important because it helps to identify other potential IRES containing transcripts in response to a particular condition.

5.4 ITAFs regulate IRES-mediated translation

Co-regulation of IRES containing mRNAs is likely mediated by specific binding proteins termed IRES trans-acting factors (ITAFs), that either enhance or inhibit translation. Recent evidence suggests that some ITAFs can regulate cohorts of mRNAs. For example, cytoplasmic accumulation of the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) in response to osmotic shock suppresses translation of XIAP (12) and Bcl-xL (13) mRNAs. This is in addition to the many other IRESes hnRNP A1 regulates including egr2 (14), cyclin D1, c-myc (15, 16), SREBP-1a (16), as well as some viral IRESes including human rhinovirus (17), enterovirus 71 and sindbis virus (18, 19) under a variety of cellular conditions. Moreover, we identified HuR as a bona fide ITAF of both the XIAP (20) and Bcl-xL (21) IRESes where it stimulates XIAP IRES activity but inhibits Bcl-xL IRES translation.

Given that XIAP and Bcl-xL are co-regulated in a variety of scenarios, this prompted us to look for other potential ITAFs that may regulate both the XIAP and Bcl-xL IRES elements. As mentioned above, XIAP and Bcl-xL expression is up-regulated in response to FGF-2 activated S6K2, which suggests that an ITAF that is regulated by S6K2 is responsible
for this increase. Therefore, we used a tandem affinity purification (TAP) approach and identified PDCD4 as a binding partner of S6K2 after treatment with FGF-2. We demonstrate that under normal conditions, PDCD4 acts to inhibit translation from these IRES elements by preventing formation of the 48S translation initiation complex. Furthermore, we show that in response to treatment with FGF-2, S6K2 phosphorylates PDCD4 leading to its degradation and the subsequent de-repression of XIAP and Bcl-xL translation. It is likely that other proteins are involved in this repression, thus it will be interesting to determine which, if any, proteins PDCD4 interacts with and what role they play in repression. Initial candidates that can be assessed are the eIF4A proteins (I, II, and III), since PDCD4 is a known eIF4A binding protein. Furthermore, other helicases have been implicated in translation such as DHX29 (22), which was shown to be required for translation of mRNAs with structured 5'UTRs including XIAP. Thus, it would be interesting to determine if this or other helicases are required for IRES-mediated translation of XIAP and Bcl-xL and if PDCD4 acts to inhibit their activity. Additionally, other potential protein partners that were identified in the TAP-tag experiment as S6K2 interactors could be tested for their role in PDCD4 repression. In particular, we identified Poly (rC)-binding protein 1 (PCBP1), which has previously been shown to regulate the IRES element of BAG-1 (23), as interacting with S6K2 after FGF-2. Interestingly, PCBP1 can also enhance the activity of XIAP upon infection with Kaposi’s sarcoma-associated herpesvirus (KSHV; (24)) and similar to PDCD4, is also a miR-21 target (25). Thus, it would be interesting to determine if PDCD4 blocks the ability of PCBP1 to enhance XIAP activity.

5.5 How does PDCD4 act to repress IRES translation?
To fully understand the function and importance of ITAFs, we need to dissect how these proteins act to enhance or repress IRES-mediated translation. One possibility is that binding to the IRES element causes a remodeling of the IRES structure, which promotes or inhibits recruitment and binding of the necessary translational apparatus. In the case of PDCD4, it would be interesting to compare the structure of the XIAP and/or Bcl-xL IRES by chemical and enzymatic probing in the presence or absence of PDCD4. These experiments would identify if structural changes could explain the inability of the ribosome to be recruited for functional translation. Furthermore, it may be possible that binding of PDCD4 to the mRNA causes sequestration of the mRNA into processing bodies (P-bodies) or stress granules (SGs) thus rendering the mRNA inaccessible for translation. RNA FISH experiments probing for XIAP or Bcl-xL mRNA after PDCD4 overexpression could identify if these mRNAs are in cytoplasmic granules. Ultimately, it would be ideal to elucidate the minimal proteins required for the function of IRES elements to use in a reconstituted in vitro translation system combined with toe-printing assays. This would provide us with the biochemical tools necessary to study how individual ITAFs affect regulation.

5.6 PDCD4 may regulate a cohort of IRES-containing transcripts

As described above, some IRESes seem to be co-regulated, and this regulation depends on the function of ITAFs. This points to the idea that ITAFs may regulate multiple mRNAs as part of an RNA-regulon in response to cellular signals. Considering the tumour suppressive functions of PDCD4, it would not be surprising to find a PDCD4 regulon consisting of mRNAs involved in cell death and survival. To identify other potential PDCD4 mRNA targets, Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP; (26)) could be used to identify the mRNAs that associate
with PDCD4 as well as the PDCD4 binding site(s). Furthermore, ribosome profiling of PDCD4 depleted cells would identify target mRNAs whose translation is normally repressed by PDCD4. These results would provide insight into novel PDCD4 regulated genes as well as identify other potential therapeutic targets. Such possible clinical implications are demonstrated in our studies of GBM tumours where we identify a strong correlation between loss of PDCD4 and an increase in expression of Bcl-xL. Furthermore, we show that this correlates with poor survival. Importantly, re-expression of PDCD4 or inhibition of Bcl-xL directly sensitizes these cells to chemotherapeutics. It would be expected that this correlation would be observed with other mRNAs in the PDCD4 operon and uncovering these targets could provide other potential therapeutic options. Initially, we could test the correlation of PDCD4 loss and XIAP expression in GBM. It would be expected that elevated levels of XIAP would be observed in GBM and treatment with a Smac mimetic (an inhibitor of XIAP) would cause sensitization of these cells to chemotherapeutics. In fact, Wagner et al (27) demonstrate that GBM cells are sensitive to a combination of Smac mimetic treatment and Temozolomide, but claim that this effect is through inhibition of cIAP1 protein. Thus, it would be interesting to monitor the effect on XIAP under these conditions.

5.7 The interplay of ITAFs determines IRES function

Many studies of ITAF-IRES interactions have indicated that overexpression or knockdown of ITAFs can positively or negatively impact IRES translation, making it clear that the intracellular concentration of ITAFs and their combined cooperation is a crucial determinant of IRES function. For example, loss of a repressive ITAF by degradation or sequestration, or translocation to the cytoplasm of an activating ITAF enhances IRES translation. On the other hand, stabilization or cytoplasmic accumulation of a repressive
ITAF could inhibit IRES activity. Many of these proteins respond to cellular stress, thus it is the combined activity of all ITAFs specific for an IRES element that are important for the resulting increase or decrease of IRES activity. The regulation of PDCD4 by HuR that we observe further supports the idea that various pathways involved in IRES translation are linked and co-regulation of the proteins involved is important for IRES activity. Therefore, it is not only important to identify all the ITAFs of a particular IRES, but it is crucial to determine how these ITAFs work together in order to get a big picture understanding of IRES translation.

5.8 PDCD4 expression is regulated at the level of stability by the RNA binding protein HuR

In chapter 4 we describe HuR’s ability to regulate microRNA (miR) -21-mediated loss of PDCD4. Most examples of HuR regulating mRNAs through miRs demonstrate that HuR has a protective role in response to cellular stresses (28). Generally, stress conditions induce HuR localization into the cytoplasm where it can bind its target mRNA and protect it from miR-induced silencing. In our case, however, treatment with H₂O₂ results in a loss of PDCD4 that is dependent on miR-21. We initially monitored HuR localization after H₂O₂ and indeed, observed a cytoplasmic accumulation of HuR. Surprisingly, this correlated with a loss of PDCD4 expression. This data suggests that it is not the increase in cytoplasmic HuR that plays a role in PDCD4 regulation, but rather that a change in post-translational modification of HuR after H₂O₂ treatment is important. This is similar to the observation by Yoon and colleagues (29) where phosphorylation of HuR by JAK3 causes a dissociation of HuR from SIRT1 and VHL target mRNAs. Future work should focus on identifying the kinase(s) responsible for modifying HuR in response to H₂O₂ to prevent PDCD4 mRNA
binding. A kinome knockdown screen with H$_2$O$_2$ can narrow down the potential kinases involved in HuR relocalization, which could then be tested using *in vitro* kinase assays for their ability to phosphorylate HuR. On the other hand, since HuR is known to function in many processes including the export of mRNAs, it would be important to rule out the possibility that HuR alters PDCD4 mRNA export thus affecting the amount of mRNA available for miR-21. To do this we could perform RNA-FISH analysis to follow PDCD4 mRNA export after HuR knockdown and H$_2$O$_2$.

The expression of HuR is increased in GBM cells and this expression is linked to the aggressive nature of these tumors (30-32). It would be expected from our data that HuR might play a protective role in stabilizing PDCD4; however, PDCD4 expression is markedly reduced in GBM. Moreover, miR-21 levels are increased in GBM so it is possible that these levels overcome the protective functions of HuR, or that HuR is post-translationally modified and is not able to bind PDCD4 mRNA. This would not be surprising since many signaling cascades are typically overactive in tumor cells (33). It would be interesting to determine if promoting the binding of HuR to PDCD4 mRNA might increase its protein levels and provide a subsequent increase in cell death and sensitivity to chemotherapeutics.

Subcellular localization of ITAFs is also particularly important in disease states because mis-localization of these proteins following chemotherapeutic treatment can contribute to the chemoresistance of cancer cells. For example, Dobbyn and colleagues (34) demonstrated that treatment of cells with vincristine causes relocalization of PTB and PCBP1, which are important ITAFs of the anti-apoptotic BAG1 protein. This leads to enhanced BAG1 translation, which unexpectedly, causes chemoresistance to vincristine. This effect should be considered in future studies of chemotoxic substances since many ITAFs may be regulated in a similar fashion, which could contribute to the cell’s acquired
chemoresistance. Furthermore, many key signaling pathways are altered in cancer cells, which could affect translational control by altering post-translational modifications of ITAFs and/or subcellular localization resulting in mis-regulation of these important proteins.

5.9 Conclusions

In summary, this dissertation identifies the tumour suppressor PDCD4 as a novel ITAF of the XIAP and Bcl-xL IRES elements. It demonstrates that PDCD4 acts to inhibit translation from these IRES elements by preventing the formation of the 48S translation initiation complex. Furthermore, it shows that FGF-2 causes activation of S6K2, which phosphorylates PDCD4 leading to its degradation and the subsequent de-repression of XIAP and Bcl-xL translation. Importantly, the clinical significance of this regulation is demonstrated in GBM tumours where the loss of PDCD4 expression correlates with an increase in Bcl-xL protein and poor patient outcome. By over-expressing PDCD4, we down-regulate Bcl-xL and decrease cell viability. Furthermore, direct inhibition of Bcl-xL by ABT-737 sensitizes GBM cells to the effects of doxorubicin. Finally, it demonstrates that the RNA-binding protein HuR regulates miR-21 induced silencing of PDCD4. It shows that HuR can bind directly to the PDCD4 3'UTR and prevent miR-21 binding and that a loss of PDCD4 expression following H2O2 treatment is mediated via miR-21. These findings highlight the importance of ITAFs in cancer progression and suggest the ability of PDCD4 to regulate an operon of IRES containing mRNAs that may lead to uncovering novel therapeutic targets.

5.10 References


Appendix A:
References for chapter 1 (sections 1.2-1.5)

References


Appendix B:
RNA-binding protein HuR mediates cytoprotection through stimulation of XIAP translation.

Preamble

“RNA-binding protein HuR mediates cytoprotection through stimulation of XIAP translation” is published in the journal Oncogene (Volume 30, March 2011). This article describes the RNA-binding protein HuR as a novel ITAF of the XIAP IRES element. It demonstrates HuR’s ability to bind the XIAP IRES and stimulate translation by promoting incorporation into polysomes. Importantly, it shows that protection from the apoptosis-inducing agent etoposide, is dependent on increased XIAP expression which is mediated by HuR.

Author List

Danielle Durie, Stephen M. Lewis, Urszula Liwak, Magdalena Kisilewicz, Myriam Gorospe, and Martin Holcik

Author Contribution

MH wrote the paper; MH, SL, MG, DD, and UL conceived the experiments; DD, SL, UL, MK conducted the experiments; UL performed experiments presented in Figures 3 and 4.
Abstract

Expression of the intrinsic cellular caspase inhibitor XIAP is regulated primarily at the level of protein synthesis. The 5’ untranslated region harbours an Internal Ribosome Entry Site (IRES) motif that supports cap-independent translation of XIAP mRNA during conditions of cellular stress. We show here that the RNA-binding protein HuR, which is known to orchestrate an anti-apoptotic cellular program, stimulates translation of XIAP mRNA through XIAP IRES. We further show that HuR binds to the XIAP IRES in vitro and in vivo, and stimulates recruitment of the XIAP mRNA into the polysomes. Importantly, protection from the apoptosis-inducing agent etoposide by overexpression of HuR requires the presence of XIAP, suggesting that HuR-mediated cytoprotection is partially executed through enhanced XIAP translation. Our data suggests that XIAP belongs to the HuR-regulated RNA operon of anti-apoptotic genes, which along with Bcl-2, Mcl-1 and ProTα, contributes to the regulation of cell survival.

Introduction

The X chromosome-linked inhibitor of apoptosis (XIAP) is the prototype member of the inhibitor of apoptosis family of proteins. XIAP fulfills several distinct roles within the cell, including the modulation of receptor-mediated signal transduction, protein ubiquitination, and primarily direct caspase inhibition and thus regulation of cell survival (reviewed in Dubrez-Daloz et al., 2008; Liston et al., 2003). Although no mutations linking XIAP to oncogenic transformation have been described, elevated levels of XIAP were reported in a number of cancers and have been linked to enhanced chemo- and radiation resistance (Gu et al., 2009; Holcik et al., 2000b; Tamm et al., 2000; Tamm et al., 2004a; Tamm et al., 2004b; Yoon et al., 2006). The therapeutic potential of targeting XIAP
expression has recently been demonstrated in several clinical trials (Dean et al., 2009; Dean et al., 2007; Schimmer et al., 2005; Schimmer et al., 2009).

Cellular levels of XIAP are regulated by several distinct mechanisms, but the predominant regulation appears to be at the level of translation initiation (Holcik, 2003). The 5’ untranslated region (5’ UTR) of XIAP mRNA harbours an Internal Ribosome Entry Site (IRES) motif that drives efficient XIAP mRNA translation during conditions of cellular stress, when global, cap-dependent translation is compromised (Gu et al., 2009; Holcik et al., 1999; Yoon et al., 2006). IRES-driven translation of cellular mRNAs has recently emerged as an important regulator of selective translation, in particular under conditions of hypoxia, endoplasmic stress, or serum starvation (Holcik & Sonenberg, 2005; Holcik et al., 2000a). These conditions are often present in tumours, and the IRES-mediated translation of specific mRNAs is required by many cancer cells for their survival (Braunstein et al., 2007; Silvera et al., 2009). While the switch from cap-dependent to IRES-dependent translation has been identified (Braunstein et al., 2007), the precise mechanism(s), and the cohort of cellular proteins that contribute to the regulation of IRES-dependent translation remain unclear.

The XIAP IRES is 162 nt long and forms a distinct RNA structure (Baird et al., 2007). We have previously characterized the sequence and structural attributes of the XIAP IRES (Baird et al., 2007; Holcik et al., 1999) and have begun to elucidate the mechanism that regulates its activity. We and others have identified IRES trans-acting factors (ITAFs), cellular proteins that specifically bind to and regulate XIAP IRES. Interestingly, while some of these factors stimulate XIAP IRES function (La autoantigen (Holcik & Korneluk, 2000); hnRNP C1/C2 (Holcik et al., 2003); mdm2 (Gu et al., 2009)), the others repress it (hnRNP A1 (Lewis et al., 2007); PTB (Baird et al., 2007)). However, the nature of the XIAP IRES ribonucleoprotein (RNP) complex and the identities of all XIAP ITAFs remain unknown.
In this work we have used RNA-affinity chromatography with XIAP IRES RNA to identify HuR as a novel XIAP ITAF. We find that HuR binds to XIAP IRES \textit{in vitro} and is part of a XIAP IRES RNP complex \textit{in vivo}. Furthermore, HuR stimulates translation of the endogenous XIAP mRNA, which contributes to the preservation of cell viability in an etoposide-mediated model of cell death.

Results

\textit{HuR interacts with the XIAP IRES.} We have shown that the La autoantigen, hnRNP C1/C2, hnRNP A1, and PTB are XIAP ITAFs (Baird et al., 2007; Holcik et al., 2003; Holcik & Korneluk, 2000; Lewis et al., 2007). We previously used an RNA affinity chromatography approach to isolate XIAP ITAFs and identified an approximately 37 kDa protein species as hnRNP A1 (ref (Lewis et al., 2007) and Figures 1A and B therein). We now report that this 37 kDa protein species is in fact a complex mixture of proteins, and in addition to hnRNP A1 we have also detected the presence of the RNA-binding protein HuR by mass-peptide fingerprinting (Figure 1A). To confirm that HuR associates with the XIAP IRES we performed RNA-affinity chromatography and subjected the isolated protein sample to Western-blot analysis using HuR antibodies. We included cIAP1 IRES RNA (Graber et al., 2010) as an affinity matrix to test the specificity of the HuR interaction with the XIAP IRES. HuR was detected in the protein eluate from XIAP IRES RNA affinity matrix, but was not detected in the protein eluate from avidin-agarose beads alone (Figure 1B, compare lane 3 to lane 2). Moreover, HuR does not associate with cIAP1 IRES RNA (Figure 1B, lane 4),
Figure 1. HuR interacts with the XIAP IRES. (A) Underlined peptide sequences were identified by mass spectrometry analysis of the 37 kDa protein species isolated by RNA-affinity chromatography using XIAP IRES RNA as an affinity matrix (Lewis et al., 2007). (B) HuR associates with XIAP IRES RNA in vitro. XIAP IRES and cIAP1 IRES RNAs were used in RNA-affinity chromatography as described in Material and Methods; isolated proteins were separated by SDS-PAGE, transferred to PDVF membrane, and probed with anti-HuR antibody. (C) HuR associates with XIAP mRNA in vivo. RNA-protein complexes were cross-linked with formaldehyde, isolated from cells, and immunoprecipitated using antibodies against HuR, TIA-1, or IgG. After immunoprecipitation, RNA-protein cross-links were reversed, and the RNA was isolated and used in an RT-PCR reaction with XIAP- and β-Actin-specific primers. (D) Recombinant HuR binds directly to XIAP IRES. GST or increasing amounts of GST-HuR were incubated with 32P-labelled XIAP IRES RNA probe, UV-cross-linked, and then separated by SDS-PAGE and visualized by autoradiography. (E) Mapping the HuR binding site. GST-HuR was incubated with 32P-labelled XIAP IRES RNA probe alone (NC) or was first pre-incubated with 100 ng of indicated unlabeled competitor oligonucleotides, UV-crosslinked, and then separated by SDS-PAGE and visualized by autoradiography. Bottom, black lines in the secondary structure model of XIAP IRES (Baird et al., 2007) indicate the successful competitor oligonucleotides; shaded circle denotes the proposed HuR binding site in domain Ib (see Results for details).
Figure 1. HuR interacts with XIAP IRES. (a) Underlined peptide sequences were identified by mass spectrometry analysis of the 37 kDa protein species, isolated by RNA-affinity chromatography using XIAP IRES RNA as an affinity matrix (Lewis et al., 2007). (b) HuR associates with XIAP IRES RNA in vitro. XIAP IRES and cIAP1 IRES RNAs were used in RNA-affinity chromatography as described in Material and methods section; isolated proteins were separated by SDS–PAGE, transferred to PDVF membrane and probed with anti-HuR antibody. (c) HuR associates with XIAP mRNA in vivo. RNA–protein complexes were crosslinked with formaldehyde, isolated from cells and immunoprecipitated using antibodies against HuR, TIA-1 or IgG. After immunoprecipitation, RNA-protein crosslinks were reversed, and the RNA was isolated and used in an RT–PCR reaction with XIAP- and actin-specific primers. (d) Recombinant HuR binds directly to XIAP IRES. GST or increasing amounts of GST-HuR were incubated with 32P-labelled XIAP IRES RNA probe, UV-crosslinked and then separated by SDS–PAGE and visualized by autoradiography. (e) Mapping the HuR-binding site. GST-HuR was incubated with 32P-labelled XIAP IRES RNA probe alone (NC) or was first preincubated with 100 ng of indicated unlabelled competitor oligonucleotides, UV-crosslinked and then separated by SDS–PAGE and visualized by autoradiography. Bottom: Black lines in the secondary structure model of Durie et al.
demonstrating that HuR does not interact non-specifically with all IRES (or RNA) sequences. Therefore, HuR specifically associates with XIAP IRES RNA in vitro.

We next assessed whether the endogenous HuR associates with endogenous XIAP mRNA in cells. We immunoprecipitated RNA-protein complexes using HuR antibodies, IgG (negative control), or TIA-1/TIAR (a known RNA-binding protein) from protein extracts in conditions that preserved RNA-protein complexes, as previously described (Lewis et al., 2007). RNA was isolated from these immunoprecipitates, and cDNA was produced by reverse transcription, followed by PCR-amplification with XIAP and β-actin coding sequence-specific primers. We were unable to amplify any significant amounts of XIAP from an IgG or TIA-1/TIAR immunoprecipitation (Figure 1C, lanes 3 and 4). However, immunoprecipitation with HuR antibodies co-precipitated XIAP mRNA (Figure 1C, lane 2), confirming that endogenous HuR associates with endogenous XIAP mRNA in cells in vivo. We were unable to amplify the high-abundance β-actin transcript from our immunoprecipitates with either antibody, indicating that our co-immunoprecipitation of XIAP mRNA is specific. Although it has been reported that HuR may associate with β-actin transcript (Dormoy-Raclet et al., 2007) we were unable to confirm this association.

*HuR binds directly to XIAP IRES RNA.* Our observations that HuR associates with XIAP IRES RNA both in vitro and in vivo suggest that HuR can bind directly to the XIAP IRES sequence. To determine if HuR binds directly to XIAP IRES RNA we performed a UV-crosslinking experiment using a radiolabeled XIAP IRES RNA probe and purified recombinant GST-HuR. Increasing amounts of recombinant GST-HuR were incubated with
We find that XIAP IRES RNA is crosslinked to GST-HuR in vitro in a dose-dependent manner (Figure 1D) indicating that HuR does indeed bind directly to XIAP IRES RNA. We further wished to determine the binding site of HuR within the XIAP IRES sequence. Therefore, we mapped the binding site of HuR using competitor oligonucleotides as described previously (Holcik & Korneluk, 2000). Two competitors, 68-88 and 115-135, which consist of sequences that are located near the central core of domain I of XIAP IRES compete for binding to GST-HuR (Figure 1E). Although HuR was initially shown to bind the AUUUA sequence (Myer et al., 1997), the HuR consensus binding site was later refined as NNUUNNUUU which must be presented as a single stranded RNA (Meisner et al., 2004). Interestingly, while both single stranded competitor oligonucleotides contain such a sequence and thus compete effectively for HuR binding, this sequence is single-stranded only in domain Ib of XIAP IRES (Figure 1E, competitor 115-135), indicating that HuR binds this region of the IRES sequence. However, our data also raise the possibility that a second potential HuR binding site exists within the XIAP IRES (the double stranded stem of domains Ic and Ia; competitor 68-88), which could be used if the XIAP IRES undergoes structural remodeling.

HuR contains three RNA recognition motifs (RRM), of which RRM1 and RRM2 are involved in RNA binding while RRM3 is needed for cooperative assembly of HuR oligomers on RNA but contributes minimally to RNA binding (Fialcowitz-White et al., 2007). We tested, using UV-crosslinking experiments with deletion mutants of HuR (Mazroui et al., 2008), whether this is also the case with binding of HuR to XIAP IRES. Purified AP-HuR-GST (full length HuR), AP-HuR(CP1)-GST (containing RRM1 and RRM2), or AP-HuR(CP2)-GST (containing RRM3) were incubated with $[^{32}\text{P}]$-labeled XIAP IRES RNA,
followed by UV-crosslinking and separation by SDS-PAGE. We find that RRM1 and RRM2 are required for HuR binding to XIAP IRES (Supplementary Figure 1A) indicating that the mechanism of HuR binding to XIAP IRES RNA is similar to other HuR target RNAs. Additionally, we tested the ability of these deletion constructs to modulate XIAP IRES activity (see below).

Cellular levels of HuR modulate XIAP IRES activity. We have shown that HuR interacts with the XIAP IRES both in vitro and in vivo, suggesting that HuR may control XIAP IRES function. We therefore examined whether modulating levels of HuR, either by overexpression or knockdown by siRNA, has any effect on the activity of the XIAP IRES. To assess XIAP IRES activity, we used a previously characterized bicistronic reporter plasmid containing the minimal XIAP IRES element (pβgal(-162)/CAT; (Holcik et al., 1999)). In this construct, expression of the first cistron (βgal) is cap-dependent, whereas expression of the second cistron (CAT) is driven by the XIAP IRES. By calculating the ratio of CAT expression to βgal expression, the relative IRES activity can be determined.

HEK293T cells were transiently co-transfected with pβgal(-162)/CAT bicistronic reporter plasmid and either a GFP- or GFP-HuR-expressing plasmid. We found that overexpression of GFP-HuR caused an approximately 3-fold increase in XIAP IRES activity compared with a GFP control (Figure 2A), suggesting that HuR has a positive effect on XIAP IRES function. Similarly, we tested the deletion mutants of HuR for their ability to enhance XIAP IRES activity. We find that as seen in the UV-crosslinking experiments (Supplementary Figure 1A) only those HuR constructs that bind XIAP IRES in vitro are able to also modulate XIAP IRES activity in transfected cells (Supplementary Figure 1B). Conversely,
Figure 2. Cellular levels of HuR regulate XIAP IRES activity. (A) HEK293T cells were cotransfected with a plasmid expressing GFP or GFP-HuR and the pβgal/5’(-162)/CAT bicistronic reporter plasmid. 24 hours after transfection βgal and CAT protein expression was assayed; relative IRES activity is expressed as a ratio of CAT/βgal. The activity of XIAP IRES in GFP-transfected cells was set as 1. Mean +/- SEM (bars) of three independent experiments performed in triplicate. Expression levels of HuR were determined by Western-blot analysis using anti-HuR antibody. (B) HEK293T cells were transfected with 100 nM non-silencing control (CTRL) or HuR-targeting siRNA; 24 hours later the cells were transfected with the pβgal/5’(-162)/CAT bicistronic reporter plasmid and βgal and CAT protein expression was assayed 24 hours later; relative IRES activity is expressed as a ratio of CAT/βgal. The activity of XIAP IRES in CTRL siRNA-transfected cells was set as 1. Mean +/- SEM (bars) of three independent experiments performed in triplicate. Expression levels of HuR were determined by Western-blot analysis using anti-HuR antibody. (C) Modulating cellular levels of HuR does not affect the integrity of the bicistronic RNA transcript produced from the pβgal/5’(-162)/CAT. Cells were treated as described above; total RNA was isolated and cDNA was generated by reverse transcription. Quantitative PCR was used to determine the levels of βgal and CAT cistrons; values are expressed as CAT relative to βgal (2^[-ΔΔCt(CAT)-ΔΔCt(βgal)]) and the ratio for GFP- or CTRL siRNA-transfected cells was set as 1. Mean +/- SEM (bars) of three independent experiments.
Figure 2. Cellular levels of HuR regulate XIAP IRES activity. (a) HEK293T cells were co-transfected with a plasmid expressing GFP or GFP-HuR and with the p\(\beta\)-gal/5\(\alpha\)(162)/CAT bicistronic reporter plasmid. At 24 h after transfection, \(\beta\)-gal and CAT protein expression was assayed; relative IRES activity is expressed as a ratio of CAT/\(\beta\)-gal. The activity of XIAP IRES in GFP-transfected cells was set as 1. Mean±s.e.m (bars) of three independent experiments performed in triplicate. Expression levels of HuR were determined by western blot analysis using an anti-HuR antibody. (b) HEK293T cells were transfected with 100 nM non-silencing control (CTRL) or HuR-targeting siRNA; after 24 h the cells were transfected with the p\(\beta\)-gal/5\(\alpha\)(162)/CAT bicistronic reporter plasmid and \(\beta\)-gal, and CAT protein expression was assayed 24 h later; relative IRES activity is expressed as a ratio of CAT/\(\beta\)-gal. The activity of XIAP IRES in CTRL siRNA-transfected cells was set as 1. Mean±s.e.m (bars) of three independent experiments performed in triplicate. Expression levels of HuR were determined by western blot analysis using anti-HuR antibody. (c) Modulating cellular levels of HuR does not affect the integrity of the bicistronic RNA transcript produced from the p\(\beta\)-gal/5\(\alpha\)(162)/CAT. Cells were treated as described above; total RNA was isolated and cDNA was generated by reverse transcription. Quantitative PCR was used to determine the levels of \(\beta\)-gal and CAT cistrons; values are expressed as CAT relative to \(\beta\)-gal (2^\(-[Ct(CAT)-Ct(\beta\text{-gal})]\)), and the ratio for GFP- or CTRL siRNA-transfected cells was set as 1. Mean±s.e.m (bars) of three independent experiments.

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we tested the effect of HuR knockdown on XIAP IRES activity. HEK293T cells were reverse-transfected with either HuR-targeting siRNA or a non-silencing control siRNA. 24 hours later, the cells were transfected with pβgal/(-162)/CAT bicistronic reporter plasmid and reporter protein levels were assayed 24 hours after this second transfection. We found that reducing the levels of HuR resulted in an approximately 50% reduction in XIAP IRES activity compared with a non-silencing control (Figure 2B). These observations suggest that HuR possesses stimulatory ITAF activity for the XIAP IRES. An alternative explanation for our observations could be that HuR affects the integrity of the reporter bicistronic RNA transcript, thus altering the ratio of CAT to βgal protein. Therefore, we determined the effect of modulating HuR levels on the integrity of the βgal/(-162)/CAT bicistronic RNA transcript using quantitative RT-PCR as described previously (Holcik et al., 2005). Total RNA was isolated from transfected cells and cDNA was produced by reverse transcription. Quantitative PCR was performed using primers that amplify a portion of the βgal coding region and a portion of the CAT coding region. As shown in Figure 2C, the ratio of CAT and βgal cistrons was unchanged in cells irrespective of the status of HuR expression.

**Cellular levels of HuR modulate translation of endogenous XIAP mRNA.** We have shown that HuR binds to and positively regulates XIAP IRES activity. We next examined whether cellular levels of HuR affect the translation of endogenous XIAP mRNA. HEK293T cells were transiently transfected with either a GFP- or GFP-HuR-expressing plasmid, and the levels of endogenous XIAP were determined by Western-blot analysis 24 hours after transfection. We found that overexpression of GFP-HuR caused an approximately 2-fold increase in XIAP protein levels compared with a GFP control (Figure 3A), without a
concomitant increase in the XIAP mRNA levels (Figure 3B). In a converse experiment, HEK293T cells were transfected with either HuR-targeting or non-silencing control siRNA and the levels of XIAP protein were determined 48 hours later by Western-blot analysis. We found that reducing the levels of HuR by siRNA resulted in approximately 50% reduction in XIAP protein levels compared with a non-silencing control (Figure 3C), while having no effect on XIAP mRNA levels (Figure 3B). These observations paralleled what we have seen with the XIAP IRES reporter construct (Figure 2), suggesting that HuR is a positive regulator of XIAP translation by modulating XIAP IRES function.

To further demonstrate that HuR indeed affects the translation of endogenous XIAP mRNA we employed polysomal profiling to examine the association of XIAP mRNA with translating ribosomes in cells that overexpress HuR. We observed that overexpression of HuR resulted in a slight loss of heavy polysomes and an increase in monosomes (Figure 3D), indicating a repression of global protein synthesis. This observation was rather surprising, since HuR is not known to affect global rates of protein synthesis. Nevertheless, RT-PCR amplification of XIAP mRNA from individual fractions showed that despite the reduction in global protein synthesis XIAP mRNA is recruited into the heavier polysomes in HuR-overexpressing cells, confirming that the translation of the XIAP mRNA is enhanced by HuR.

**HuR-mediated induction of XIAP protects cells from etoposide-induced cell death.** We have shown that increased levels of HuR stimulate translation of XIAP mRNA, resulting in increased XIAP protein expression. Since XIAP is a potent inhibitor of apoptosis, and since
Figure 3. Cellular levels of HuR regulate endogenous XIAP mRNA translation. (A) HEK293T cells were transfected with a plasmid expressing GFP or GFP-HuR, and the expression levels of HuR and XIAP were determined 24 hours later by Western-blot analysis using anti-HuR and anti-XIAP antibodies. Representative blot is shown on left. Densitometric analysis of three independent experiments is shown on right. The expression of XIAP relative to Nucleolin in GFP-transfected cells was set as 1. Mean +/- SEM (bars). (B) Modulating cellular levels of HuR does not affect XIAP mRNA levels. Cells were treated as A or C; total RNA was isolated and cDNA was generated by reverse transcription. Quantitative PCR was used to determine the levels of XIAP and GAPDH mRNA; values are expressed as XIAP relative to GAPDH \(2^{[-\Delta\Delta CT]}\), and the ratio for GFP- or CTRL siRNA-transfected cells was set as 1. Mean +/- SEM (bars) of three independent experiments. (C) HEK293T cells were transfected with 100 nM non-silencing control (CTRL) or HuR-targeting siRNA; 48 hours later the expression levels of HuR and XIAP were determined by Western-blot analysis using anti-HuR and anti-XIAP antibodies. Representative blot is shown on left. Densitometric analysis of three independent experiments is shown on right. The expression of XIAP relative to Nucleolin in GFP-transfected cells was set as 1. Mean +/- SEM (bars). (D) Overexpression of HuR enhances translation of XIAP mRNA. HEK293T cells were transfected with a plasmid expressing GFP or GFP-HuR and polysomal profiling was performed 24 hours later. Representative RNA profile (indicated by absorbance at 254 nm) is shown on top for GFP- and GFP-HuR-transfected cells. Individual fractions were probed for the presence of XIAP mRNA by qPCR, which is shown as the percent distribution of specific XIAP mRNAs relative to GAPDH mRNA across the gradient (LMWP – low molecular weight polysomes; HMWP – high molecular weight polysomes). Mean +/- SEM (bars) of three independent experiments.
Figure 3. Cellular levels of HuR regulate endogenous XIAP mRNA translation. (a) HEK293T cells were transfected with a plasmid expressing GFP or GFP-HuR, and the expression levels of HuR and XIAP were determined 24 h later by western blot analysis using anti-HuR and anti-XIAP antibodies. The representative blot is shown on the left. Densitometric analysis of three independent experiments is shown on the right. The expression of XIAP relative to Nucleolin in GFP-transfected cells was set as 1. Mean±s.e.m (bars). (b) Modulating cellular levels of HuR does not affect XIAP mRNA levels. Cells were treated as A or C; total RNA was isolated and cDNA was generated by reverse transcription. Quantitative PCR was used to determine the levels of XIAP and GAPDH mRNA; values are expressed as XIAP relative to GAPDH (2^{-\Delta\Delta Ct(XIAP)-\Delta\Delta Ct(GAPDH)}) and the ratio for GFP- or CTRL siRNA-transfected cells was set as 1. Mean±s.e.m (bars) of three independent experiments. (c) HEK293T cells were transfected with 100 nM non-silencing control (CTRL) or HuR-targeting siRNA; after 48 h the expression levels of HuR and XIAP were determined by western blot analysis using anti-HuR and anti-XIAP antibodies. The representative blot is shown on the left. Densitometric analysis of three independent experiments is shown on the right. The expression of XIAP relative to Nucleolin in GFP-transfected cells was set as 1. Mean±s.e.m.
the cellular levels of HuR were shown to be regulated by a variety of cellular stress events, we wished to investigate if the HuR-mediated increase in XIAP offers protection against cell death. HEK293T cells were reverse transfected with siRNA targeting XIAP or a non-silencing scrambled control and 24 hours later with either a GFP or a GFP-HuR overexpressing plasmid. 24 hours post transfection the cells were treated with increasing doses of etoposide and the viability of cells was determined 24 hours later by Alamar blue assay. As expected, we observed that treatment of GFP-transfected cells with etoposide resulted in a significant amount of cell death. In contrast, cells overexpressing HuR were protected against etoposide-induced cell death (Figure 4). Importantly, siRNA-mediated knock down of XIAP abolished the protective effect of HuR overexpression. These results indicate that the induction of XIAP expression through HuR contributes to protection against cell death.

**Discussion**

IRES-dependent translation initiation of cellular mRNAs has emerged as an important regulatory step in fine-tuning the cellular response to distinct physiological conditions. Yet the precise mechanism of how cellular IRES-containing mRNAs engage the ribosomal machinery, and the mechanisms by which this process is regulated, remain unclear. In this work we have identified HuR as a novel XIAP IRES *trans*-acting factor. We show here that HuR binds to XIAP IRES RNA *in vitro* and is associated with XIAP mRNA in cells. Furthermore, we show that HuR stimulates both XIAP IRES activity as well as translation of endogenous XIAP mRNA, and thus contributes to enhanced cytoprotection through elevation of XIAP protein levels.
Figure 4. Overexpression of HuR protects cells against etoposide-induced cell death in a XIAP-dependent manner. HEK293T cells were reverse transfected with a non-silencing control (CTRL) or XIAP-targeting siRNA and 24 hours later with a plasmid expressing GFP or GFP-HuR. 24 hours post-transfection the cells were treated with indicated doses of etoposide for 24 hours and the cell viability was determined by an Alamar blue assay. Mean +/- SEM (bars) of two independent experiments performed in triplicate (* p<0.05, ** p<0.01; unpaired t-test). The expression levels of XIAP and HuR were assessed by Western-blot analysis using anti-HuR and anti-XIAP antibodies, and a representative blot is shown above the graph.
Figure 4. Overexpression of HuR protects cells against etoposide-induced cell death in a XIAP-dependent manner. HEK293T cells were reverse transfected with a non-silencing control (CTRL) or XIAP-targeting siRNA and 24 h later with a plasmid expressing GFP or GFP-HuR. At 24-h post–transfection, the cells were treated with indicated doses of etoposide for 24 h and cell viability was determined by an Alamar blue assay. Mean±s.e.m (bars) of two independent experiments performed in triplicate (*P<0.05, **P<0.01; unpaired t-test). The expression levels of XIAP and HuR were assessed by western blot analysis using anti-HuR and anti-XIAP antibodies, and a representative blot is shown above the graph.
The role of HuR in the posttranscriptional regulation of XIAP expression was reported recently (Zhang et al., 2009). It was shown that HuR binds to the XIAP coding region and the 3’ UTR to stabilize XIAP mRNA, thus contributing to increased expression of XIAP protein. In our experimental system (HEK293T cells) we did not observe any stabilization effect of HuR on the XIAP mRNA (Figure 3B). In contrast, we observed that increased levels of HuR enhance recruitment of XIAP mRNA into heavier polysomes, suggesting that HuR affects the translation of XIAP mRNA. Indeed, using reporter constructs we show that HuR acts as a positive regulator of XIAP IRES activity.

HuR, a member of the ELAV family of RNA binding proteins, is a multifunctional protein that has been implicated in the regulation of various aspects of RNA metabolism (Hinman & Lou, 2008). Although primarily known for binding to the 3’ UTRs of mRNAs and increasing their intrinsic stability, several recent reports identified HuR as a bona fide translational regulator as well. We have shown previously that HuR enhances translation of HIF1α in response to treatment with the hypoxia mimetic CoCl2 (Galban et al., 2008). Interestingly, although HIF1α translation is constitutively enhanced by the HIF1α IRES, HuR does not act through this IRES, but rather stimulates HIF1α translation in concert with another RNA-binding protein, PTB, which binds the 3’ UTR of HIF1α. In contrast, HuR was shown to directly regulate translation of viral as well as some cellular IRES elements. While the effect of HuR on RNA stability is invariably to increase it, with the exception of HCV IRES the consequence of HuR binding to the IRES is to repress its translation (Kullmann et al., 2002; Meng et al., 2005; Rivas-Aravena et al., 2009; Yeh et al., 2008). Although the mechanism of how HuR represses IRES-dependent translation is not clear, it has been suggested that in some cases, such as with the p27(Kip1) IRES, the binding of HuR to the
IRES may block ribosome entry (Coleman & Miskimins, 2009). In contrast, we find that XIAP IRES activity and XIAP mRNA translation are both stimulated by HuR. We have previously identified both positive (hnRNP C1/C2 (Holcik et al., 2003); La (Holcik & Korneluk, 2000)) and negative (hnRNP A1 (Lewis et al., 2007); PTB (Baird et al., 2007)) modulators of XIAP IRES activity. While we do not fully understand how distinct ITAFs regulate XIAP IRES function, we envision that the binding of ITAFs to the XIAP IRES is required for structural remodeling of the IRES so that it is amenable to ribosome recruitment.

XIAP is a member of the family of the intrinsic inhibitors of apoptosis (IAP) proteins. While a number of divergent cellular functions are regulated by XIAP, its primary role in the cell appears to be caspase inhibition (Dubrez-Daloz et al., 2008). Importantly, increased expression of XIAP is seen in a number of human cancers and correlates with enhanced chemo- or radiation resistance (Holcik et al., 2000b; Pardo et al., 2003; Tamm et al., 2000; Wilkinson et al., 2004). We find that overexpression of HuR markedly preserves the viability of cells treated with etoposide in a XIAP-dependent manner. In this respect it is interesting to note that HuR was proposed to orchestrate an anti-apoptotic cellular program (Abdelmohsen et al., 2007). mRNAs of several key members of the cellular apoptotic network are known targets of posttranscriptional regulation by HuR, either by affecting their stability or translation, and for the most part HuR enhances expression of the anti-apoptotic molecules, and represses expression of the pro-apoptotic molecules. Indeed, numerous reports have established a link between elevated levels of HuR and cancer. For example, the expression of HuR correlates with malignancy and poor clinical outcome in breast (Denkert et al., 2004b; Heinonen et al., 2005; Heinonen et al., 2007), ovarian (Denkert et al., 2004a; Erkinheimo et al., 2005), and colorectal (Denkert et al., 2006; Yoo et al., 2009) cancers. Our data suggest that caspase inhibitor XIAP is also a member of the anti-apoptotic program that
is orchestrated by HuR, and along with additional anti-apoptotic proteins such as Bcl-2 (Abdelmohsen et al., 2007), ProTα (Lal et al., 2005), and Mcl-1 (Abdelmohsen et al., 2007) contributes to HuR-mediated protection against cell death.

Materials & Methods

Cell culture, expression constructs and transfection. Human embryonic kidney (HEK293T) cells were maintained in standard conditions in serum- and antibiotic-supplemented Dulbecco's modified Eagle's medium (DMEM). The bicistronic reporter plasmid pβgal/5′(-162)/CAT was previously described (Holcik et al., 1999) and contains the minimal functional region of the human XIAP IRES. The 6myc-XIAP, GFP-HuR, GFP-HuR(CP1), GFP-HuR(CP2), GST-HuR, Ap-HuR-GST, AP-HuR(CP1)-GST, and AP-HuR(CP2)-GST expression plasmids were described previously (Liston et al., 1996; Mazroui et al., 2008). Transient transfections were performed using LipofectAMINE 2000 according to the protocol provided by the manufacturer (Invitrogen). Cells were seeded at a density of 5x10^5 cells per well in 6-well plates and were transfected 24 h later with 2 µg of plasmid DNA per well. Cells were collected for analysis 24 h post-transfection. siRNA transfections were performed using RNAiFect according to the protocol provided by the manufacturer (Qiagen). Briefly, cells were seeded at a density of 5 × 10^5 cells/well in 6-well plates and were transfected 24 h later in serum-free D-MEM with a 100 nM final concentration of HuR siRNA (Santa Cruz Biotechnology) or a non-silencing control siRNA (Qiagen). Cells were collected for analysis 48h post-transfection.

RNA-affinity chromatography. Isolation of XIAP IRES-binding proteins was performed using a RNA-affinity chromatography protocol as described (Lewis et al., 2007). Briefly,
XIAP IRES RNA and cIAP1 IRES RNA were transcribed *in vitro* with the MEGAShortscript transcription kit according to the manufacturer’s protocol (Ambion), and were biotinylated at the 5’ end with the 5’ EndTag Nucleic Acid Labeling System according to the manufacturer’s instructions (Vector Laboratories). The biotinylated RNAs (60 µg) were conjugated to Avidin-agarose beads (Sigma) in the presence of incubation buffer (10 mM Tris-Cl [pH 7.4], 150 mM KCl, 1.5 mM MgCl\(_2\), 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.05% [v/v] Nonidet P-40) at 4°C for 2 h with continuous rotation. Unbound RNAs were removed by washing beads twice with incubation buffer. 2 mg of HEK293T protein extract (in incubation buffer) was added to the coated beads, along with 120 µg yeast tRNA (Sigma) and 800 units of Prime RNase inhibitor (Eppendorf). Reactions were incubated at room temperature with continuous rotation for 30 minutes, followed by incubation at 4°C with continuous rotation for 2 h. Beads were washed five times with incubation buffer, resuspended in 50 µl of 1× SDS-PAGE loading dye, and boiled for 5 minutes to elute bound proteins. Proteins were separated by 10% SDS-PAGE and visualized using Sypro Ruby stain (Genomic Solutions). Protein bands were excised and identified by in-gel trypsin digestion and mass peptide fingerprinting at the Protein Function Discovery Centre (Queen’s University, Kingston, ON, Canada).

**RNA-protein complex immunoprecipitation.** *In vivo* crosslinking and co-precipitation of RNA-protein complexes was performed as described previously (Lewis et al., 2007). Crosslinked RNA-protein complexes were immunoprecipitated using anti-HuR (Santa Cruz Biotechnology), or anti-TIA-1/TIAR (clone 3E6; generous gift from P. Anderson) antibodies at 1:50 dilution. Following immunoprecipitation and crosslink reversal, RNA was isolated.
using Trizol reagent following the manufacturer’s protocol (Invitrogen). cDNA was generated from the isolated RNA using an oligo dT₁₈ primer and Superscript II (Invitrogen). The partial coding sequences of XIAP and β-actin were PCR amplified from the resulting cDNA using the primers 5’-gcggtgccttagtttgtc (XIAP forward), 5’-tcgggtatatgtgtctgata (XIAP reverse), 5’-ctggaacggtgaaggtgaca (β-actin forward), and 5’-aagggactctgtaacaagtc (β-actin reverse). PCR products were visualized on a 1.5% agarose gel by ethidium bromide staining.

**UV-crosslinking of RNA-protein complexes.** RNA-protein UV-crosslinking experiments and oligonucleotide competition experiments were performed as previously described (Holcik et al., 2003; Lewis et al., 2007).

**β-galactosidase and CAT analysis.** Transiently transfected cells were washed in 1 ml PBS and harvested in 300 µl CAT ELISA kit lysis buffer according to the protocol provided by the manufacturer (Roche Molecular Biochemicals). β-galactosidase (βgal) enzymatic activity was determined by spectrophotometric assay using o-nitrophenyl-β-D-galactopyranoside as previously described (MacGregor et al., 1991). CAT levels were determined using the CAT ELISA kit according to the protocol provided by the manufacturer (Roche Molecular Biochemicals). Relative IRES activity was calculated as the ratio of CAT/βgal.

**Western-blot analysis.** Cells were washed in 1 ml PBS and lysed in 150 µl RIPA buffer for 30 minutes at 4°C, followed by centrifugation at 12,000 × g for 10 minutes to pellet debris. Protein concentration was assayed by BCA Protein Assay Kit (Pierce) and equal amounts of
protein extract were separated by 10% SDS-PAGE and transferred to PVDF or nitrocellulose membranes. Samples were analyzed by Western blotting using mouse monoclonal anti-HuR (Santa Cruz Biotechnology), rabbit polyclonal anti-XIAP (AEgera), mouse monoclonal anti-Nucleolin (Santa Cruz Biotechnology), or rabbit polyclonal anti-GST antibodies followed by secondary antibody (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG; Amersham Biosciences). Antibody complexes were detected using the ECL or ECL Plus systems (Amersham Biosciences) and were quantified using Odyssey densitometry software (LiCor).

**Quantitative RT-PCR analysis.** Total RNA was isolated from transfected cells using the Absolutely RNA miniprep kit according to the manufacturer’s instructions (Stratagene). cDNA was generated using an oligo dT18 primer and the Bulk 1st-Strand Synthesis kit according to the protocol provided by the manufacturer (Amersham Biosciences). The synthesized cDNA was used as the template for quantitative PCR using the QuantiTect SYBR Green PCR kit (Qiagen) and analyzed on a Stratagene Mx3005P™ real-time thermocycler. Relative expression levels were determined using the standard curve method. Controls lacking RT demonstrated no significant genomic DNA amplification (>10 cycle difference). Quantitative PCR reactions were carried out to detect βgal (forward: 5’-actatccgacccgccttacct; reverse: 5’-ctgtacgcgtgatgttgaa), CAT (forward: 5’-gcgttgacctgcgtaaacc; reverse: 5’-gggccaagaaccttccata), XIAP (forward: 5’-gcggttagtgtagttgaa; reverse: 5’-tcgggtatatggtgtcata), or GAPDH (forward: 5’-acagtcaggcgcatcttt; reverse: 5’-acgaccaaatccgttgactc).
**Polysome profiling.** HEK293T cells from three 15 cm plates per condition were lysed in cold polysome lysis buffer (15mM Tris-HCl, pH7.4, 15mM MgCl2, 300mM NaCl, 1%(v/v) Triton X-100, 0.1mg/ml cycloheximide, 100U/ml RNasin). Equal OD254 units were loaded onto 10-50% linear sucrose gradients and centrifuged at 39000rpm for 90 minutes at 4°C. Gradients were fractionated from the top (Densi-Flow, Labconco) and RNA was monitored at 254nm using a HPLC system (Äkta Explorer, GE Biosciences). 1 ml fractions were collected and flash frozen in liquid nitrogen. After thawing on ice, the fractions were spiked with 10 µl of a 10 ng/µl solution of *in vitro*-transcribed CAT RNA, to ensure technical consistency in RNA extraction. RNA was isolated from individual fractions by proteinase K digestion followed by phenol/chloroform extraction and was recovered by ethanol precipitation. Equal volumes of RNA from each fraction were used to generate cDNA using oligo-dT primers and a reverse transcription kit (First-Strand cDNA synthesis kit, GE Biosciences). PCR primers specific for XIAP isoforms, CAT, or GAPDH were used to amplify messages using quantitative PCR as described above.

**Cell viability.** HEK293T cells were transfected with 2 µg of either GFP or GFP-HuR expressing plasmids and 100 nM final concentration of XIAP siRNA (Santa Cruz Biotechnology) or a non-silencing control siRNA (Qiagen) as described above. 24 hours post-transfection cells were treated with indicated doses of etoposide. Cell viability was determined 24 hours later by Alamar blue assay according to the protocol provided by the manufacturer (Invitrogen). Statistical analysis was performed by GraphPad Prism 5 software.

**Conflict of interest.** The authors have no competing financial interests in relation to the work described in this manuscript.
Acknowledgments.

We thank Drs. Nehal Thakor and Stephen Baird for critical discussions. We are grateful to Drs. P. Anderson and R. Screaton for the gift of anti-TIA-1/TIAR and anti-GST antibodies, respectively, and Dr. I. Gallouzi for the GFP-HuR, GFP-HuR(CP1), GFP-HuR(CP2), AP-HuR-GST, AP-HuR(CP1)-GST, and AP-HuR(CP2)-GST overexpression constructs. This work was supported by an operating grant from the Canadian Institutes for Health Research to M.H. (CIHR; MOP 89737); M.G. was supported by the Intramural Research Program of the National Institute on Aging, National Institutes of Health. M.H. is the CHEO Volunteer Association Endowed Scholar.
Supplementary Figure. Distinct HuR domains modulate XIAP IRES activity. (A) Purified recombinant AP-GST, AP-HuR-GST, AP-HuR(CP1)-GST, or AP-HuR(CP2)-GST were incubated with $^{32}$P-labelled XIAP IRES RNA probe, UV-crosslinked, and then separated by SDS-PAGE and visualized by autoradiography (top). Equal amounts of recombinant proteins in the binding assay were verified by Western-blot analysis using anti-GST antibody and is shown below. (B) HEK293T cells were cotransfected with a plasmid expressing GFP, GFP-HuR(CP1), or GFP-HuR(CP2), and the pβgal/5′(-162)/CAT bicistronic reporter plasmid. 24 hours after transfection βgal and CAT protein expression was assayed; relative IRES activity is expressed as a ratio of CAT/βgal. The activity of XIAP IRES in GFP-transfected cells was set as 1. Mean +/- SEM (bars) of four independent experiments performed in triplicate.
References


Appendix C:
Curriculum vitae

Urszula Liwak-Muir, M.Sc.

EDUCATION

Doctorate in Biochemistry (Ph.D.) 09/2009-Present
University of Ottawa/Children's Hospital of Eastern Ontario Research Institute, Ottawa, ON

Master's in Biochemistry (M.Sc.) 09/2007-04/2009
University of Windsor, Windsor, ON

Bachelor of Science in Biochemistry (B.Sc.) [Honors], Minor in Psychology 09/2003-04/2007
University of Windsor, Windsor, ON

ANALYTICAL CHEMISTRY EXPERIENCE

Maxxam Analytics 05/2009-09/2009
CNRL Horizon Project, Main Laboratory, Fort McMurray, Alberta
- Determined pH levels, Total Suspended Solids, and Sulfur levels
- Required to follow standard operating procedures and record my findings to ensure quality control

MOLECULAR BIOLOGY/BIOCHEMISTRY RESEARCH EXPERIENCE

Ph.D. Biochemistry 09/2009-Present
University of Ottawa, Ottawa, ON

Supervisor: Dr. Martin Holcik
- Investigating the role of RNA binding proteins in regulating translation of cell death proteins in Glioblastoma Multiforme tumors
- Specialized in Western blot, ELISA, quantitative RT-PCR, cloning, tissue culture, RNA interference, confocal microscopy, recombinant protein purification, \textit{in-vitro} transcription of radiolabelled RNA

University of Windsor, Windsor, ON
Thesis: “*Toxoplasma gondii* Differentiation: Effects of Modifying Lactate Dehydrogenase and Argonaute Expression Patterns”

Supervisor: Dr. Sirinart Ananvoranich

- Investigated the function of argonaute and lactate dehydrogenase in the parasite, *Toxoplasma gondii*, with respect to an RNA interference-like pathway as well as in the differentiation process
- Specialized in techniques including Western blotting, cloning, tissue culture, parasitology, confocal microscopy, radioactivity, mass spectrometry


University of Windsor, Windsor, ON

Thesis: “Determining the Localization of Argonaute within *Toxoplasma gondii* using a Fluorescent Fusion Protein”

Supervisor: Dr. Sirinart Ananvoranich

- Generated and characterized the fusion between argonaute and a cherry fluorescent protein to determine the localization and function within *Toxoplasma gondii*.

LABORATORY SKILLS

Translation assays: polysome profiling, reporter assays

RNA assays: RNA immunoprecipitation, *in vitro* transcription of $^{32}$P labeled RNA, UV-crosslinking RNA binding assays, REMSA, quantitative RT-PCR

DNA techniques: plasmid/genomic DNA purification, cloning, primer design, site-directed mutagenesis

Protein assays: ELISA, Western blotting, recombinant protein purification, immunoprecipitation, mass spectrometry, flow cytometry, confocal microscopy

Cell based techniques: tissue culture, RNA interference, plasmid overexpression, cell viability assays, cytotoxicity and apoptosis assays, establishing stable cell lines, immunofluorescence

Research Skills: critical thinking, experimental design, data analysis, peer-reviewed publications, grant writing, aseptic techniques, statistical analyses

COMMUNICATION AND LEADERSHIP

Teaching Assistantships, Laboratory
University of Windsor, Windsor, Ontario, Canada

Chemistry 380 – Biotechnology Laboratory  Fall 2007, Fall 2008

- Demonstrated proper laboratory techniques, discussed theoretical and practical concepts
- Evaluated student’s laboratory reports, assignments, and exams
• Demonstrated good interpersonal skills and the ability to interact with and train students

Teaching Assistantship, Tutorial
University of Windsor, Windsor, Ontario, Canada
Chemistry 191 - Organic/Biological Chem./Health Sciences  Winter 2009

• Responsible for classroom instruction to review the material taught in lectures
• Fielded student’s questions in preparation for weekly quizzes
• Evaluated quizzes and exams

PUBLICATIONS


CONFERENCE PRESENTATIONS

Ontario, Canada. Oral Presentation.


BIOINFORMATICS TRAINING

3rd Annual ApiDatabase/EuPathDatabase Workshop
06/2008
University of Georgia, Athens, Georgia, USA

ACADEMIC TRAINING and OTHER SKILLS

TRAINING

Graduate courses in RNA metabolism, molecular biology of human diseases, cell death and diseases, and advanced DNA science.
Basic and graduate courses in biochemistry: molecular biology, proteins, lipids, metabolism, and microbiology.
Basic courses in chemistry: organic, inorganic, physical, and analytical.
Basic statistics.
Advanced laboratories in biochemical techniques and in analytical and organic chemistry synthesis.

COMPUTER SKILLS

Microsoft Office Suite, EndNote, GraphPad Prism, Photoshop

AWARDS and SCHOLARSHIPS

Ontario Graduate Scholarship Program Research Award, $60,000 05/2010-05/2014
Excellence Scholarship, University of Ottawa, Ottawa, ON, $26,000 05/2010-05/2014
Admission Scholarship, University of Ottawa, Ottawa, ON, $6,500 09/2009-05/2010
Admission Scholarship, University of Windsor, Windsor, ON, $500 09/2003-12/2003