CHARACTERIZING THE INTERACTION BETWEEN PDCD4 AND eIF3 WITH RESPECT TO TRANSLATION REGULATION

DIVYA SHARMA KHANDIGA
Master of Science, Bangalore University, India 2009

A Thesis/Project
Submitted to the School of Graduate Studies
of the University of Lethbridge
in Partial Fulfillment of the
Requirements for the Degree

MASTER OF SCIENCE

Department of Chemistry and Biochemistry
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

CHARACTERIZING THE INTERACTION BETWEEN PDCD4 AND eIF3 WITH RESPECT TO TRANSLATION REGULATION

DIVYA SHARMA KHANDIGA

Date of Defense: December 12, 2017

Dr. N. Thakor Thesis Supervisor	Assistant Professor	Ph.D.
Dr. M. Roussel Thesis Co-supervisor	Professor	Ph.D.
Dr. U. Kothe Thesis Examination Committee Member	Associate Professor	Ph.D.
Dr. R. Golsteyn Thesis Examination Committee Member	Associate Professor	Ph.D.
Dr. R. Fahlman External Examiner University of Alberta Edmonton, Alberta	Professor	Ph.D.
Dr. M. Gerken Chair, Thesis Examination Committee	Professor	Ph.D.

Dedication

To my beloved family and friends,

My inspiration, my parents Subraya Sharma and Kamala Sharma

My dearly loved husband Samarth, sister Dr. Lakshmi and brother-in-law Dr. Pradeep

My cute little niece Mithali and nephew Aathreya

My adorable brother Dr. Ganesh, sister Dr. Sharadha, Silly Vidya and little angels

My loving cousins and in-laws

I am grateful to have them in my life, it is their well wishes, teachings, support and love that have enabled me to achieve success and happiness in life.

Abstract

Programmed cell death protein 4 (PDCD4) inhibits IRES-mediated translation of anti-apoptotic proteins such as XIAP. PDCD4 was shown to directly interact with the XIAP IRES element and inhibit translation initiation. Additionally, our lab reported that a eukaryotic initiation factor, eIF3 interacts with the XIAP IRES to facilitate ribosome recruitment. Interestingly, the activity of PDCD4 and eIF3 are regulated by common regulatory kinases called S6K1 and 2. Therefore, to investigate the possibility of interaction between PDCD4 and eIF3 as well as their co-regulation by S6K1 and 2, I have performed co-immunoprecipitation assays in glioblastoma and S6K double knockout MEFs. The results of *in cellulo* assays demonstrate RNA-independent PDCD4-eIF3 interactions. In addition, eIF3F, one of the 13 eIF3 subunits has been demonstrated to interact directly with PDCD4. This study suggests that the interaction of PDCD4 with eIF3F may have a role in regulating global and/or transcript-specific translation.

Acknowledgments

I would like to thank my supervisor Dr. Nehal Thakor for providing me an opportunity to pursue my master's in his laboratory. He has been a constant support and helped me to work my way through the master's program.

I would also like to thank my committee members, Drs. Roy Golsteyn, Marc Roussel and Ute Wieden-Kothe for their valuable feedback and guidance.

Lastly, I would like to thank my lab mates for helping me with my experiments and for all the encouraging words when my experiments were a total disaster.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

4E-BP	eIF4F-binding protein
Apaf1	Apoptotic protease activating factor 1
Bcl-xL	B-cell lymphoma extra-large
Bicis	Bi-cistronic
BIR	Baculovirus IAP repeat
CARD	Caspase activation recruitment
c-FLIP	FADD-like IL-1β-converting enzyme-inhibitory protein
cIAP1	Cellular inhibitor of apoptosis 1
CST	Cell signaling technology
Deptor	DEP domain containing mTOR-interacting protein
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DR4/5	Death receptor 4/5
dsDNA	Double-stranded deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
FADD	Fas-associated protein with death domain
FasR	Fas-receptor
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
hnRNP	Heterogenous ribonucleoprotein
HuR	Human antigen R
IAP	Inhibitor of apoptosis protein
tRNAi	Initiator transfer RNA
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRES	Internal ribosome entry site
ITAF	IRES trans-acting factor
LB	Luria-Bertani
MDM2	Murine double minute 2
mLST8	Mammalian lethal with sec-13
mTOR	Mammalian target of rapamycin
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAIP1	Polyadenylate-binding protein-interacting protein 1
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline tween 20
PDCD4	Programmed cell death 4
PERK	PKR-like ER kinase
PI3K	Phosphatidylinositol 3-kinase
PKR	Protein kinase R
PMSF	Phenylmethanesulfonyl fluoride
Pras40	Proline rich Akt substrate 40 kDa
PTEN	Phosphatase and tension homolog

Raptor	Regulatory associated protein of mTOR
RING	Really interesting new gene
RIP	Receptor interacting protein
RIPA	Radioimmunoprecipitation assay
RT	Room temperature
S6K	Ribosomal protein S6 kinase
S6K	P70 ribosomal protein S6 kinase
SCF	SKP1–CUL1–F-box protein
SDS	Sodium dodecyl sulphate
siRNA	Small interfering ribonucleic acid
Smac	Second mitochondria-derived activator of caspases
TCS2	Tuberous sclerosis protein 2
TNFR1	Tumor necrosis factor receptor 1
TNFα	Tumor necrosis factor alpha
TRADD	Tumor necrosis factor receptor type 1-associated death domain
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRAIL-R	TRAIL-receptor
UBA	Ubiquitin-associated domain
UTR	Untranslated region
XIAP	X-linked inhibitor of apoptosis

Chapter 1

Introduction

In this chapter, I have described eukaryotic translation initiation and the different types of initiation mechanisms. I have also discussed the regulatory mechanisms and factors affecting translation initiation and their downstream effects on cell survival. To conclude, I have described my hypothesis and research plan.

1.1 Description of eukaryotic translation initiation

The genetic information stored in the double-stranded deoxyribonucleic acid (dsDNA) is transcribed into the messenger ribonucleic acid (mRNA) by enzymes called RNA polymerases (Kireeva, Kashlev, & Burton, 2013). These enzymes utilize the DNA as a template to synthesize complementary mRNA by a process called transcription. In eukaryotes, the mRNA is synthesized as precursor mRNA that is processed by the addition of a 7-methylguanosine cap structure onto the 5' end of the mRNA (capping), addition of polyadenosine onto the 3' end of the mRNA (polyadenylation) and removal of introns (splicing) to form mature mRNA (Halbeisen, Galgano, Scherrer, & Gerber, 2008). The mature mRNA is transported into the cytoplasm. In the cytoplasm, the translation machinery utilizes the mRNA as a template to synthesize proteins by a process called translation. Translation is divided into three steps, which occur in the order of initiation, elongation, and termination (Hinnebusch & Lorsch, 2012; R. J. Jackson, Hellen, & Pestova, 2010). Translation initiation is the most regulated and rate-limiting step of the three. The process requires about 12 eukaryotic initiation factors (eIFs), cellular proteins (also called *trans*-acting factors), mature mRNA, ribosomal subunits and initiator transfer RNA (tRNAi). The process of initiation is further classified into three steps i) 43S pre-

initiation complex ii) 48S pre-initiation complex, and iii) 80S pre-initiation complex formation. With the assembly of the 80S pre-initiation complex, the ribosome is prepared to move into the next step of translation and begin protein synthesis (Hinnebusch & Lorsch, 2012; R. J. Jackson et al., 2010).

In eukaryotes, the translation initiation process is broadly classified into canonical and non-canonical initiation. In canonical translation initiation, the 5' cap structure of the mRNA plays an essential role to begin protein synthesis. Alternatively, in non-canonical translation, the 5' cap structure is no longer essential and the translation machinery can be recruited onto the mRNA without the involvement of a 5' cap or initiation factors interacting with the cap structure.

1.1.1 Cap-dependent translation initiation

Most eukaryotic mRNAs are translated by canonical or cap-dependent translation initiation mechanism (Figure 1.1). In this process, it utilizes a set of about 12 eIFs to initiate translation. eIF2 is one of the 12 eIFs required for the cap-dependent translation and it consists of 3 distinct subunits (eIF2α, eIF2β, and eIF2γ). eIF2 directly interacts with guanosine triphosphate (GTP) and initiator met-tRNAi to form the ternary complex (Figure 1.1) (Asano, Clayton, Shalev, & Hinnebusch, 2000; Kimball, 1999). Specifically, the eIF2γ subunit interacts with GTP, while eIF2β and eIF2γ have regions interacting to the met-tRNAi (Kimball, 1999). The active ternary complex then positions the initiator met-tRNAi to the P site of the 40S ribosomal subunit associated with eIF3, eIF1A, and eIF1 (R. J. Jackson et al., 2010; Maag, Fekete, Gryczynski, & Lorsch, 2005). The ternary complex interacts with the 40S ribosomal subunit associated with eIF3, eIF1, and eIF1A to form the 43S pre-initiation complex (Figure 1.1). Separately, eIF4B and eIF4F complex interact with the mRNA (Figure 1.1) (R. J. Jackson et al., 2010; Maduzia,

Moreau, Poullet, Chaffre, & Zhang, 2010). eIF4F is a cap-binding complex and is composed of eIF4E, eIF4A and eIF4G. eIF4E is the cap-binding protein that interacts with modified guanosine present on the 5' end of mRNA, eIF4A is a helicase that helps in unwinding of secondary structures present on the mRNA and facilitates scanning of mRNA by the ribosome, and eIF4G acts as the scaffold protein enabling protein-protein interaction between eIF4G and other eIFs such as eIF4G, eIF4E, and eIF3 (Andreev et al., 2017; R. J. Jackson et al., 2010). eIF4F bound mRNA complex associates with the 43S pre-initiation complex (Figure 1.1). This interaction is mediated by eIF3 present in the 43S pre-initiation complex and eIF4G of the eIF4F-complex (Hinnebusch & Lorsch, 2012). At this stage, the translation machinery is referred to as an open conformation. Upon interaction of mRNA and the 40S ribosome, eIF1 works along with eIF1A to support ribosome scanning in order to recognize the start AUG codon (Maduzia et al., 2010). The complex to is believed to scan the mRNA in the 5' to 3' direction to locate the initiation codon (Hinnebusch & Lorsch, 2012). When the first start codon (AUG) in the context of Kozak sequence is recognized by the translation initiation complex, eIF5 promotes the GTPase activity of eIF2 to release the eIFs form the 48S pre-initiation complex (Figure 1.1). After the release of eIFs from the complex, eIF5B helps the joining of the 60S subunit to the smaller ribosomal subunit takes place as mentioned in Figure 1.1. This forms the 80S pre-initiation complex and initiates translation elongation (Figure 1.1) (R. J. Jackson et al., 2010). At the end of translation, ribosomal subunits are dissociated by the function of eukaryotic release factors (Dever & Green, 2012). The molecules involved in translation are recycled and fed back into the next round of the translation cycle. The cycle of translation is an energy consuming process and hence it is

essential for the cells to regulate this mechanism and control the expression of all cellular proteins.

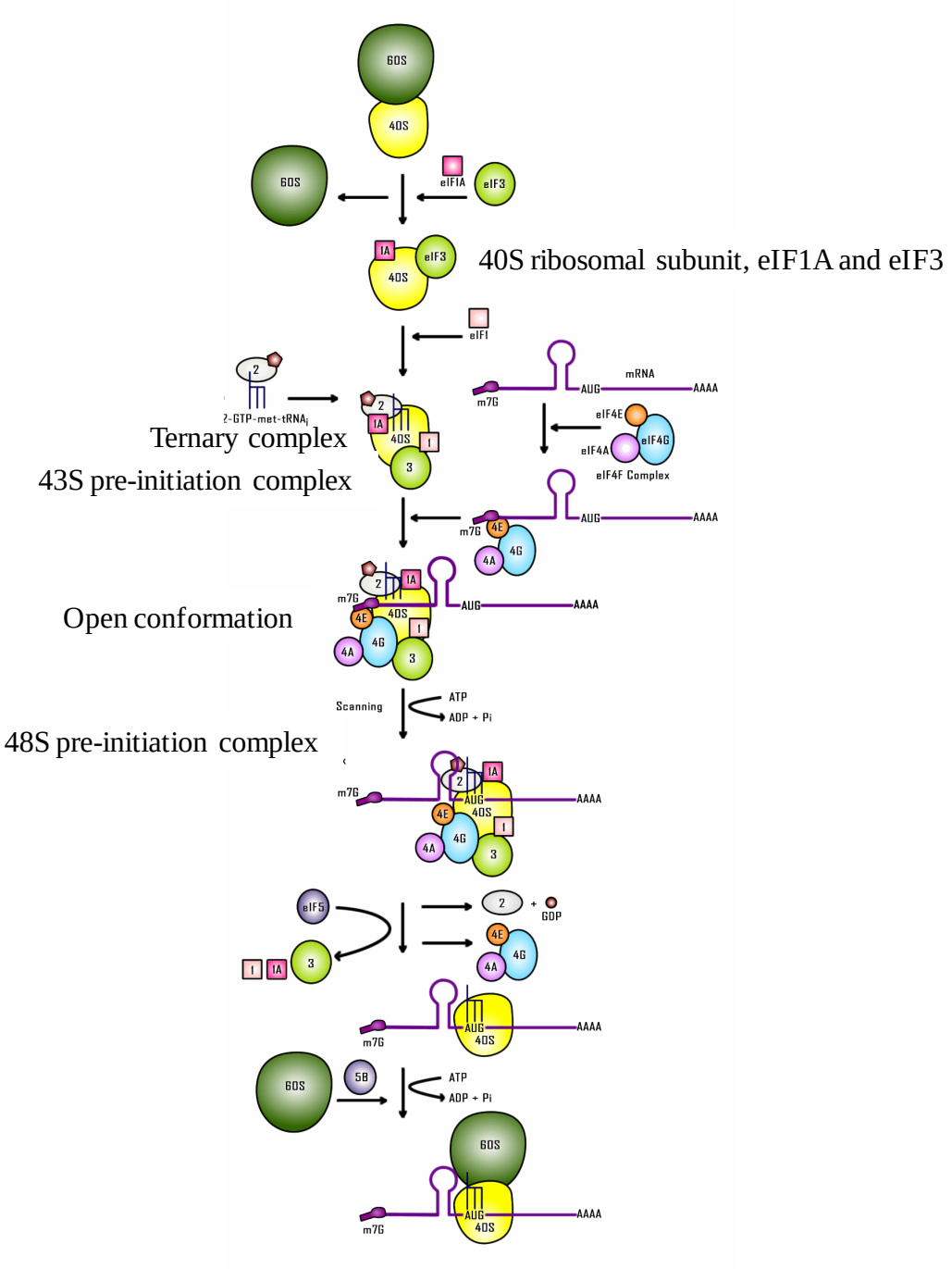


Figure 1.1: Schematic representation of cap-dependent translation initiation. met-tRNAi-eIF2-GTP form the ternary complex, and this complex interacts with the 40S ribosomal subunit (associated with eIF3, eIF1, and eIF1A) to form the 43S pre-initiation complex. The mRNA associated with the eIF4F complex interacts with the 43S pre-initiation

complex. The complex scans mRNA in the 5' to 3' direction, and when met-tRNAi is delivered into the P site of the ribosome, it is called the 48S pre-initiation complex. Following this event, eIF5 induces GTP hydrolysis by eIF2. Upon the GTP hydrolysis, eIFs that are associated with the complex is released. This then leads to the joining of the 60S ribosomal subunit to the 40S ribosomal to initiate translation. The figure was adapted from Sharma D.K *et al*, Role of eukaryotic initiation factors during cellular stress and cancer progression, *Journal of Nucleic Acids* (2016).

1.1.2 Cap-independent translation initiation

During stress conditions, eIFs required for cap-dependent translation can undergo post-translational modification or proteolytic cleavage. As a result of the cleavage, they are unavailable to form an active initiation complex and hence attenuate canonical translation initiation (Komar & Hatzoglou, 2011; Marissen, Gradi, Sonenberg, & Lloyd, 2000). In such conditions, the cell switches from the cap-dependent to a cap-independent mode of translation initiation. A cap-independent mechanism of translation initiation can utilize a subset of eIFs and IRES *trans*-acting factors (ITAFs) to recruit the ribosome and to form an active initiation complex (Komar & Hatzoglou, 2011).

During non-canonical translation, the mRNA sequence and/or structure is recognized by a subset of eIFs and ITAFs. The interaction of the subset of eIFs and ITAFs with the mRNA helps in the ribosome recruitment without the involvement of 5' cap structure. Such RNA structure and sequences are termed as *cis*-acting elements. Some of the well-characterized *cis*-acting elements are internal ribosome entry site (IRES), upstream open reading frames and cap-independent translation enhancers (Komar & Hatzoglou, 2011; Morris & Geballe, 2000; Terenin, Andreev, Dmitriev, & Shatsky, 2013). Of the different types of non-canonical translation mechanisms, IRES-mediated translation is well characterized and is explained in the later part of this section.

1.1.2.1 Significance of cap-independent translation

During physiological stress conditions such as hypoxia, DNA damage, nutritional deprivation or viral infection, downstream cellular pathways are activated. Stress inducers can be an external factor or internally induced in the cell (Fulda, Gorman, Hori, & Samali, 2010). Nutritional deprivation, hypoxia (oxygen deficiency), and viral infection are categorized as external inducers. On the other hand, DNA damage, imbalance in calcium homeostasis, and increase in reactive oxygen species are classified as internal inducers (Krampe & Al-Rubeai, 2010; Liang et al., 2017; Pugh, Gleadle, & Maxwell, 2001; Xu, Bailly-Maitre, & Reed, 2005). In presence of the internal stress inducers or external stress inducers, downstream cellular pathways are activated. For example, when DNA is damaged due to the stress inducers (e.g. oxidative stress or DNA damaging agent), cellular DNA damage response is activated (S. P. Jackson & Bartek, 2009). As a result of this response, an increased expression of the p53 protein and its downstream target p21 is observed. Increased levels of the p53 and p21 proteins promote cell cycle arrest (Benchimol, 2001). The cell cycle arrest aids the DNA repair mechanism (Branzei & Foiani, 2008). In case of a failure in the DNA repair mechanism, p53 activates the gene expression of proteins such as Bax and PUMA to promote cell death (Benchimol, 2001). However, the function of p53 can be inhibited by Murine double minute 2 (MDM2) to promote cell survival (Nag, Qin, Srivenugopal, Wang, & Zhang, 2013). Thus, the determining factor of cell status is the dominating active protein pool of the cell. If the proteins that are a part of a pro-survival pathway are predominantly expressed, the cell survives or continues to proliferate. If not, the cell is inclined towards the cell death mechanism. Hence, regulation of protein translation plays a critical role in deciding the cell fate during stress conditions. Interestingly, during stress conditions, the cap-

dependent translation is compromised by cleavage or unavailability of eIFs (Choo, Yoon, Kim, Roux, & Blenis, 2008; Haghighat et al., 1996; Showkat, Beigh, & Andrabi, 2014).

As mentioned later in the sections 1.1.2.2 and 1.4, when the cell death pathways are activated, it affects the eIF4F cap-binding complex which is crucial for canonical translation. In the absence of a mitogenic stimulation, eIF4E-binding protein (4E-BP) interacts with eIF4E and inhibits its association with eIF4G, thus interrupting the cap-binding complex formation (Choo et al., 2008; Showkat et al., 2014). On the other hand, when a pro-survival/pro-growth pathway such as the mammalian target of rapamycin (mTOR) is activated it results in phosphorylation of 4E-BP and dissociation of 4E-BP from eIF4E to promote cap binding activity (Choo et al., 2008; Showkat et al., 2014). Thus, these data suggest that external stimuli leading to hyperphosphorylation or hypophosphorylation of 4E-BP regulate the efficiency of cap-dependent translation. During physiological stress conditions such as DNA damage or hypoxia, programmed cell death is induced and as a result, cysteine-aspartic proteases (caspases) are activated. Caspases are endoproteases designed to systematically cleave downstream target proteins (McIlwain, Berger, & Mak, 2013). Stress-activated executioner caspases cleave eIF4G by various mechanisms to inhibit canonical (cap-dependent) translation initiation. Moreover, global translation can also be inhibited by eIF2α phosphorylation. During viral infection, amino acid starvation, nutritional deprivation, and endoplasmic reticulum (ER) stress conditions, protein kinases such as heme-regulated inhibitor kinase, general control nonderepressible 2, protein kinase R (PKR), or PKR-like ER kinase (PERK) are activated. Any of these activated kinases can phosphorylate eIF2α subunit to decrease the availability of the ternary complex and attenuate canonical translation (Clemens, 2001;

Komar & Hatzoglou, 2011; Rojas, Arias, & Lopez, 2010; R. Yang, Wek, & Wek, 2000). In such conditions, when the cap-dependent translation is compromised, the cell transitions from cap-dependent translation to a cap-independent mode of translation to synthesize a subset of proteins (Lacerda, Menezes, & Romao, 2017). Many of the cell fate regulating mRNAs that code for growth factors, oncogenes, RNA-binding proteins, heat shock proteins, tumor suppressors, cyclins, translation factors and apoptosis-related genes can be translated by a cap-independent mechanism (Lacerda et al., 2017). For example, in response to genotoxic stress, the level of a tumor suppressor protein p53 is increased (Walters & Thompson, 2016). The increase in p53 protein level is attributed to the increased protein stability and p53 mRNA translation (Walters & Thompson, 2016). Also, many of the p53 isoforms that have a pro-apoptotic role are translated by a cap-independent mechanism (Ray, Grover, & Das, 2006; Walters & Thompson, 2016). This is also demonstrated by point mutation in the IRES element that decreased p53 expression in comparison to wild-type in the presence of a DNA damaging agent (Grover, Sharathchandra, Ponnuswamy, Khan, & Das, 2011). This mechanism of translation is fairly common as many mRNAs coding for proteins such as c-Jun, B-cell lymphoma extra-large (Bcl-xL), X-linked inhibitor of apoptosis (XIAP), and cellular inhibitor of apoptosis protein 1 (cIAP1) utilize an IRES element in the 5’ UTR for translation initiation (Graber, Baird, Kao, Mathews, & Holcik, 2010; Holcik & Korneluk, 2000; Sherrill, Byrd, Van Eden, & Lloyd, 2004; Walters & Thompson, 2016). The oncoprotein c-Jun is known to function as a transcription factor by affecting the expression of cell cycle regulators and tumor suppressors. c-Jun protein levels are increased in cancer cells without any evidence of a corresponding increase in transcription of c-Jun mRNA. Hence, the increased protein levels are correlated to an increase in IRES-mediated translation of

c-Jun mRNA (Walters & Thompson, 2016). Moreover, IRES-mediated translation of anti-apoptotic proteins such as Bcl-xL, XIAP, and cIAP1 is responsible for the decreased apoptotic cell death (Graber et al., 2010; Holcik & Korneluk, 2000; Sherrill et al., 2004). These examples further highlight the importance of cap-independent translation or IRES-mediated translation during stress conditions and apoptosis. In general, the cap-dependent translation is more efficient than a cap-independent translation (Thompson, 2012). However, IRES-mediated translation has an advantage during stress conditions (Thompson, 2012).

1.1.2.2 Discovery and classification of IRESes

IRESes were initially discovered in viruses that are a part of the family picornaviridae (Martinez-Salas, Francisco-Velilla, Fernandez-Chamorro, Lozano, & Diaz-Toledano, 2015; Pelletier & Sonenberg, 1988). Poliovirus and foot-and-mouth disease virus belong to this family. The genomes of the latter two viruses encode proteases that cleave eIF4G upon infection (Belsham, McInerney, & Ross-Smith, 2000; Novoa & Carrasco, 1999). They also increase 4E-BP activity upon infection resulting in enhanced 4E-BP and eIF4E association and inhibition of eIF4F complex formation (Gingras, Svitkin, Belsham, Pause, & Sonenberg, 1996). As a result, translation of cellular mRNAs is affected, but not the viral mRNA translation. These observations where the viruses utilized the eukaryotic translation machinery in the absence of cap-binding complex led to the discovery of the IRES-mediated translation initiation of the viral genome (Hellen & Sarnow, 2001; Pelletier & Sonenberg, 1988). Following this, IRES elements were identified in cellular mRNAs. mRNA coding for immunoglobulin heavy chain binding protein was the first cellular IRES to be discovered (Hellen & Sarnow, 2001; Sarnow, 1989). After this discovery, extensive studies have been conducted to identify the eukaryotic mRNAs

containing IRES elements. Recent studies using a high throughput bicistronic assay indicated that about 10% of the total eukaryotic cellular mRNAs do have IRES elements (Weingarten-Gabbay et al., 2016).

Based on the RNA sequence and structural similarities, viral IRESes are classified into four types (Hellen & Sarnow, 2001; R. J. Jackson et al., 2010). IRES elements present in the picornavirus mRNA are classified into type I IRES (e.g. poliovirus) or type II IRES (e.g. encephalomyocarditis virus). Hepatitis C virus (HCV) like IRES element and similar IRES elements are classified into type III IRES. Lastly, dicistrovirus intergenic regions IRES elements (e.g. cricket paralysis virus) are classified as type IV IRES (Gross et al., 2017; Jaafar, Oguro, Nakamura, & Kieft, 2016; R. J. Jackson et al., 2010; Martinez-Salas, Pacheco, Serrano, & Fernandez, 2008; Sweeney, Abaeva, Pestova, & Hellen, 2014). Type I and type II IRESes elements do not require eIF4E but do require eIF4G and eIF4A for translation initiation (Martinez-Salas et al., 2008). The picornaviruses code for proteases that cleave eIF4G and utilize the cleaved part of eIF4G for translation initiation (Hellen & Sarnow, 2001). Type III or HCV-like IRESes can directly recruit the ribosome onto an mRNA without the involvement of eIF4F, eIF4B, eIF1, and eIF1A. In type IV IRESes, initiation can occur in the absence of all eIFs and initiator tRNA (Hellen & Sarnow, 2001; R. J. Jackson et al., 2010). Contrarily to viral IRESes, cellular IRESes do not display structural or sequence similarities and hence are grouped into a separate class of IRESes called cellular IRESes (R. J. Jackson et al., 2010).

1.1.2.3 Mechanism of IRES-mediated translation

The detailed mechanism of IRES-mediated translation remains unclear. As mentioned earlier, cellular IRESes do not have a structural similarity making it difficult to deduce a common mechanism of translation initiation for all mammalian IRESes (Hellen &

Sarnow, 2001; R. J. Jackson et al., 2010). Some of the cellular IRESes have been found to have a short nine-nucleotide Shine-Dalgarno-like sequence. This sequence is complementary to 18S rRNA and promotes ribosome loading onto the mRNA (Komar & Hatzoglou, 2011). Another possible mechanism is that the ribosomes land in the vicinity of the start codon and then scan down to recognize the first AUG sequence. This is called the 'land and scan' mechanism and is mostly observed in picornaviruses (Komar & Hatzoglou, 2011; Spriggs et al., 2009). In both these mechanisms, IRESes do not require an eIF4F complex or the entire repertoire of eIFs to recruit the ribosome.

Chemical inhibitors and siRNA-mediated knockdown studies have demonstrated that cellular IRESes can initiate translation in the absence of eIF4E or full-length eIF4G, but do require eIF4A of the eIF4F complex (Komar & Hatzoglou, 2011; Tsai et al., 2014). The requirement for eIF4A can be attributed to eIF4A helicase activity required to unwind the secondary structure of the IRESes and allow scanning of mRNAs by the ribosome (Parsyan et al., 2011). eIF3 can also interact with the cellular IRESes in a conformation-specific manner to directly recruit the ribosome onto the 5' UTR of the mRNA (Thakor et al., 2017). In stress conditions, IRES-mediated translation is not affected by eIF2α phosphorylation as IRES-mediated translation can utilize eIF5B as an alternative. eIF5B is an ortholog of prokaryotic initiation factor 2 and it delivers initiator tRNA into the P site of ribosome without the involvement of the ternary complex (Komar & Hatzoglou, 2011). In general, cellular IRESes harness only a subset of eIFs to recruit ribosomes onto the mRNA and tRNAi into the ribosome and initiate translation in a cap-independent fashion.

Besides eIFs, IRES-mediated translation initiation is affected by ITAFs such as poly-pyrimidine tract binding protein (PTB), heterogeneous ribonucleoproteins (hnRNPs),

programmed cell death 4 protein (PDCD4), human antigen R (HuR) and La autoantigen (Hellen & Sarnow, 2001; Komar & Hatzoglou, 2011). Each ITAF has a characteristic role to playand can positively or negatively regulate translation. The possible modes of action of the ITAFs are i) modify mRNA structure on binding, ii) interact with ribosomes or other proteins, acting as molecular bridges and iii) function as molecular chaperones (Komar & Hatzoglou, 2011; Sharma, Bressler, Patel, Balasingam, & Thakor, 2016). These mechanisms can be elaborated with examples of human La, PDCD4, HuR, and MDM2 protein. Human La autoantigen acts as a molecular RNA chaperone, aiding in RNA folding to enhance IRES-mediated translation (Gao, Li, Zhu, & Jin, 2016; A. Kumar, Ray, & Das, 2013; Naeeni, Conte, & Bayfield, 2012). Similarly, HuR and MDM2 proteins are positive regulators of translation. The binding of HuR increases the stability of mRNA and thus enhances translation (Zhang et al., 2009). The interaction of HuR and MDM2 with XIAP mRNA promotes its IRES activity, while another protein, heterogeneous ribonucleoprotein A1 (hnRNPA1), interacts with the XIAP mRNA to inhibit its IRES activity (Gu et al., 2009; Roy et al., 2014; Zhang et al., 2009). Like hnRNPA1, PDCD4 is a negative regulator of XIAP IRES-mediated translation (Suzuki et al., 2008). PDCD4 sequesters eIF4A by directly interacting with eIF4A. This results in the displacement of eIF4A and ultimately inhibition of translation (Pelletier, Graff, Ruggero, & Sonenberg, 2015; Suzuki et al., 2008). PDCD4 can also directly interact with mRNAs such as XIAP and Bcl-xL to inhibit the translation initiation complex formation (Biyanee, Singh, & Klempnauer, 2015; Liwak et al., 2012). Interaction of XIAP mRNA with PDCD4 or hnRNPA1 inhibits its translation, while the interaction of XIAP mRNA with HuR or MDM2 enhances its IRES-mediated translation. This suggests that IRES elements can interact with more than one ITAFs and the interaction with each one of

them can have a different outcome with respect to the IRES-mediated translation. It is possible that these ITAFs compete with each other to bind to their target IRESes. Therefore, the levels and/or activity of ITAFs can influence the cap-independent mechanism of translation.

Non-canonical translation, although not dependent on all eIFs, is regulated by a subset of eIFs and ITAFs. Hence, the availability of eIFs and ITAFs determine the efficiency of IRES-mediated translation (Sharma et al., 2016). As IRES-mediated translation has an upper hand during stress, it can affect the types of proteins expressed in such condition and thus determine if the cell will survive or undergo apoptosis.

1.2 Apoptosis

Apoptosis is a systematic process of cell death that is also called programmed cell death. It is a process where a cell methodically undergoes death without any deleterious effect on the neighboring cells (Taylor, Cullen, & Martin, 2008). During this process, there are several morphological changes observed, such as shrinkage in cell size, tightly packed organelles, condensation of chromatin, blebbing of the plasma membrane and formation of apoptotic bodies. These apoptotic bodies are then engulfed by the immune cells without triggering an inflammatory response (Elmore, 2007). The apoptotic mechanisms are broadly classified into two pathways, the intrinsic apoptotic pathway, and extrinsic apoptotic pathway (Koff, Ramachandiran, & Bernal-Mizrachi, 2015). Additionally, there is the granzyme pathway that is induced by granzyme A or B. The intrinsic apoptotic pathway, extrinsic apoptotic pathway, and granzyme B pathway converge to activate caspases, while granzyme A functions *via* a caspase-independent mechanism (Elmore, 2007).

There are two main classes of caspases, initiator and executioner caspases. Initiator caspases are at the receiving end of the stress signals while executioner caspases are downstream molecules that cleave cellular proteins influencing cell survival (McIlwain et al., 2013). The caspases are present as inactive procaspases that are activated by cleavage (Y. Shi, 2004). The initiator procaspases are inactive monomers and upon activation, they form dimers (Y. Shi, 2004). The formation of dimers stimulates autocatalytic cleavage of caspases to form active initiator caspases. On the other hand, executioner caspases are present as homodimers with reduced activity (Y. Shi, 2004). The activated initiator caspases cleave executioner procaspases. As a result of the cleavage, the conformation of the executioner caspase's active sites are modified and the enzyme activity is enhanced (Y. Shi, 2004).

1.2.1 Extrinsic apoptosis pathway

In the extrinsic apoptotic pathway, death receptors, which are transmembrane proteins present on the plasma membrane, interact with their corresponding ligands to trigger apoptosis. Some of the examples of death receptors are Fas-R, TNFR1, TRAIL-R, CD95, Apo-3, and DR4/DR5; and their corresponding ligands are Fas-L, TNFα, TRAIL, CD95-L, Apo-3L, and TRAIL respectively (Eggert et al., 2002; Elmore, 2007; Fulda & Debatin, 2006; Ren et al., 2004). Interaction of TRAIL-R–TRAIL, CD95–CD95-L, and Fas-R–Fas-L stimulates Fas-associated death domain (FADD) recruitment to the cytosolic domain of the receptor (Elmore, 2007). FADD is an adaptor protein and is essential to transfer the signals from the death receptor to the initiator caspases (Elmore, 2007; Fulda & Debatin, 2006). In the case of the TNFR1–TNFα interaction, in addition to FADD, TRADD and RIP are recruited to the cytosolic domain of TNFR1 (Figure 1.2) (Elmore, 2007). Similar to FADD, TRADD and RIP are death receptor interacting proteins

essential to trigger apoptosis (Elmore, 2007). Upon FADD interaction with the death receptor, it forms a death-inducing signaling complex (DISC) along with procaspase 8/10 and leads to oligomerization and autolytic cleavage of procaspase 8/10 to active caspase 8/10 (Fulda & Debatin, 2006; J. Li & Yuan, 2008). Active caspases 8/10 then cleaves procaspases 3/7 to form active caspase 3/7, inducing apoptosis (Elmore, 2007).

1.2.2 Intrinsic apoptosis pathway

The intrinsic apoptosis pathway is also called the mitochondrial apoptosis pathway as it mainly depends on the release of pro-apoptotic molecules such as cytochrome c and second mitochondria-derived activator of caspase (smac) from the intermembrane space of mitochondria (Koff et al., 2015). Under sustained genotoxic stress conditions or nutrient deprivation, the mitochondrial membrane pores are opened (Elmore, 2007). As a result of the mitochondrial membrane depolarization, cytochrome c and smac proteins are released into the cytoplasm. The cytochrome c thus released forms an apoptosome complex by interacting with apoptotic protease activating factor 1 (Apaf1) and procaspase 9. The apoptosome leads to cleavage of procaspase 9 and formation of active caspase 9. Activated caspase 9 then activates the downstream effector caspases 3/6/7, which induce apoptosis (Elmore, 2007). The smac released from the mitochondria into the cytoplasm can interact with the inhibitor of apoptosis proteins (IAPs) such as XIAP. Due to the smac interaction with the IAPs, IAPs are unavailable to interact with caspases and inhibit caspase function. Therefore, in presence of smac, caspases are available to induce apoptosis in cells (Figure 1.2) (Chai et al., 2000). In general, the mitochondrial membrane integrity plays a critical role in the intrinsic apoptosis pathway. The integrity of the mitochondrial membrane is modulated by the presence and absence of anti-apoptotic

proteins and pro-apoptotic proteins as well as receptor-mediated signaling (Elmore, 2007).

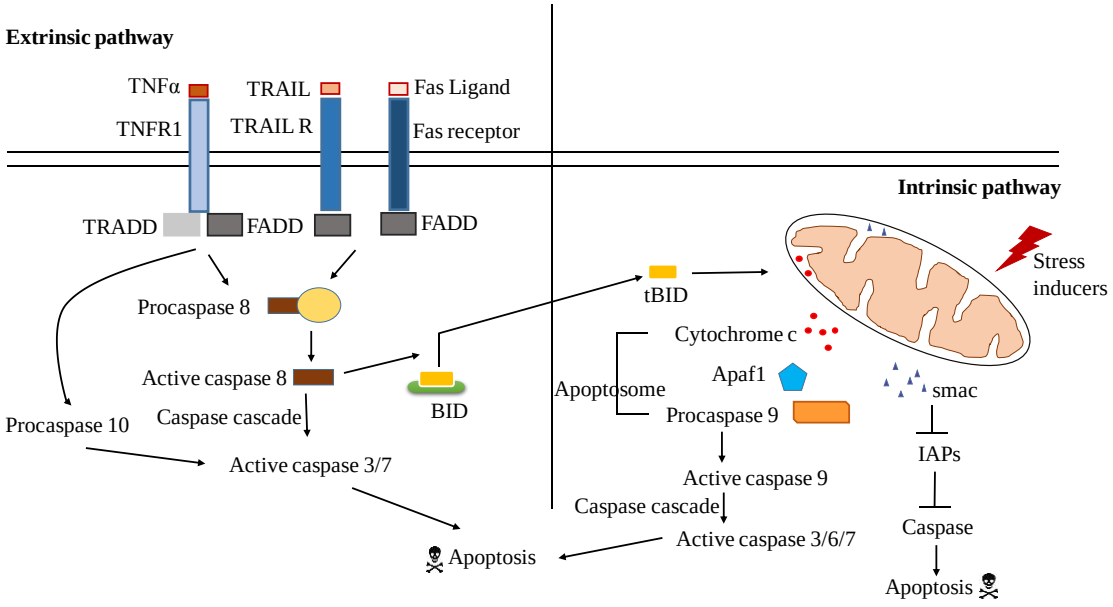


Figure 1.2: Schematic representation of the extrinsic and intrinsic pathways of apoptosis. i) Extrinsic pathway: In presence of extracellular inducers (ligands), TRADD and FADD are localized to the cytosolic domain of the death receptor. This leads to the formation of DISC and activation of procaspase 8/10. Activated caspase 8/10 triggers the caspase cascade; as a result, caspase3/7 is activated and induces apoptosis. ii) Intrinsic pathway: Stress-induced internal factors alter the membrane integrity of mitochondria that causes the release of cytochrome c and smac. Cytochrome c interacts with Apaf1 and procaspase 9 to form the apoptosome. The formation of the apoptosome leads to the activation of caspase 9.This then triggers the caspase cascade resulting in active caspase 3/6/7 and execution of apoptosis.

1.2.3 Anti-and pro-apoptotic proteins

Proteins that inhibit apoptosis are called anti-apoptotic proteins. The Bcl-2 family of proteins and FADD-like IL-1β-converting enzyme-inhibitory protein (c-FLIP) are examples of anti-apoptotic proteins (Elmore, 2007; Safa, 2013). Overexpression of Bcl-xL, which is a member of Bcl-2 family of proteins, inhibits the opening of mitochondrial membrane pore and prevents the release of cytochrome c. The inhibition of cytochrome c release inhibits apoptosome formation and downstream induction of apoptosis. Hence

Bcl-xL inhibits the intrinsic apoptotic pathway (Carthy et al., 2003). cFLIP has different splice variants such as long (c-FLIPL), short (c-FLIPS), and c-FLIPR (Ewald et al., 2011). These variants of cFLIP are dysregulated in cancer (Gaidos, Panaitiu, Guo, Pellegrini, & Mierke, 2015; Safa & Pollok, 2011). cFLIP interacts with FADD and inhibits activation of caspases 8 and 10. As a result of caspase 8 and 10 inhibition, the extrinsic pathway of apoptosis is inhibited (Safa, 2013).

In addition to the Bcl-2 family of proteins and c-FLIP, there is a sub-group of anti-apoptotic proteins called IAPs. IAPs were initially discovered in baculoviruses (Owens, Gilmore, Streuli, & Foster, 2013). In humans, the IAPs are classified into 8 groups based on the conserved baculovirus IAP repeat (BIR) domain(s) and a zinc-binding domain (Oberoi-Khanuja, Murali, & Rajalingam, 2013). The protein must contain a BIR domain to be grouped under IAPs, but not all proteins with a BIR domain are inhibitors of apoptosis proteins (Schimmer, 2004). Besides the BIR domain/s and a zinc-binding domain, IAPs can also have an ubiquitin-associated domain (UBA), a really interesting new gene (RING) domain or a caspase activation recruitment (CARD) domain (Schimmer, 2004; Silke & Meier, 2013). Furthermore, IAPs are characterized by their ability to bind with caspases and to inhibit their activity (Schimmer, 2004). Due to this function of inhibiting caspase activity, IAPs are regarded as crucial factors in deciding cell fate during apoptosis. Other than apoptosis, IAP-IAP complexes facilitate cell differentiation, inflammation, cell migration and oncogenesis (Oberoi-Khanuja et al., 2013). cIAP1, cIAP2, survivin, XIAP, BRUCE/Apollon, and Livin are some of the well-characterized IAPs (Owens et al., 2013; Silke & Meier, 2013). cIAP1 and cIAP2 interact with TNF receptor-associated factors 1 and 2 and inhibit the extrinsic pathway of apoptosis (Guicciardi et al., 2011; Labbe, McIntire, Doiron, Leblanc, & Saleh, 2011).

Livin interacts with active caspase 3/7 and inhibits both the extrinsic and the intrinsic pathways of apoptosis (Kasof & Gomes, 2001). XIAP belongs to the BIRC4 class of IAPs. XIAP consists of 3 BIR domains and a RING domain. XIAP interacts with caspases 3, 7, and 9 *via* the BIR domain and inhibits caspase activity. The RING domain is important for ubiquitination of its substrate such as caspase 7 as well as autoubiquitination (Creagh, Murphy, Duriez, Duckett, & Martin, 2004; Deveraux & Reed, 1999; Oberoi-Khanuja et al., 2013). Ubiquitination of the substrate, promotes proteasomal degradation of the substrate. Autoubiquitination leads to proteasomal degradation of XIAP and hence the level of XIAP protein can be regulated by autoubiquitination (Galban & Duckett, 2010). To summarize, IAPs interact with initiator and executioner caspases and inhibit caspase-dependent apoptosis by occluding caspases or by ubiquitinating them.

In contrast to anti-apoptotic proteins, pro-apoptotic proteins are positive regulators of apoptosis. PDCD4 and a subset of Bcl-2 family proteins (BID, Bax, and Bak) are examples of pro-apoptotic proteins (Kluck et al., 1999; Tsujimoto, 1998; Wigington et al., 2015; Zhen et al., 2013). BID and Bax interact with the outer membrane of mitochondria, open the mitochondrial membrane pore and facilitate the release of cytochrome c (Kluck et al., 1999). There are also reports suggesting internalization of BID and Bax from the cytoplasm into mitochondria to interact with the adenine nucleotide translocator present on inner mitochondrial membrane to release cytochrome c (Kluck et al., 1999). Upon TNFα-mediated activation of apoptosis, cytosolic Bid is cleaved to truncated BID (tBID). tBID induces cytochrome c release by inducing the oligomerization of Bax or Bak (Grinberg et al., 2002; Sutton et al., 2000). Besides TNFα, other receptor-mediated

activation of apoptosis and granzyme B pathway can trigger the formation of tBID to promote the release of cytochrome c (H. Li, Zhu, Xu, & Yuan, 1998). In general, the activity and levels of anti-apoptotic and pro-apoptotic proteins regulate the mechanism of cell survival. The synthesis and/or the activity of anti-apoptotic and pro-apoptotic proteins can be regulated by pathways such as mTOR pathway.

1.3 mTOR pathway

mTOR is a serine/threonine kinase that is a part of the phosphatidylinositol 3-kinase (PI3K) pathway. mTOR interacts with several other proteins to form two types of complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Zarogoulidis et al., 2014). mTORC1 and mTORC2 are extensively studied for their role in cell growth as they converge external signals such as growth factors, nutrients, and oxygen towards catabolic processes in cells (Laplante & Sabatini, 2012). mTORC2 plays a role in cell survival and cytoskeletal organization while mTORC1 influences protein translation, energy metabolism, lipid biosynthesis and autophagy (Laplante & Sabatini, 2012). Due to the relevance of mTORC1 in the regulation of protein translation, I have focused on mTORC1 and not mTORC2 in this section.

mTORC1 is composed of six different proteins namely mTOR, a regulatory associated protein of mTOR (raptor), proline-rich Akt substrate 40 kDa (pras40), DEP domain containing mTOR-interacting protein (deptor), mammalian lethal with sec-13 (mLST8), and the Tti1/Tel2 complex (Laplante & Sabatini, 2012; Zarogoulidis et al., 2014). Each of these six proteins has a distinct function as a part of the complex and mTOR is the core of the complex. Deptor and pras40 inhibit the function of mTOR. Raptor and Tti1/Tel2 complex act as scaffold proteins and influence the substrate binding (Laplante & Sabatini,

2012). mLST8 is essential for the kinase activity of mTOR (Kakumoto, Ikeda, Okada, Morii, & Oneyama, 2015). Functionally, mTORC1 is regulated by upstream positive and negative regulators. Events leading to the phosphorylation of mTORC1 activate the complex, and conversely, dephosphorylation events inhibit the activity of mTORC1. mTORC1 is negatively regulated by phosphatases like PTEN and positively by kinases like AKT. AKT phosphorylates pras40 and TCS2 and relieves mTORC1 from the inhibitory effect of these proteins (Hay & Sonenberg, 2004; Porta, Paglino, & Mosca, 2014; M. S. Song, Salmena, & Pandolfi, 2012). TSC1 and 2 are present upstream to mTORC1. Many of the external or internal signals phosphorylate or dephosphorylate TSC1 and TSC2 to regulate mTORC1 activity (Laplante & Sabatini, 2012). Upon mTORC1 phosphorylation, it hyperphosphorylates 4E-BPs, p70 ribosomal proteinS6 kinase 1 (S6K1) and S6K2 and maf1 (Laplante & Sabatini, 2013). Phosphorylation of 4E-BP and S6K1 and 2 directly enhance translation, while phosphorylation of maf1 promotes transcription of tRNAs and rRNA genes required for translation (Laplante & Sabatini, 2012). Maf1 is a repressor of RNA polymerase III. Phosphorylation of maf1 by mTORC1 relives RNA polymerase III from maf1 repression and promotes ribosome biogenesis and tRNA synthesis (Laplante & Sabatini, 2012). In particular, S6K1 and 2 have several downstream targets that can influence apoptosis by regulating translation.

1.3.1 S6K

S6K1 and 2 are serine-threonine kinases (Tavares et al., 2015). S6K1 and S6K2 share an 83% homology in the kinase domain and hence S6K2 was considered as a redundant protein in mammals. Extensive research to study the differences between S6K1 and S6K2 indicated that they do have similarities as well as distinct roles to play in cells (Karlsson

et al., 2015; Pardo & Seckl, 2013; Tavares et al., 2015). S6K1 and 2 function by phosphorylating and modifying the function of the substrate such as PDCD4 and eIF3 (Liwak et al., 2013; Martineau et al., 2014). S6K1 and 2 contain nuclear localization signals and can shuttle between the cytoplasm and nucleus (Pardo & Seckl, 2013). p85S6K1 and p70S6K1 are the two isoforms of S6K1. p85S6K1 includes an additional 23 amino acids in the N-terminus in comparison to p70S6K1. This additional sequence of amino acids is considered to contain the nuclear localization signal, making p85S6K1 a nuclear protein and p70S6K1 a cytoplasmic protein (Kim, Akcakanat, Singh, Sharma, & Meric-Bernstam, 2009). S6K1 targets the nuclear pool of ribosomal protein S6 and transcription factor cAMP-response element modulator in the nucleus, and to modulate the translational and transcriptional efficiency of cells (Edelmann, Kuhne, Petritsch, & Ballou, 1996).

Like S6K1, S6K2 has two isoforms called p56S6K2 and p54S6K2. p56S6K2 contains two nuclear localization signals and has been found to be localized in the nucleus (Pardo & Seckl, 2013). The second isoform, p54S6K2, is a shorter isoform and is found to shuttle between the nucleus and the cytoplasm (Pardo & Seckl, 2013). The translocation of the S6K2 protein is observed in the presence of growth factors (Pardo & Seckl, 2013).

In presence of nutrition or growth signals, S6K1 and 2 phosphorylate S6 protein and enhance canonical and non-canonical translation initiation. Besides S6, S6K1 and 2 can also phosphorylate PDCD4 and eIF3 subunits to regulate translation initiation as mentioned below in section 1.3.2 (Martineau et al., 2014; Pardo & Seckl, 2013).

1.3.2 Regulation of protein translation by S6K

In the presence of mitogens, mTORC1 is activated and this, in turn, activates p70S6K1 (Fang, Meng, Vogt, Zhang, & Jiang, 2006). Activated p70S6K1 phosphorylates eIF4B, eIF3, eukaryotic elongation factor 2 kinase and PDCD4 to enhance translation (Dennis, Jefferson, & Kimball, 2012; Martineau et al., 2014). With the focal point being translation initiation, I have only explained the S6K-mediated regulation of eIF4B, eIF3 and PDCD4 and not elongation factor 2 kinase. eIF4B is a eukaryotic initiation factor involved in the cap-dependent translation (Lindqvist, Imataka, & Pelletier, 2008). eIF4B acts as a co-factor important for the helicase activity of eIF4A and increases mRNA translation (Shahbazian et al., 2006). eIF4B consists of two RNA binding domains and a protein binding domain. The RNA binding domain is essential for mRNA and rRNA binding, and thus forms a bridge between the mRNA and ribosome (van Gorp et al., 2009). The protein binding domain interacts with eIF3, aiding initiation complex formation (van Gorp et al., 2009). In the presence of serum or mitogens, eIF4B is phosphorylated on Serine-422 by p70S6K1 (Kuang, Fu, Liang, Myoung, & Zhu, 2011; Mendoza, Er, & Blenis, 2011). FLAG-immunoprecipitation assays have demonstrated that phosphorylation of eIF4B augments the eIF4B and eIF3A subunit interaction (Shahbazian et al., 2006). Mutations impairing the phosphorylation of eIF4B have a negative effect on translation initiation (van Gorp et al., 2009). Thus, p70S6K1-mediated phosphorylation of eIF4B has an effect on general cap-dependent translation and cell proliferation. The effect on cell proliferation could be due to the requirement for eIF4B for the mRNA translation of pro-survival mRNAs such as Bcl-2 and XIAP (Shahbazian et al., 2006).

eIF3 is an 800 kDa multi-subunit complex consisting of 13 subunits. The subunits are non-identical and denoted by letters A to M. Each subunit has a designated role in translation initiation (Hinnebusch, 2006). eIF3A, eIF3C, eIF3E, eIF3F, eIF3H, eIF3K, eIF3L and eIF3M form the structural core in human eIF3 (Cate, 2017). eIF3A, eIF3B, eIF3C and eIF3J play roles in 40S binding; eIF3A, eIF3B and eIF3C in mRNA recruitment; eIF3B is critical for mRNA scanning and eIF3C for AUG recognition. eIF3A, eIF3B, and eIF3C help in the recruitment of ternary complex to the 40S subunit to form the 43S pre-initiation complex (Hinnebusch, 2006). Many of the eIF3 subunits undergo post-translational modifications that promote their interactions with other proteins. eIF3 subunits contain multiple phosphorylation sites that are regulated by mTOR-dependent and mTOR-independent signaling pathways (Martineau et al., 2014; Miyamoto, Patel, & Hershey, 2005). Inactive S6K1 remains associated with the eIF3 complex. mTORC1 activation phosphorylates S6K1 and abrogates the eIF3-S6K1 interaction (Ma & Blenis, 2009). Activated S6K1 phosphorylates multiple proteins including eIF3 subunits. S6K1 directly interacts with eIF3F and phosphorylates eIF3G to regulate translation (Martineau et al., 2014). Phosphorylation of eIF3G by S6K1 promotes the eIF3-polyadenylate-binding protein-interacting protein 1 (PAIP1) interaction (Martineau et al., 2014). Under amino acid starvation condition or treatment with mTOR inhibitors, the interaction between eIF3 and PAIP1 is interrupted (Martineau et al., 2014). This suggests the requirement for S6K1 for the interaction. The interaction between eIF3 and PAIP1 promotes mRNA circularization and, in turn, enhances translation (Martineau et al., 2014). Immunoprecipitation assays conducted using human embryonic kidney (HEK) cells have shown that S6K1 interacts with eIF3B, eIF3C, eIF3E and eIF3F, and

mTOR with eIF3C to regulate translation (Holz, Ballif, Gygi, & Blenis, 2005; Martineau et al., 2014).

S6K1 phosphorylates PDCD4 on the serine-67 residue, this phosphorylation triggers additional phosphorylation on the serine-71 and serine-76 residues of the protein (Dorrello et al., 2006; Magnuson, Ekim, & Fingar, 2012). Phosphorylated PDCD4 was co-immunoprecipitated with $SCF^{\beta TRCP1}$ and $SCF^{\beta TRCP2}$, suggesting that PDCD4 could be ubiquitinated by the ubiquitin ligase $SCF^{\beta TRC}$. In addition, PDCD4 contains a $SCF^{\beta TRC}$ binding domain, further supporting the possibility of PDCD4 ubiquitination by $SCF^{\beta TRC}$ (Dorrello et al., 2006). The interaction between PDCD4 and the ubiquitin ligase $SCF^{\beta TRC}$ is lost when similar immunoprecipitation assays were conducted with serine-71 and serine-76 mutants. Hence, it is important for all three phosphorylation reactions to occur on PDCD4 for the protein to be recognized by $SCF^{\beta TRC}$ and degraded (Dorrello et al., 2006; Magnuson et al., 2012). Downregulation of PDCD4 increases translation of a subset of mRNAs including XIAP and Bcl-xL mRNAs. As a result, the increase in translation of XIAP and Bcl-xL promotes cell survival (N. Kumar, Wethkamp, Waters, Carr, & Klempnauer, 2013; Liwak et al., 2012; Schmid et al., 2008).

Like S6K1, S6K2 phosphorylate S6, PDCD4, and eIF3 to have a similar effect on translation (Liwak et al., 2012; Martineau et al., 2014; Pardo & Seckl, 2013). S6K2 mostly affects the cap-independent translation of Bcl-xL, XIAP, and a subset of mRNAs consisting of an IRES element. S6K2 does not affect the cap-dependent translation of housekeeping genes. XIAP mRNA expression is also positively affected by the phosphorylation of PDCD4 mediated by S6K2 (Liwak et al., 2012; Pardo & Seckl, 2013). Hence, S6K1 and 2 mediated post-translational regulation of PDCD4 and eIF3 can

modulate both cap-dependent and cap-independent translation and therefore affect cell survival.

1.4 PDCD4

PDCD4 is found to have a reduced expression in many cancer types such as lung cancer, melanoma, and glioblastoma (Y. Chen et al., 2003; Liwak et al., 2013; Vikhreva & Korobko, 2014). The decrease in PDCD4 expression is due to epigenetic silencing, post-transcriptional and post-translational regulation (Liwak et al., 2013). Post-transcriptionally, miR-21 and miR-499 target the 3' UTR of PDCD4 mRNA and silence gene expression. The levels of miR-21 and miR-499 are inversely proportional to PDCD4 protein levels, emphasizing the antagonistic effect of these miRNAs on PDCD4 in cancer cells (Asangani et al., 2008; Zhang et al., 2016). As mentioned earlier, PDCD4 is post-translationally modified by S6K1 and 2. The phosphorylated form of PDCD4 protein is susceptible to ubiquitin-mediated degradation (Dorrello et al., 2006; Galan et al., 2014; Liwak et al., 2012). The loss of PDCD4 is correlated with increased cell proliferation and resistance to apoptosis (Vikhreva & Korobko, 2014). Due to its role in the enhancement of apoptosis and inhibition of cancer cell proliferation, PDCD4 is commonly known as a tumor suppressor protein (Z. Chen et al., 2015; Zhen et al., 2016).

PDCD4 contains a nuclear localization signal and can shuttle between the nucleus and the cytoplasm (Bohm et al., 2003). In the nucleus, it affects the function of transcription factors such as twist1 and c-Jun, and in the cytoplasm, it modulates the translation efficiency of specific mRNAs (Jo, Kim, Clocchiatti, & Dotto, 2016; N. Kumar et al., 2013; Liwak et al., 2012). Other than the nuclear localization signal, PDCD4 contains two MA3 binding domains and an RNA binding domain (Chang et al., 2009). It utilizes both

the MA3 binding domains to interact with eIF4A and to inhibit its helicase activity or to compete with eIF4G and prevent eIF4F complex formation. By inhibiting eIF4A helicase activity and eIF4F complex formation, PDCD4 abrogates cap-dependent translation (Chang et al., 2009). In addition to inhibiting cap-dependent translation, PDCD4 directly interacts with XIAP and the Bcl-xL IRESes *via* the RNA binding domain and inhibits 48S initiation complex formation (Liwak et al., 2012). As a result, during apoptosis, PDCD4 can potentially inhibit global as well as transcript-specific translation. In many cancers, such as glioblastoma and ovarian cancer, the PDCD4 expression is downregulated and as a consequence of this, the levels of anti-apoptotic proteins are increased, resulting in higher chemoresistance and cell migration (Liwak et al., 2013; Wei, Liu, Chan, & Ngan, 2012).

1.5 Role of eIF3 in translation and apoptosis

The expression of all 13 subunits in the eIF3 complex may not be dysregulated at the same time. Nevertheless, even variation in any one of these eIF3 subunits can have implications in cancer, apoptosis and cell growth (Sharma et al., 2016). eIF3A is upregulated in breast cancer and lung cancer (Dong & Zhang, 2006; Hershey, 2015). This, in turn, upregulates ribonucleotide reductase M2 during S phase of the cell cycle to promote DNA synthesis and malignancy (Sharma et al., 2016). Another subunit, eIF3C is upregulated in tumor cells to facilitate cell proliferation (N. Song, Wang, Gu, Chen, & Shi, 2013). This activity of eIF3C can be restricted by direct interaction with a tumor suppressor protein schwannomin (Scoles, Yong, Qin, Wawrowsky, & Pulst, 2006). Similarly to eIF3A and eIF3C subunits, eIF3D is upregulated in gastric and mesothelial tissue cancer. Knockdown of the eIF3D subunit in human colon cancer cells decreased

cell proliferation and colony formation, promoted activity of pro-apoptotic proteins such as Bad by phosphorylation, and cleaved PARP (Spilka, Ernst, Mehta, & Haybaeck, 2013; Yu, Zheng, & Chai, 2014). Besides, eIF3A, eIF3C and eIF3D, eIF3E is found to be dysregulated in many cancer types (Grzmil et al., 2010; Sesen et al., 2014). Partial silencing of eIF3E specifically inhibited the expression of a subset of mRNAs related to tumorigenesis but not a global translation (Grzmil et al., 2010). A reduction in eIF3E promoted epithelial-to-mesenchymal transition by stabilizing and increasing expression of epithelial-to-mesenchymal transition regulators such as Snail1 and Zeb2 (Gillis & Lewis, 2013). Also, a decrease in eIF3E further decreased Bcl-xL (anti-apoptotic protein) and urokinase-type plasminogen activator (activator of metastasis) (Grzmil et al., 2010; Sharma et al., 2016). Unlike other eIF3 subunits, eIF3F has been mostly shown to function as a tumor suppressor protein (Sharma et al., 2016). eIF3F stabilizes p53 (tumor suppressor protein), activates Bax, promotes rRNA degradation and inhibits tumor growth in mice (J. Y. Lee, Kim, Rho, & Lee, 2016). eIF3F sequesters hnRNP K. As a result, rRNA molecules are no longer protected by hnRNPK. The unprotected rRNAs become susceptible to nuclease degradation inhibiting protein synthesis (Wen et al., 2012). eIF3F is shown to affect both cap-dependent and cap-independent translation using luciferase reporter constructs in immortalized cell lines (Wen et al., 2012). Collectively, these data suggest that eIF3 plays a role in translating the mRNAs that influence apoptosis.

To summarize, eIF3 being a large multi-protein complex (Hershey, 2015), it is not clear if the subunits play a role independent of one another. The function of these subunits can be influenced by one another or other cellular proteins. Also, the interaction of eIF3 with one another and with other cellular protein may affect translation (Hershey, 2015; Martineau et al., 2014; Thakor et al., 2017).

1.6 Objectives

PDCD4 has been demonstrated to function as a transition inhibitor (Liwak et al., 2013; Liwak et al., 2012). As mentioned earlier, PDCD4 interacts with eIF4A and inhibits cap-dependent translation (Liwak et al., 2012). Besides the negative effect on cap-dependent translation, PDCD4 directly interacts with XIAP and Bcl-xL mRNAs to downregulate their IRES-mediated translation (Liwak et al., 2012). Besides these, our group has recently shown that the eIF3 complex directly interacts with XIAP mRNA to enhance its translation (Thakor et al., 2017). These observations suggest that PDCD4 binding negatively regulates XIAP translation, while eIF3 binding positively regulates the expression of the XIAP mRNA (Figure 1.3). Interestingly, both PDCD4 and eIF3 are regulated by a common mTOR/S6Ks pathway (Figure 1.3). S6K1 and 2, downstream kinases in the mTOR pathway, are known to phosphorylate PDCD4 leading to its degradation (Figure 1.3). S6K1 and 2 also phosphorylate eIF3 to enhance its role in translation (Figure 1.3). Therefore, I hypothesize that PDCD4 interacts with eIF3 to inhibit translation initiation, and that the interaction of eIF3 and PDCD4 is regulated via S6K1 and/or 2. To this end, I focused my study on the following objectives.

Objective i) To investigate if PDCD4 interacts with eIF3.

Objective ii) To demonstrate if the inhibition of S6K1 and 2 activation affects the interaction between PDCD4 and eIF3.

Objective iii) To study the effect of eIF3F and PDCD4 on cap-dependent translation in glioblastoma cell line (U343).

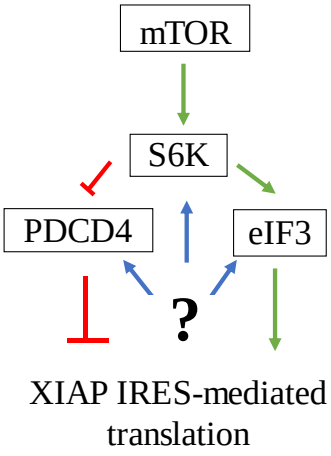


Figure 1.3: Schematic representation of S6K mediated translation regulation. mTOR activates S6K1 and 2. Activated S6K1 and 2 inhibits PDCD4 and enhances the function of the eIF3 complex. PDCD4 inhibits XIAP translation, while, eIF3 enhances XIAP translation.

eIF3 is a common factor required for both canonical and IRES-mediated translation initiation. Therefore, the interaction between PDCD4 and eIF3 can have an impact on cap-dependent and cap-independent translation. The results of the project will provide us further insights into the PDCD4-mediated regulation of translation during apoptosis.

Chapter 2

Materials and methods

2.1 Cell lines and constructs

Human glioblastoma cell line U343, S6K double knockout mouse embryonic fibroblast cells (MEFs) and wild-type MEFs were used for the *in cellulo* assays. Both MEFs lines were obtained from Dr. Tommy Alain, CHEO Research Institute, University of Ottawa. The plasmids used for expression of proteins and reporter constructs to monitor cap-dependent and cap-independent translation are tabulated below.

Sl. No	Construct	Application	Source	Reference
1	2G-T.f	Bacterial expression of GST-eIF3F	Dr. Jamie H. D. Cate, University of California, Berkeley.	(Sun et al., 2011)
2	His-PDCD4	Bacterial expression of His-PDCD4	Dr. Martin Holcik, CHEO Research Institute, University of Ottawa	(Liwak et al., 2012)
3	pcDNA3-PDCD4-FLAG	Mammalian expression of FLAG-PDCD4		
4	GST-PDCD4	Bacterial expression of GST-PDCD4		
5	pcDNA3-FLAG	Mammalian expression of FLAG-tag		
6	pRL-CMV	Monitor cap-dependent translation in mammalian cells	Promega	-
7	pGEX-4T1-GST	Bacterial expression of GST protein	Dr. Hans-Joachim Wieden, University of Lethbridge	-

Table 2.1: List of constructs, application of the constructs and their source.

2.2 Transfection and transformation

All mammalian cell lines were maintained in the Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone) supplemented with 10% v/v fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin. Before transfection, cells were trypsinized and counted using the trypan blue staining. $3X10^6$ cells were then seeded into a 100-mm tissue culture plate for transfection. 48 hours after seeding the cells, the plate was used for transfection. Mammalian cells were transiently transfected with the pcDNA3-PDCD4-FLAG and pcDNA3-FLAG plasmid for the expression of FLAG-PDCD4 and FLAG peptide respectively using Lipofectamine™ 2000 transfection reagent (ThermoFischer Scientific). The DNA, transfection reagent, and reduced-serum opti-MEM media were mixed in proportions as per the manufacturer's protocol and incubated at ambient temperature for 20 minutes. The transfection mix was then added into the 100-mm tissue culture dish containing a monolayer of mammalian cells. The cells were incubated in the 37 ^{0}C CO_2 incubator for 48 hours before lysis.

In case of siRNA and pRL-CMV transfection, the cells from a 100-mm tissue culture plate were trypsinized. The number of cells present in the suspension was counted by the trypan blue staining. $3X10^5$ cells from that cell suspension were then seeded into three wells of a 6-well plate. After seeding the cells, siPDCD4 (Dharmacon) and sieIF3F (Dharmacon) were transfected using Lipofectamine® RNAiMAX transfection reagent from Thermo FischerScientific. Transfection mix containing Lipofectamine® RNAiMAX transfection reagent, siRNA and opti-MEM media (Thermo FischerScientific) were mixed and incubated for 20 minutes at ambient temperature. The transfection mix was added into the tissue culture dish containing a mammalian cell suspension. The plate was incubated in the 37 ^{0}C CO_2 incubator for 24 hours for the monolayer formation and

depletion of the target mRNA. Post 24 hours, cells were transfected with the pRL-CMV construct. The pRL-CMV plasmid, Lipofectamine^{TM} 2000 transfection reagent, and reduced-serum opti-MEM media were mixed in proportions as per the manufacturer's protocol and incubated at an ambient temperature for 20 minutes. During the incubation period, cells in the 6-well plate were washed with opti-MEM and 750µL of opti-MEM was added into each well. After the incubation of the transfection mix, it was added to the opti-MEM media in the 6-well plate. The cells were incubated with the transfection mix for 4 hours and then the opti-MEM media was replaced with DMEM containing 10% v/v FBS. The cells were incubated for 24 hours before lysis.

Escherichia coli (*E. coli*) strains such as DH5α (NEB), BL21 DE3 (NEB) and BL21 ROSETTA 2 (DE3) PLYSS (Millipore Sigma) were used for transformation. DH5α was used for plasmid preparation, BL21 DE3 for PDCD4 and GST protein purification, and BL21 ROSETTA 2 (DE3) PLYSS for eIF3 subunit protein purification. 50ng of plasmid DNA was added to the 25µL aliquot of *E.coli* competent cells in a 1.5mL tube. Cells were incubated on ice for 45 minutes followed by a heat shock at 42 ^{0}C for 60 seconds. The cells were then placed on ice for 5 minutes and transferred into a culture tube containing 1mL LB broth. The culture tube was incubated in a 37 ^{0}C shaker for 30 minutes. The cells were then plated onto LB agar plates containing 100µg/mL final concentration of ampicillin and incubated overnight in a 37 ^{0}C incubator.

2.3 Plasmid purification

A single colony of *E. coli* containing pcDNA3-FLAG plasmid or pcDNA3-PDCD4-FLAG plasmid from the LB agar was inoculated into a 3mL LB broth containing 100µg/mL final concentration of ampicillin. The 3mL starter culture was incubated at 37 ^{0}C shaker for 10 hours. 1mL from the starter culture was then inoculated into 200mL

LB broth containing 100µg/mL final concentration of ampicillin. The 200mL culture was then incubated overnight at 37 ^{0}C with a constant shaking at 150 rpm. The overnight culture was used for the purification of the plasmid with the Qiagen plasmid maxiprep kit. The cells were harvested by centrifuging the 200mL culture at 6,000g for 15 minutes and the plasmid was purified as per the manufacturer's recommendation. The quality of the plasmid purified was analyzed on a 0.8% w/v agarose gel and quantified using the BioDrop spectrophotometer.

2.4 Optimization of protein purification

Various concentrations of IPTG were tested to optimize the induction of His-PDCD4, GST-eIF3F and GST proteins. To begin with, 3mL of the *E. coli* culture was grown to 0.6 OD and induced with a 0.1mM, 0.5mM, and 1mM final concentration of IPTG. The tubes were then incubated for 3 hours at 37 ^{0}C post-induction. 1mL of the culture was centrifuged and the pellet was re-suspended in 0.25mL of the lysis buffer used for recombinant protein purification. To the 0.25mL of the cell suspension, 60µL of 5X concentration SDS loading dye was added and incubated for 5 minutes at 98 ^{0}C. The samples were centrifuged at 10,000g for 5 minutes. 20µL of the supernatant was analyzed on a 10% w/v SDS-PAGE followed by Coomassie staining to detect the proteins.

To confirm His-PDCD4 and GST-eIF3F expression Western blot assay was performed. The samples were processed like in the case of Coomassie-stained gels but were diluted by 10-fold and 100-fold before loading in to the 10% w/v SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane by Western blotting. The nitrocellulose membrane was then stained using Ponceau Stain to ensure uniform protein loading in all

the lanes. The membrane was then de-stained by washing for 10 minutes with 1X concentration PBS solution. The membrane was then blocked with 10% w/v milk for 1 hour at ambient temperature and then probed with primary antibody (anti-eIF3F/anti-PDCD4) overnight at 4 ^{0}C. After the overnight incubation, the blot was washed for 10 minutes with 1X PBST (twice) and 10 minutes with 1X PBS (once) to remove the unbound primary antibody. The blot was then probed with anti-rabbit secondary antibody and visualized using an Amersham Imager 600.

2.5 Recombinant protein purification

A single colony of *E. coli* containing His-PDCD4, GST-eIF3F, or GST bacterial expression construct was inoculated into a 3mL LB broth (containing 100µg/mL final concentration of ampicillin) and incubated overnight at 37 ^{0}C. 1mL of the starter culture was inoculated into 200mL of LB broth containing ampicillin and incubated at 37 ^{0}C with constant shaking at 150 rpm. The culture was induced with 0.5mM IPTG at 0.6 OD. The culture was incubated for 3 hours after induction and then pelleted by centrifuging at 6,000g for 30 minutes. The pellet was suspended in 20mL of lysis buffer (50mM Tris-HCl pH 8, 1M NaCl, 5% v/v glycerol, 0.5mM PMSF, and 1mM β-mercaptoethanol) and then lysed using a French press. The lysate was centrifuged at 16,000g for 30 minutes and the supernatant was added into a 50mL tube containing 500µL of equilibrated Glutathione Sepharose 4 Fast Flow affinity medium (GE) or Ni-NTA agarose (Qiagen). The lysate was incubated with affinity matrix for 1 hour at 4 ^{0}C. The lysate was centrifuged at 1,000g for 2 minutes and the supernatant was discarded. The matrix was washed with 20mL of lysis buffer three times. In case of His-PDCD4 protein purification, 40mM of imidazole was added to the lysis buffer during the wash step. GST and GST-

tagged protein were eluted using 10mM glutathione in the lysis buffer. His-PDCD4 was eluted with 500mM imidazole in the lysis buffer. 20µL of each of the eluates were separated using SDS-PAGE and visualized by Coomassie staining. The eluates were pooled based on the purity and concentrated for *in vitro* assays.

2.6 Western blot assay

Cells were harvested in RIPA (50mM Tris-HCl pH 7.4, 1mM EDTA, 150mM NaCl, 1% v/v NP 40, 0.5% w/v deoxycholic acid, 0.05% w/v SDS, protease inhibitors and phosphatase inhibitors). The lysate was centrifuged at 10,000g for 10 minutes to remove the cell debris. The protein concentration of the supernatant was quantified by the Bradford assay, using the 1X Bradford assay reagent from BioRad. 5µL of the cell lysate was added into 500µL of the 1X Bradford assay reagent and incubated for 5 minutes at an ambient temperature. Likewise, 5µL of the protein standards ranging from 2mg/mL to 0.125mg/mL bovine serum albumin solution was added into 500µL of the 1X Bradford assay reagent and incubated for 5 minutes at an ambient temperature. The absorbance of the samples was measured at 595 nm using the BioTek® luminescence plate reader. The protein concentration of the cell lysate was deduced based on the absorbance values obtained for the protein standards. After protein quantification, 10ng of each lysate was analyzed on SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane, incubated with 10% w/v milk in 1X PBST (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, and 1% tween 20) for 1 hour followed by incubation with primary antibody overnight at 4 ^{0}C. The blots were then incubated with secondary antibody for one hour and visualized using an Amersham Imager 600. The primary and secondary antibodies were commercially available and the dilutions used were as per the manufacturer's recommendation. The list of antibodies used are, PDCD4 (Rockland),

eIF3 subunits (Abcam), S6 (CST), pS6 (CST), actin (Bio-Rad), secondary rabbit (Abcam), secondary mouse (CST) and conformation-specific secondary anti-rabbit (CST).

2.7 FLAG-immunoprecipitation

U343 cells were transfected with pcDNA3-FLAG or pcDNA3-PDCD4-FLAG plasmids. Post 48 hours of transfection the cells were treated with 3.4% v/v formaldehyde for 10 minutes followed by 10 minutes incubation with 0.02mM glycine to remove the excess formaldehyde. The cells were washed with PBS and then the cells were lysed in 1.1mL of RIPA buffer. The lysate was centrifuged at 10,000g for 10 minutes and the supernatant was transferred into a 1.5mL tube. 1mL of the lysate was added into a 1.5mL tube containing 40µL of equilibrated ANTI-FLAG® M2 Affinity Gel (Sigma). The lysate was incubated with the affinity gel in the cyclomixer for 4 hours at 4 ^{0}C. After incubation, the beads were isolated by centrifuging at 5,000g for 30 seconds. The matrix was washed with 500µL of 1X PBS four times. The proteins were eluted by addition of 40µL of 2X concentration Laemmli sample buffer (Bio-Rad) and heating for 10 minutes at 98 ^{0}C. The samples were analyzed using the Western blotting technique. 1% of the total cell lysate used for IP was loaded in the SDS-PAGE along with co-IP samples as input control.

2.8 FLAG-IP to detect RNA-independent protein-protein interaction

Two sets of U343 cells containing 100-mm tissue culture dish were transfected with the pcDNA3-FLAG or pcDNA3-PDCD4-FLAG plasmids. Each of the two sets had a 100-mm plate containing U343 transfected with the pcDNA3-FLAG and a 100-mm plate containing U343 transfected with the pcDNA3-PDCD4-FLAG plasmid. After 48 hours of transfection, set 1 was harvested in 1mL of the RIPA buffer supplemented with 250µg of RNaseA and set 2 was harvested in 1mL of the RIPA buffer supplemented with 200 units

of RNase inhibitor. The lysate was then centrifuged at 10,000g for 10 minutes at 4 ^{0}C to separate out the cell debris. Then the supernatant after the centrifugation was used to perform FLAG-IP in a similar fashion as mentioned in section 2.7.

2.9 eIF3F immunoprecipitation

40µL of protein G Dynabeads^{TM} (Invitrogen) was incubated with 1µg of eIF3F or PAIP1 (negative control) specific antibody for 20 minutes at room temperature, followed by 1 hour at 4 ^{0}C. Unbound antibody was washed with PBS and incubated with cell lysate prepared as mentioned above for FLAG-immunoprecipitation. The lysate and beads were incubated for 4 hours at 4 ^{0}C. Post incubation the beads were separated using a magnetic rack and washed with 500µL of 1XPBS. The wash step was repeated five times to avoid any false positive signals. Proteins bound to beads were eluted with 40µL of 2X concentration of Laemmli sample buffer and analyzed by Western blotting. 1% of the total lysate volume used for IP was used as input control during Western blot analysis.

2.10 *In vitro* protein pull-down assay

40µL of Glutathione Sepharose 4 Fast Flow affinity medium was equilibrated with TGEM (0.1) buffer (20mM Tris-HCl pH 7.9, 20% v/v glycerol, 1mM EDTA, 5mM $MgCl_2$, 0.1% v/v NP 40, 1mM DTT, 0.2mM PMSF, 0.1M NaCl). Purified recombinant proteins were quantified by the Bradford assay. The procedure for the Bradford assay was similar to the protocol mentioned in section 2.6. 500ng of the quantified bait protein (GST-eIF3F) was diluted to a final volume of 250µL in TGEM (0.1) buffer. The diluted sample was added to the equilibrated Glutathione Sepharose 4 Fast Flow affinity medium and incubated at 4 ^{0}C for 2 hours. The matrix was then washed once with ice cold TGEM (1.0) buffer (20mM Tris-HCl pH 7.9, 20% v/v glycerol, 1mM EDTA, 5mM $MgCl_2$, 0.1% v/v NP 40, 1mM DTT, 0.2mM PMSF, 1M

NaCl) and twice with ice cold TGMC (0.1) buffer (20mM Tris-HCl pH 7.9, 20% v/v glycerol, 5mM $CaCl_2$, 5mM $MgCl_2$, 0.1% v/v NP 40, 1mM DTT, 0.2mM PMSF, 0.1M NaCl) to remove the unbound bait protein. 500ng of PDCD4 was diluted to a final volume of 50µL in TGMC (0.1) buffer and added to the matrix coated with the bait protein. The prey protein was incubated with the bait protein at 4 ^{0}C for 2 hours. The matrix was washed four times with TGEM (0.1) buffer to remove the unbound prey protein. The proteins bound to the matrix were eluted by addition of 2X concentration Laemmli Sample Buffer and heated at 98 ^{0}C for 5 minutes. 50ng of His-PDCD4 and eIF3F was loaded into 10% w/v SDS-PAGE along with the pull-down samples as 10% input control. Western blot analysis was then performed to identify the interaction between PDCD4 and eIF3F.

2.11 Luciferase assay

Cells were lysed in the lysis buffer provided in the renilla luciferase assay system (Promega). The lysate was centrifuged at 10,000g for 10 minutes to separate the cell debris. 10µL of the supernatant was added into 50µL of luciferase substrate in a 96 well plate and relative light unit (RLU) was measured using a BioTek® luminescence plate reader.

Chapter 3

Results

3.1 FLAG-IP to detect the interaction between eIF3 and PDCD4

To investigate whether PDCD4 interacts with eIF3, I expressed FLAG-PDCD4 in the U343 cells and performed FLAG-IP. The proteins co-immunoprecipitated along with FLAG-PDCD4 were then analyzed by Western blotting. eIF3 is a dynamic complex of 13 subunits and hence, it is possible that the PDCD4 could interact with the entire eIF3 complex or one or more specific subunits of eIF3. Therefore, I probed the FLAG-IP blot with eIF3F, eIF3G, eIF3B, eIF3D, eIF3E, and eIF3H specific antibodies. As a result of this experiment, eIF3F, eIF3G, eIF3B, eIF3D, and eIF3E were found to be co-immunoprecipitated with FLAG-PDCD4 (Figure 3.1). eIF3F, eIF3G, eIF3B, eIF3D, and eIF3E did not co-immunoprecipitate similarly with the FLAG-tag lysate, suggesting a specific interaction between FLAG-PDCD4 and eIF3 subunits (Figure 3.1). The band intensity of the eIF3H protein in the FLAG-PDCD4 IP and the FLAG-tag IP control was similar (Figure 3.1). This result suggests eIF3H non-specifically interacts with the FLAG-tag or the affinity matrix (Figure 3.1). In addition to the eIF3H protein, a lower molecular weight protein was detected in the blot probed for eIF3H with anti-eIF3H mouse-raised antibody. This additional band corresponded to the molecular weight of mouse IgG from the anti-FLAG antibody used for FLAG-IP.

Earlier work from Dr. Klempnauer's group has shown that PABP interacts with PDCD4 (Fehler et al., 2014). Hence, probing for PABP served as a positive control for the FLAG-PDCD4 IP. On the other hand, PAIP1 is shown to interact with eIF3 and the PAIP1-eIF3 interaction is regulated by S6K1. The PAIP1-eIF3 interaction has been demonstrated to

have a possible role in translation (Martineau et al., 2014). Hence, I probed the blot with PAIP1 specific antibody to check if PAIP1 is co-immunoprecipitated with FLAG-PDCD4. As observed in the result, PAIP1 did not co-immunoprecipitate with FLAG-PDCD4 or the FLAG-tag (Figure 3.1). 1% of the total cell lysate was analyzed to ensure the difference in the interaction between FLAG-PDCD4 and FLAG-tag lysates with eIF3 was not due to the variation in eIF3 expression. The level of eIF3 subunits, PABP and PAIP1 were similar in the FLAG-tag and FLAG-PDCD4 expressing lysates (Figure 3.1). This further emphasizes the specificity of the interaction between FLAG-PDCD4 and eIF3 subunits.

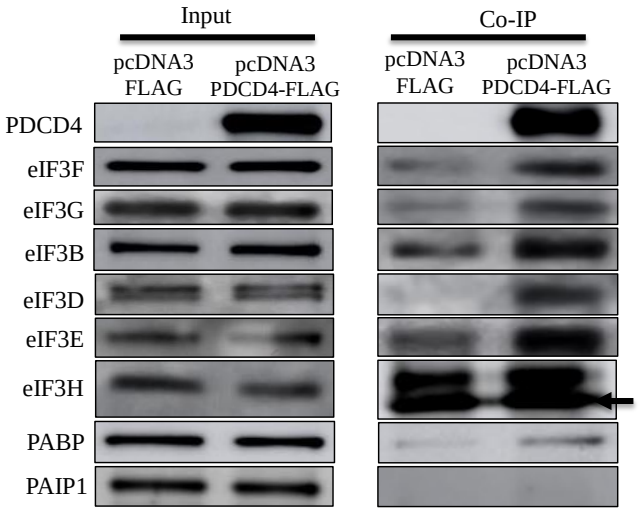


Figure 3.1: PDCD4 interacts with eIF3. 1% input from the total cell lysate was probed with antibodies against PDCD4, eIF3 subunits, PABP and PAIP1 (left panel). The level of eIF3 subunits, PABP and PAIP1 were similar in both the lysates. co-IP samples were probed with the same antibodies as the inputs. eIF3F, eIF3G, eIF3B, eIF3D, eIF3E and PABP were co-immunoprecipitated with FLAG-PDCD4 (right panel). There was no interaction between PAIP1 and FLAG-PDCD4. There was no enhanced pull down of the eIF3H subunit in the presence of FLAG-PDCD4 protein. Based on the molecular weight, the band indicated in the eIF3H co-IP could be the mouse IgG of the anti-FLAG antibody. This was detected as the secondary antibody used to detect eIF3H was an anti-mouse antibody. This image is a representation of the three repeats of the FLAG-IP.

3.2 eIF3F IP to detect the interaction between PDCD4 and eIF3F

eIF3F has been reported to directly interact with S6K1 and aid phosphorylation of eIF3G (Martineau et al., 2014). Therefore, as the later objective was to study the effect of S6K1 and 2 on eIF3-PDCD4 interaction. I chose to perform reciprocal IP with eIF3F antibody to further confirm the PDCD4-eIF3 interaction. The protein G Dynabeads^{TM} were coated with anti-eIF3F antibody and used for eIF3F subunit IP from a 100-mm tissue culture dish. In the SDS-PAGE, the migration of the PDCD4 protein overlapped with the migration of the heavy chain of rabbit IgG antibody. Therefore, a conformation specific secondary anti-rabbit antibody raised in the mouse was used to detect PDCD4 and eIF3F instead of the Abcam anti-rabbit secondary antibody.

In the reciprocal IP, PDCD4 was observed to be co-immunoprecipitated with the eIF3F subunit (Figure 3.2). When the co-IP blot was probed with an anti-PDCD4 antibody, an additional band of a higher molecular weight was observed along with PDCD4 in the eIF3F immunoprecipitated sample (Figure 3.2). The additional band observed could be the ubiquitinated form of PDCD4. PDCD4 is known to be degraded by the ubiquitin ligase-mediated mechanism (Dorrello et al., 2006). eIF3F may interact with post-translationally modified and unmodified PDCD4 protein. Alternatively, PDCD4 associated with eIF3F could be post-translationally modified by ubiquitination. The higher molecular weight protein was not observed in the input lanes. This suggests that the higher molecular weight protein has been enriched during the process of immunoprecipitation. PDCD4 was not detected in the lanes with elution from beads coated with non-specific antibodies (PAIP1) or lysates without FLAG-PDCD4 expression (Figure 3.2). As PDCD4 was not pulled-down with the non-specific antibody, these data

support the hypothesis that PDCD4 specifically interacts with the eIF3F subunit. However, endogenous PDCD4 was not detected in this experiment and this could be attributed to the low expression of PDCD4 in U343 cells. To circumvent this problem and to be able to perform endogenous PDCD4 IP, three other human glioblastoma cell lines were tested for PDCD4 expression. Of the different cell lines tested, SF767 and A172 were found to have higher expression of PDCD4 in comparison to U343 (Figure 3.3). Therefore, these cell lines can be used to perform endogenous IP to study PDCD4-eIF3 interaction. The current data on FLAG IP and reciprocal IP with eIF3F antibody indicate a specific interaction between eIF3F and PDCD4.

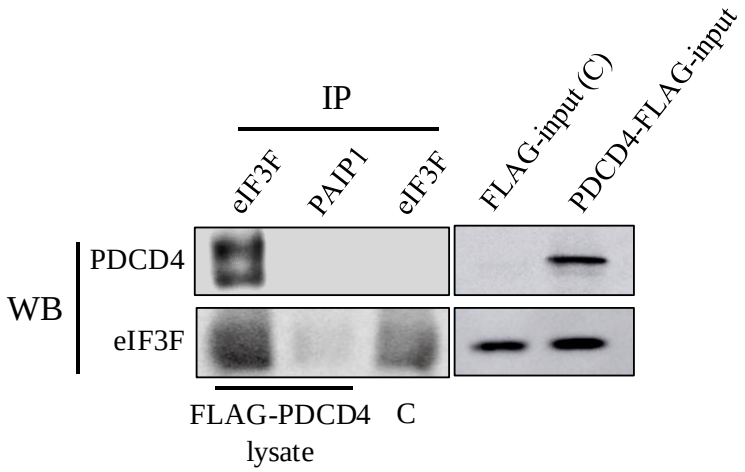


Figure 3.2: eIF3F interacts with PDCD4. Reciprocal-IP performed using eIF3F antibody in U343 cell line (left panel). PDCD4 is co-immunoprecipitated along with eIF3F. PDCD4 was not detected when the IP was conducted with non-specific antibody (PAIP1) or the lysate without FLAG-PDCD4 protein (C). 1% input control for FLAG-PDCD4 and FLAG-tag containing lysates (right panel). The image is a representative image of the IP performed in duplicate.

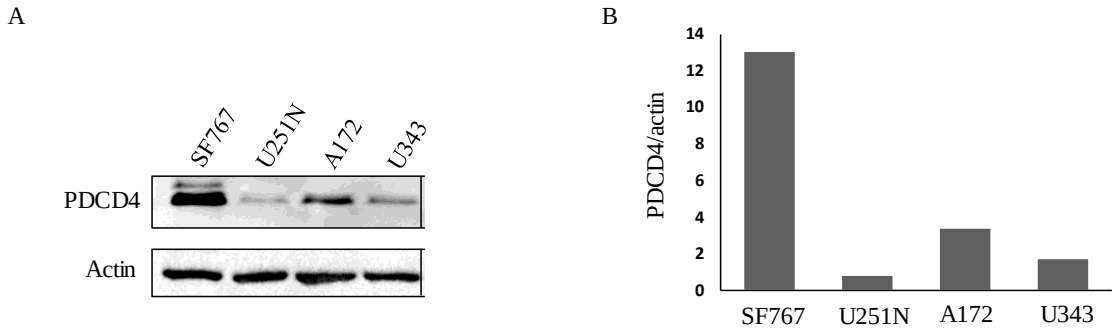


Figure 3.3: PDCD4 protein levels are higher in S7F767 and A172 in comparison to the other cell lines. A) A Western blot assay was performed with 10µg of the total cell lysates obtained from $3X10^5$ cells of different cell lines. The different cell lines used are indicated. The membrane was probed with anti-PDCD4 and anti-actin antibodies. The protein level of PDCD4 was higher in SF767 and A172 in comparison to U251N and U343 cells. B) Bar graph representing the densitometry values for the band intensity of PDCD4 normalized with their respective actin band intensity. This is a representation of a single experiment.

3.3 Co-IP in the presence and absence of RNaseA to detect if the eIF3 and PDCD4 interaction is RNA-dependent

The interaction of proteins demonstrated by IP can be a result of three major types of protein association in cells. In type I, PDCD4 and eIF3 may interact with the same RNA molecule without interfering with each other's interactions. In type II, PDCD4 and eIF3 can be a part a common protein complex and as a result be co-immunoprecipitated with each other. Lastly, in type III, PDCD4 and eIF3 subunits can directly interact with each other as depicted in the schematic diagram (Figure 3.4).

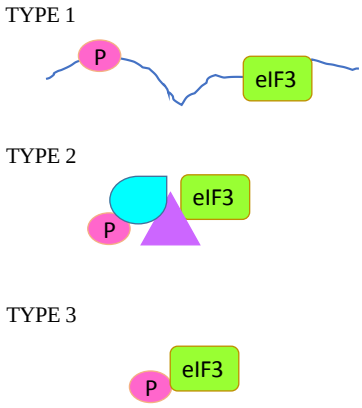


Figure 3.4: Schematic diagram representing the types of interactions detected by IP. The proteins detected in the IP assay could be a consequence of any one of the three types of interaction. P stands for PDCD4 in this schematic diagram. Firstly, PDCD4 and eIF3F can be interacting to the same RNA molecule with no interaction with each other (Type I). Secondly, PDCD4 and eIF3F may be a part of a common protein complex (Type II). Lastly, the two proteins could directly interact with each other (Type III).

To identify if the interaction between PDCD4 and eIF3 was mediated by an RNA molecule, FLAG IP was performed with RNaseA-treated lysates. eIF3F and eIF3D were co-immunoprecipitated with FLAG-PDCD4 in both RNaseA-treated and RNaseA-untreated samples (Figure 3.5). This suggests that PDCD4 interacts with eIF3F and eIF3D in an RNA-independent manner. There was no effect on the protein levels of PDCD4 upon RNaseA treatment as observed from the Western blot image of 1% total lysate (Input, Figure 3.5). The protein levels of eIF3F and eIF3D were similar in all four lysates as seen in the input blot (Figure 3.5). PDCD4 protein was not detected in the FLAG tag control lysate (Figure 3.5). Also, there was no significant interaction of eIF3 subunits with FLAG-tag or the matrix used for IP. To conclude, this data demonstrates that the PDCD4-eIF3F and PDCD4-eIF3D interaction is specific and RNA-independent.

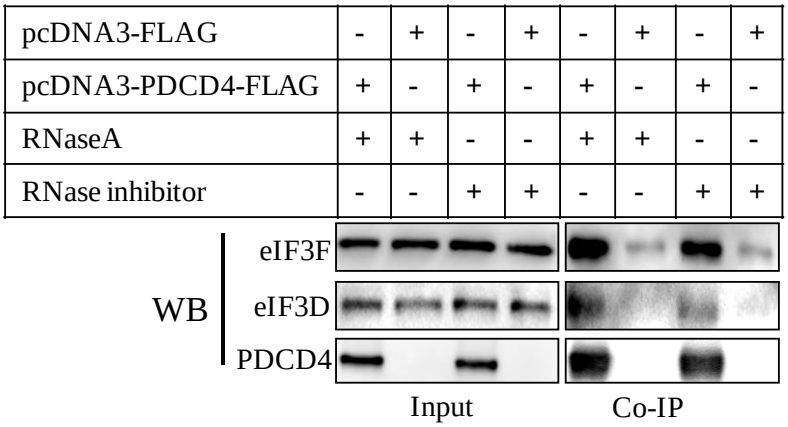


Figure 3.5: The PDCD4-eIF3 interaction is RNA-independent. 1% input from the total cell lysate was analyzed to compare the protein levels upon different treatment (left panel). Similar levels of eIF3 subunits were observed across samples. PDCD4 was below detectable levels in lysate from cells transfected with the pcDNA3-FLAG plasmid. PDCD4 was co-immunoprecipitated with eIF3 subunits (right panel). There was no difference in interaction upon RNaseA treatment. This indicates that the interaction between PDCD4 and eIF3 is not dependent on RNA.

3.4 Optimization of recombinant protein purification

The PDCD4-eIF3 interaction is RNA-independent, however, it is unclear whether this is a direct or an indirect interaction. These two types of interactions can be differentiated by *in vitro* protein pull-down. To perform this assay, His-PDCD4, GST-eIF3F, and GST expressing plasmids were obtained from the labs as mentioned in table 2.1.

The sequence of the gene inserted in the plasmid was confirmed by sequencing. A varied concentration of IPTG was investigated to optimize the protein purification as mentioned in section 2.4. Based on the results from the Coomassie-stained gels, the intensity of a 25 kDa protein band was increased upon IPTG induction for *E. coli* DE3 transformed with GST expression plasmid (Figure 3.6B). The band intensity did not vary with 0.5mM and 1mM IPTG concentration and hence a final concentration of 0.5mM IPTG was used for further purification of GST protein (Figure 3.6B). The band pattern of the proteins in the

SDS-PAGE was analyzed to check for the induction of proteins. The protein band pattern in the bacterial lysates from *E. coli* transformed with His-PDCD4 and GST-eIF3F plasmids were similar in both un-induced as well a culture induced with varying concentration of IPTG. However, the protein profile varied across samples transformed with different expression constructs. When compared to the protein standard, migration of the proteins indicated in Figure 3.6 corresponds to the molecular weight of His-PDCD4 (52kDa, Figure 3.6B) and GST-eIF3F (62 kDa, Figure 3.6A). This led to the speculation that the bands indicated in Figure 3.6 could be of His-PDCD4 and GST-eIF3F proteins. The GST-eIF3F expressing bacterial lysates were run along with GST-PDCD4 expressing bacterial lysates (Figure 3.6A). The 62 kDa band observed in the GST-eIF3F expressing bacterial lysate was not observed in the GST-PDCD4 expressing lanes. Instead, a band of about 75 kDa corresponding to GST-PDCD4 was observed (Figure 3.6A). The Coomassie-stained gels did not show any enhanced expression with the addition of IPTG. The vector backbone of these recombinant plasmids may not code for *lac* repressor and result ina leaky expression of the gene. Further confirmation of the proteins expressed was done prior to the *in vitro* pull-down assay.

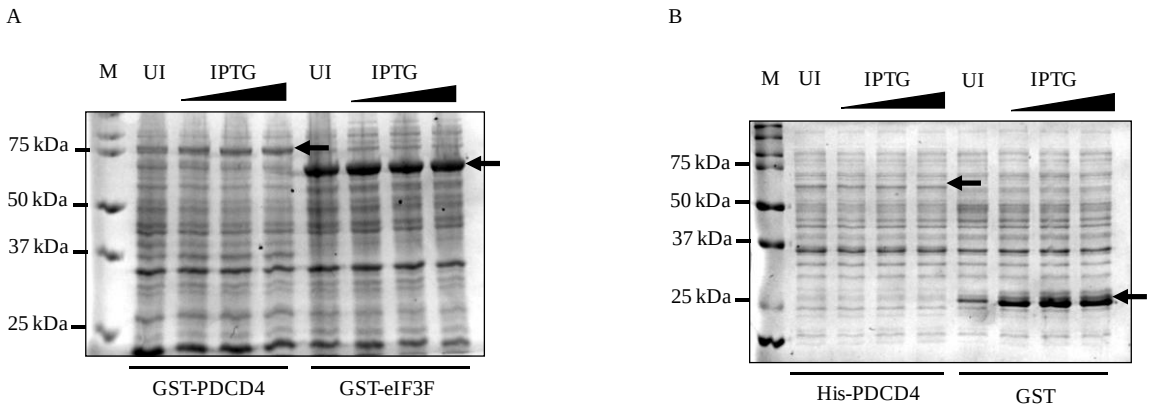


Figure 3.6: Coomassie staining to detect protein induction. A) BL21 DE3 strains as mentioned in section 2.2 were transformed with GST-PDCD4 and GST-eIF3F plasmid was induced at varying concentration of IPTG for 3 hours at 37 ^{0}C. No change in protein profile between the un-induced and induced was observed. However, the band indicated by an arrow was unique in the GST-PDCD4 and GST-eIF3F lanes. The molecular weights of the bands indicated correspond to the GST-PDCD4 and GST-eIF3F proteins. B) Similar to panel A, the unique bands in the cell lysates are indicated for His-PDCD4 and GST protein. The molecular weights of the indicated bands correspond to the molecular weights of the His-PDCD4 and GST proteins.

3.5 Western blotting to confirm His-PDCD4 and GST-eIF3F expression

Coomassie staining detects all the proteins in the cell lysate. The proteins can be differentiated from one another only based on their molecular weight or migration properties in SDS-PAGE. To confirm if the indicated bands in Coomassie-stained gels (Figure 3.6) corresponds to PDCD4 and eIF3F, a Western blot assay was performed.

Lysates were prepared as for the Coomassie staining experiment. However, as the Western blot technique is more sensitive in comparison to Coomassie staining, the samples were diluted by 10-fold or 100-fold and analyzed as in the case of Figure 3.7B. In Figure 3.7A, 10-fold diluted samples were loaded for analysis. In Figure 3.7A, un-induced and induced samples with a final concentration of 0.1mM, 0.5mM, and 1mM IPTG were analyzed on SDS-PAGE followed by Western blot. The nitrocellulose

membrane was probed with a PDCD4-specific antibody. PDCD4 protein was detected in the un-induced as well as in the induced *E. coli* cultures (Figure 3.7). PDCD4 was detected in the cell lysate with His-PDCD4 plasmid and not in the lysate obtained from the bacteria transformed with the GST expression construct (Figure 3.7). This confirmed the His-PDCD4 expression in the *E. coli* BL21 DE3 culture. In Figure 3.7B, BL21 DE3 strains transformed with the GST-PDCD4 (BL21 DE3) and GST-eIF3F (BL21 DE3 ROSETTA 2 PLYSS) expression constructs as well as cells without any expression construct were investigated for protein expression. The membrane was probed with the anti-eIF3F antibody. eIF3F protein was detected in both induced and un-induced culture lysates of the bacteria with GST-eIF3F plasmid, and not in the bacteria without any plasmid or bacteria with a GST-PDCD4 plasmid. This confirmed the expression of His-PDCD4 and GST-eIF3F in *E. coli*.

Figure 3.7: Western blot assay to confirm protein expression. A) PDCD4 protein was detected in the lysates with the His-PDCD4 plasmid. PDCD4 was not observed for lysates from bacteria with GST expression plasmid. B) eIF3F was specifically detected in the bacterial lysates with GST-eIF3F expression plasmid. These results confirm the expression of His-PDCD4 and GST-eIF3F.

3.6 *In vitro* pull-down of GST-tagged protein to detect direct interaction between PDCD4 and eIF3F

As mentioned earlier, to investigate if PDCD4 can directly interact with eIF3F, *in vitro* pull-down was performed using glutathione sepharose matrix. The assay was performed as mentioned in section 2.8. Western blot analysis of the pulled-down protein was performed using anti-eIF3F and anti-PDCD4 antibody. His-PDCD4 was detected in the presence of GST-eIF3F but not with GST or glutathione sepharose matrix (Figure 3.8). These results suggest direct protein-protein interaction between eIF3F and PDCD4.

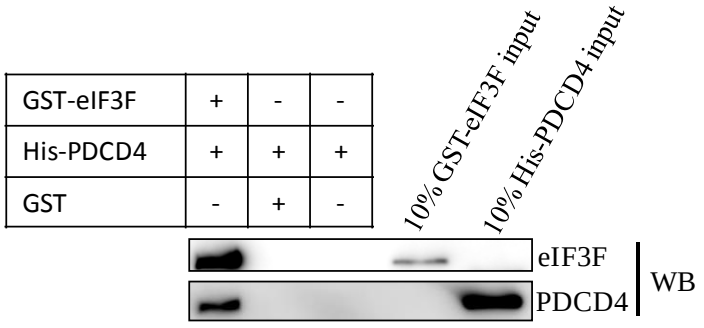


Figure 3.8: PDCD4 directly interacts with eIF3F.The proteins were separated on SDS-PAGE and was analyzed by Western blotting using specific antibodies. His-PDCD4 was observed to be pulled-down with GST-eIF3F and not with GST or only glutathione sepharose matrix.

3.7 Co-IP in S6K wild-type and knockout MEFs

PDCD4 and eIF3G subunits are post-translationally modified by S6K1 and 2 (Dennis et al., 2012; Liwak et al., 2012; Martineau et al., 2014). As mentioned in section 1.3.2 post-translation modification can affect the function and intermolecular interactions of the protein (Duan & Walther, 2015). Therefore, S6K1 and/or 2-mediated modifications of PDCD4 or eIF3G subunits could have an effect on the PDCD4-eIF3 interaction. To test if S6K1 and/or 2 have any role in the PDCD4-eIF3 interaction, FLAG-IP was performed in the S6K1 and 2 double knockout MEFs. S6K1 and 2 double knockout MEFs lacked S6K1 and 2 activity, which was confirmed by investigating the phosphorylation status of theS6 protein. The S6 protein is phosphorylated by S6K1 and 2 and this was detected using S6 and pS6 specific antibodies. As expected, phosphorylation of S6 was not detected in the S6K double knockout cell line (Figure 3.9). This confirmed the absence of S6K1 and 2 activity in the S6K knockout cell line.

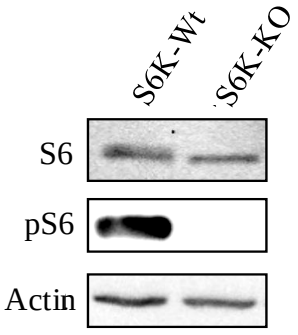


Figure 3.9: Phosphorylation of S6 is not detected in S6K double knockout MEFs. S6 protein was equally expressed in wild-type and knockout. Phosphorylation of S6 was not observed in the S6K double knockout.

To check if PDCD4 and eIF3F interact in the S6K1 and 2 double knockout background, FLAG-PDCD4and FLAG-tag were expressed in S6K1 and 2 expressing MEFs (wild-type) and S6K1 and 2 double knockout MEFs. After 48 hours of transfection, the cells were lysed and an equal volume of the lysate was used to perform FLAG-IP followed by Western blot. The membrane was probed with anti-PDCD4, anti-eIF3F, anti-S6 and anti-pS6 antibodies.

There was no detectable difference in the level of co-immunoprecipitated eIF3F in FLAG-PDCD4 and FLAG-tag expressed MEFs (Figure 3.10). Interestingly, pS6 was found to be co-immunoprecipitated with FLAG-PDCD4 in the S6K wild-type cell lysate. However, this interaction of PDCD4 with pS6 has not been reported earlier and needs to be validated. On the other hand, S6 protein did not show an interaction with FLAG-PDCD4 either in the S6K wild-type or in the knockout MEFs (Figure 3.10). PDCD4, eIF3F, and S6 had similar levels of expression in all the samples. pS6 was not observed in the knockout MEFs due to the absence of S6K1 and 2 activity. This suggests that the interaction between PDCD4 and eIF3 may not occur in these MEFs. Therefore, S6K1 and 2 double knockout and wild-type MEFs may not be suitable to test the effect of S6K1 and 2 on the PDCD4-eIF3 interaction as there was no PDCD4-eIF3 interaction detected in these cells.

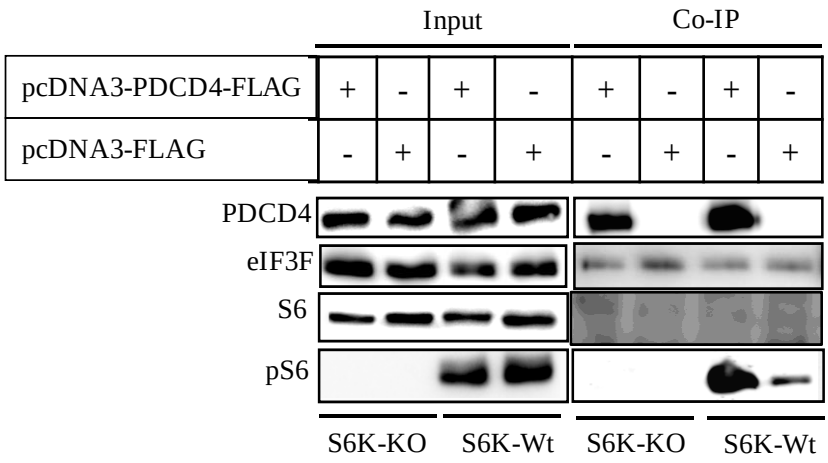


Figure 3.10: PDCD4 and eIF3 do not interact in MEFs. PDCD4, eIF3F and S6 protein levels were similar in all four input lanes (left panel). pS6 was not observed in the S6K knockout cell line. The absence of S6 phosphorylation further confirms the absence of S6K1 and 2 activity in the cell line. In the co-IP lanes, eIF3F band intensity was similar in all four lanes, indicating no specific interaction with PDCD4 (right panel).

3.8 FLAG-IP during serum starvation

mTORC1 is inhibited in the absence of growth factors. Hence, mTORC1 activity is blocked during serum starvation (R. Chen et al., 2014). Lack of mTORC1 activity inhibits the downstream activation of S6K1 and 2 (Holz et al., 2005). Thus, I conducted FLAG-IP in the serum starvation condition to study the effect of S6K1 and 2 on PDCD4-eIF3 interaction. Inhibition of S6K1 and 2 activity in U343 after 24 hours of serum starvation was tested by immunoblotting. Upon serum starvation, reduced phosphorylation of S6 protein was observed in U343 (Figure 3.11). The expression of S6 protein remained unaffected during serum starvation. This indicates that the decrease in the phosphorylated form of S6 is due to the loss in S6K1 and 2 activity.

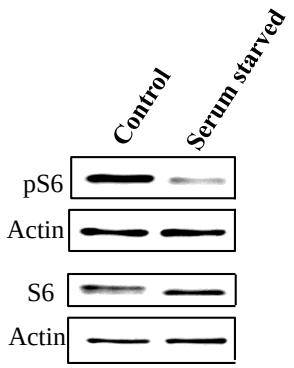
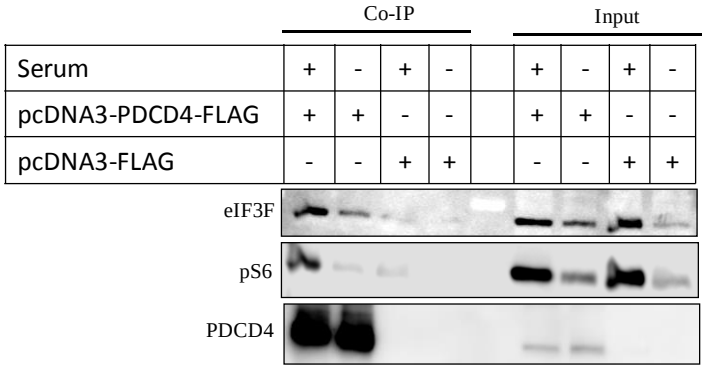


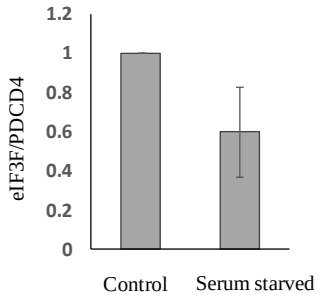
Figure 3.11: Serum starvation inhibits S6K1 and 2 activity. Protein level of S6 was not affected. The level of the phosphorylated form of S6 decreased with serum starvation.

pS6 and eIF3F were found to co-immunoprecipitate with FLAG-PDCD4 (Figure 3.12A). The eIF3F-PDCD4 interaction was found to be decreased by about 25% in serum-starved condition when normalized with the amount of PDCD4 immunoprecipitated (Figure 3.12B). However, there was a decrease in the levels of eIF3F upon serum starvation. The decrease in PDCD4-eIF3F interaction observed could be due to the decrease in eIF3F protein level and not the difference in the PDCD4-eIF3F interaction. Similarly, a decrease in the PDCD4-pS6 interaction was seen and this also is likely due to a decrease in the phosphorylation of S6 upon serum starvation (Figure 3.12A).

A



B



C

	Trial 1	Trial 2	Trial 3
eIF3F-control	1	1	1
eIF3F-serum starvation	0.699	0.336	0.763

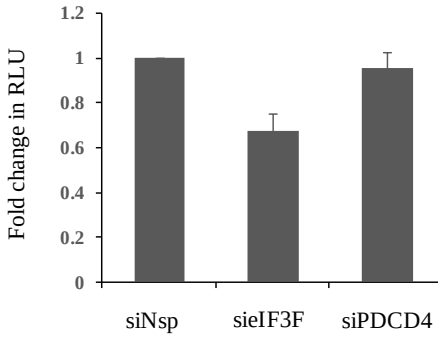
Figure 3.12: ThePDCD4-eIF3F interaction is not affected by serum starvation. A) eIF3F and pS6 were co-immunoprecipitated with PDCD4. This interaction decreased with serum starvation. However, the input lanes indicated a decrease in eIF3F and pS6 protein levels in the total cell lysate. This suggests there may not be anet difference in the PDCD4-eIF3F interaction during serum starvation. B) The amount of eIF3F immunoprecipitated from 3 independent experiments was normalized to PDCD4 pull-down and plotted as a fold change. C) Tabulation of the fold change values calculated for PDCD4-eIF3F interaction during serum starvation in comparison to the control. The eIF3F band intensity was normalized to the amount of PDCD4 pull-down. Then the control was normalized to one and the fold change during serum starvation was calculated in comparison to the control.

3.9 Luciferase reporter assay to investigate the role of eIF3F in cap-dependent translation

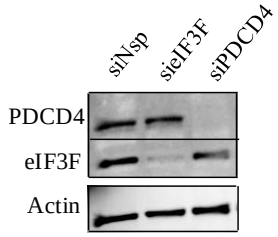
We do not know how the PDCD-eIF3F interaction would impact on global and transcript-specific translation. In order to identify the role of PDCD4-eIF3F interaction, it is first important to understand the independent role of eIF3F and PDCD4 on translation. The eIF3F subunit and PDCD4 can have a positive or negative effect on mRNA translation. In

order to investigate their specific role in cap-dependent translation in U343, a luciferase reporter assay was conducted in conjunction with the partial knockdown of eIF3F or PDCD4. The partial knockdown of eIF3F and PDCD4 was achieved by treating cells with siRNA specifically targeting eIF3F and PDCD4 mRNAs. The protein levels of eIF3F and PDCD4 after silencing were examined by the Western blotting assay. The Western blot data showed a decrease in eIF3F and PDCD4 level in comparison to the cells treated with non-specific siRNA (Figure 3.13B). Upon confirmation of partial knockdown, luciferase reporter assay was conducted with the same lysates. Partial silencing of eIF3F in U343 decreased cap-dependent translation by about 25%. There was no effect on the cap-dependent translation due to the partial knockdown of PDCD4 mRNA expression (Figure 3.13A). The decrease in luciferase expression was calculated in comparison to the cells treated with non-specific siRNA. Hence, the reduction of luciferase expression is a direct result of the loss of eIF3F protein and not an artifact of siRNA treatment. These results confirm the importance of eIF3F in cap-dependent translation.

A



B



C

	Trial 1	Trial 2	Trial 3
siNsp	1	1	1
sieIF3F	0.674	0.594	0.756
siPDCD4	1.024	0.880	0.969

Figure 3.13: Partial knockdown of eIF3F decreases cap-dependent translation. A) The luciferase readings obtained for every experiment were normalized with their respective total protein concentrations. The normalized RLUs were compared with the values for the cells treated with non-specific siRNA to derive the fold change in RLUs as represented. The average fold change of three independent experiments demonstrates a 25% decrease in luciferase expression upon eIF3F knockdown. B) Western blot to confirm partial knockdown of PDCD4 and eIF3F. The blots indicate more than 50% knockdown of eIF3F and PDCD4 proteins. C) Tabulation of the fold change values calculated for luciferase activity upon partial silencing of PDCD4 and eIF3F in comparison to the control. The control was normalized to one and the fold change during test conditions was calculated in comparison to the control.

Chapter 4

Discussion

4.1 Discussion

Genomic DNA, mRNAs, and proteins are the three key regulatory molecules in eukaryotic cells. These three are the indispensable components of the molecular biology central dogma. DNA is transcribed into mRNA which is then translated into protein. Each of these steps is regulated to control the gene expression as per the cell requirements. For example, there are several biological factors affecting the efficiency of transcription like promoter sequence, transcription factors, RNA polymerases, cytokines, and epigenetic modifications (T. I. Lee & Young, 2013). mRNA thus transcribed relies on eIFs, *cis*-acting elements, mRNA *trans*-acting factors, availability of tRNAs and ribosomes, along with many other cellular factors (Hershey, Sonenberg, & Mathews, 2012). Therefore, it is a time and energy consuming process for the cells to start with transcription every time that it requires a protein. In case of stress, it is observed that the mRNA profile of the cell does not match with the proteome profile of the cell (Cheng et al., 2016; Lackner, Schmidt, Wu, Wolf, & Bahler, 2012; Liu, Beyer, & Aebersold, 2016). This observation could be a result of the altered protein stability. Moreover, this could also indicate that the cell strategically regulates the translation of the available mRNAs to conserve energy. In order to enhance the mRNA translation, it utilizes beneficial *trans*-acting factors, eIFs, alternative translation initiation mechanism or increases mRNA stability (Cheneval, Kastelic, Fuerst, & Parker, 2010; Hershey et al., 2012; Nevins, Harder, Korneluk, & Holcik, 2003). While to decrease protein expression, the cell reduces the mRNA stability, forms P-bodies, employs small and long non-coding RNAs to degrade or suppress mRNA expression, cleaves eIFs, sequesters eIFs or stabilizes

trans-acting factors with an adverse effect on mRNA expression (Cheneval et al., 2010; Hershey et al., 2012). Investigating these post-transcriptional regulations is critical as they could be the immediate option for the cells during any stress condition.

The mTOR pathway is a classical example that is extensively studied for post-transcriptional or translation regulation. PDCD4 and eIF3 studied in this project are the targets of the mTOR pathway (Laplante & Sabatini, 2009). They are phosphorylated by S6K1 and/or 2, a downstream target of mTORC1 (Laplante & Sabatini, 2009). PDCD4 phosphorylation promotes PDCD4 degradation while eIF3 phosphorylation enhances eIF3 activity (Laplante & Sabatini, 2009; Martineau et al., 2014). Degradation of PDCD4 and phosphorylation of eIF3 enhances the translation of target mRNAs. One of the known common target mRNAs for PDCD4 and eIF3 is XIAP mRNA (Liwak et al., 2012; Thakor et al., 2017). PDCD4 and eIF3 directly interact with the XIAP mRNA with opposite effects on XIAP mRNA translation (Liwak et al., 2012; Thakor et al., 2017). Hence, as mentioned earlier, I wanted to investigate if there is an interaction between PDCD4 and eIF3 because this could be a possible mechanism of repressing XIAP or any other target mRNA expression.

The results of my experiments demonstrate a specific RNA-independent interaction between PDCD4 and the eIF3 complex. eIF3 is a 13-subunit complex (Hershey, 2015) and of the 13 subunits, eIF3B, eIF3D, eIF3E, eIF3G, and eIF3F subunits were shown to interact with PDCD4 in the co-IP I performed. Based on my results, I hypothesized that the co-immunoprecipitation of these eIF3 subunits could be a result of three types of interactions. i) PDCD4 interacts with the eIF3 complex and therefore multiple eIF3 subunits were co-immunoprecipitated with PDCD4. The PDCD4-eIF3 interaction could inhibit eIF3 association with the mRNA or other cellular proteins and thereby inhibit

translation. ii) eIF3 may be present as subcomplexes that do not contain all the 13 subunits. eIF3 could consist of a core complex made up of specific subunits and the remaining subunits may be involved only in regulatory mechanisms (Hershey, 2015). PDCD4 may interact with subcomplex that include eIF3B, eIF3D, eIF3E, eIF3G, and eIF3F subunits to inhibit translation. iii) PDCD4 may directly interact with each of the subunits as demonstrated for eIF3F in this project. The interaction of PDCD4 with one of the subunits could either block its association with other eIF3 subunits to form the functional complex or inhibit its independent activity.

eIF3F, which is shown to bind PDCD4 in my experiments, has also been earlier reported to interact with S6K1 (Martineau et al., 2014). The eIF3F and S6K1 interaction was demonstrated by an *in vitro* experiment (Martineau et al., 2014). S6K1 binds to eIF3F in order to phosphorylate the eIF3G present in the eIF3 complex (Martineau et al., 2014). However, the *in cellulo* experiments suggested that both the S6K1 and 2 have a role in eIF3 phosphorylation (Martineau et al., 2014). PDCD4 interacting with eIF3F can interfere with eIF3F and S6K1/2 association to prevent phosphorylation of the eIF3 complex. Similarly, PDCD4 binding to eIF3B and eIF3E can affect their function to interact with eIF4G and inhibit ribosome recruitment to the mRNA. Additionally, eIF3B is essential for mRNA recruitment and eIF3D can bind to the cap structure (Hinnebusch, 2006; A. S. Lee, Kranzusch, Doudna, & Cate, 2016). These canonical and specialized functions of the eIF3 subunits can be affected by its interaction with PDCD4. Besides eIF3, PDCD4 is known to interact with PABP and eIF4A to suppress their role in translation and downregulate protein synthesis (Fehler et al., 2014; Suzuki et al., 2008). Therefore, I believe that the PDCD4-eIF3 interaction will also have a similar effect and inhibit translation.

In cells, the protein-protein interactions can be regulated by post-translational modifications. There are different types of post-translational modifications and phosphorylation of proteins is one among them (Prabakaran, Lippens, Steen, & Gunawardena, 2012). Phosphorylation of proteins can alter the charge, structure and binding affinity of the proteins (Nishi, Hashimoto, & Panchenko, 2011). This particular protein modification is commonly executed by kinases (Nishi et al., 2011). Interestingly, both PDCD4 and eIF3 are phosphorylated by S6K1 and/or 2 (Dennis et al., 2012; Martineau et al., 2014). Therefore, it is possible that the PDCD4 and eIF3 interaction can be affected by the activation of S6K1 and/or 2. To validate this hypothesis, I tested if inhibiting S6K1 and/or 2 activity would affect PDCD4 and eIF3 interaction.

To inhibit the S6K1 and 2 activity, cells were incubated with serum-free media for 24 hours. Most likely, mTORC1 and its downstream target S6K1 and 2 were inhibited in the absence of growth factors. eIF3F was equally co-immunoprecipitated with PDCD4 from cells incubated with serum-free media or media containing 10% v/v serum. This implies that S6K1 and/or 2 activity do not affect the PDCD4-eIF3F interaction (Figure 3.12). However, treatment with serum-free media could trigger multiple effects besides S6K1 and 2 inhibition. It is important to perform similar co-IP experiments using chemical inhibitors that specifically target S6K1 and 2 activity. This result of eIF3F and PDCD4 interaction during serum-starved condition may not be extrapolated to all the eIF3 subunits, as the interaction between each of the eIF3 subunit and S6Ks may vary (Martineau et al., 2014). Apart from S6K, other kinases such as cyclin-dependent kinase 11 can phosphorylate eIF3F (J. Shi, Hershey, & Nelson, 2009). Such phosphorylation may also affect the PDCD4-eIF3F interaction. To conclude, the PDCD4-eIF3F interaction

is not affected by serum starvation and further testing needs to done to study the effect of S6K-independent phosphorylation on the PDCD4-eIF3F interaction.

In addition to eIF3 subunits, for the first time, I observed the interaction of pS6 with PDCD4. pS6 is a part of translating 40S ribosomes (Biever, Valjent, & Puighermanal, 2015) and interaction with PDCD4 can inhibit translation initiation. The interaction was not tested in the lysates treated with RNaseA and thus the pS6 co-immunoprecipitated with PDCD4 could be a result of an RNA-dependent or an RNA-independent interaction. Further investigations need to be conducted to validate the pS6 and PDCD4 interaction. In general, PDCD4 appears to interact with eIFs or *trans*-acting factors to sequester them and inhibit translation.

PDCD4 is characterized as a translation inhibitor while its interacting partner eIF3F has contradictory reports of promoting and inhibiting translation (Gutierrez-Fernandez, Higareda-Mendoza, Gomez-Correa, & Pardo-Galvan, 2015; Liwak et al., 2012; Wen et al., 2012). eIF3F is shown to interact with mTORC1 and activate S6K1 in skeletal muscle cells (Csibi et al., 2010). The activation of S6K1 in muscle cells leads to increased protein synthesis (Csibi et al., 2010). Similarly, the eIF3F protein level was established to peak in the S phase and mitosis of the A549 cell cycle to enhance cell proliferation (Higareda-Mendoza & Pardo-Galvan, 2010). Conversely, partial silencing of eIF3F in a human pancreatic cell line promoted cap-dependent and cap-independent translation (Wen et al., 2012). As the eIF3F subunit can either have a positive or a negative effect on translation depending on the cell line. I wanted to identify if the eIF3F subunit had a positive or an inhibitory effect on translation in U343 cells. Therefore, I partially silenced eIF3F and checked the expression of a luciferase reporter construct. A reduction in luciferase expression was observed reflecting the positive role of eIF3F in luciferase expression

likely at the translational level. Alongside eIF3F, I also performed a similar experiment with PDCD4 to check its role in the cap-dependent translation in U343 cells. PDCD4 was showed to inhibit cap-dependent translation (Suzuki et al., 2008; H. S. Yang et al., 2003). However, there was no effect on the luciferase expression upon a partial silencing of PDCD4. This observation was probably due to the type of luciferase construct used in this experiment. PDCD4 is known to interact with eIF4A and preferentially inhibit the cap-dependent translation of the mRNAs containing a structured 5' UTR (H. S. Yang et al., 2004). The pRL-CMV construct used in this experiment does not have a long structured 5' UTR. Thus, it may not require eIF4A for its expression and therefore is not inhibited by PDCD4.

The results of the luciferase assay conducted in the U343 cells suggest that eIF3F has a positive role likely in the cap-dependent translation. Based on the literature, the eIF3 complex is an essential component of both cap-dependent and independent initiation process (Cate, 2017; Thakor et al., 2017). Therefore, the interaction between PDCD4 and eIF3 subunits can potentially inhibit most translating mRNAs. This mechanism may have a regulatory role during stress conditions when a subset of mRNAs is translated by an IRES-mediated mechanism.

4.2 Future directions

4.2.1 Short-term vision

eIF3 and PDCD4 are known to interact with multiple cellular mRNAs (A. S. Lee, Kranzusch, & Cate, 2015; Liwak et al., 2012). XIAP mRNA is a common target for the binding of eIF3 and PDCD4 (Figure 4.1). XIAP is an anti-apoptotic protein. Studying the effect of the PDCD4-eIF3 interaction on XIAP mRNA translation will help us obtain further details on cell proliferation during stress. All the 13 subunits of eIF3 may not

interact with XIAP mRNA. eIF3A, eIF3B, and eIF3C are involved in mRNA recruitment and hence are the most likely candidates to interact with XIAP mRNA. It will be interesting to identify the specific eIF3 subunits interacting with XIAP mRNA and PDCD4. Identifying the eIF3 subunit interacting with PDCD4 will give us information on the probable function of eIF3 inhibited by PDCD4. The data on eIF3 subunit interacting with XIAP mRNA will help us understand the role of eIF3 in XIAP mRNA translation. After obtaining the information on eIF3 subunit interaction with XIAP mRNA, the nucleotides in the XIAP mRNA interacting with eIF3 subunits and PDCD4 can be identified by a toeprinting assay. This will tell us if it is possible that PDCD4 and eIF3 subunits compete with each other for the binding site on XIAP mRNA and inhibit each other's function. Alongside the toeprinting assay, a UV crosslinking assay could be performed to investigate if the binding of one protein affects the interaction of the other to the XIAP mRNA. These experiments will be able to give us an idea of the significance of the PDCD4-eIF3 interaction on XIAP mRNA translation. This mechanism can be extrapolated and validated for other target cellular mRNAs.

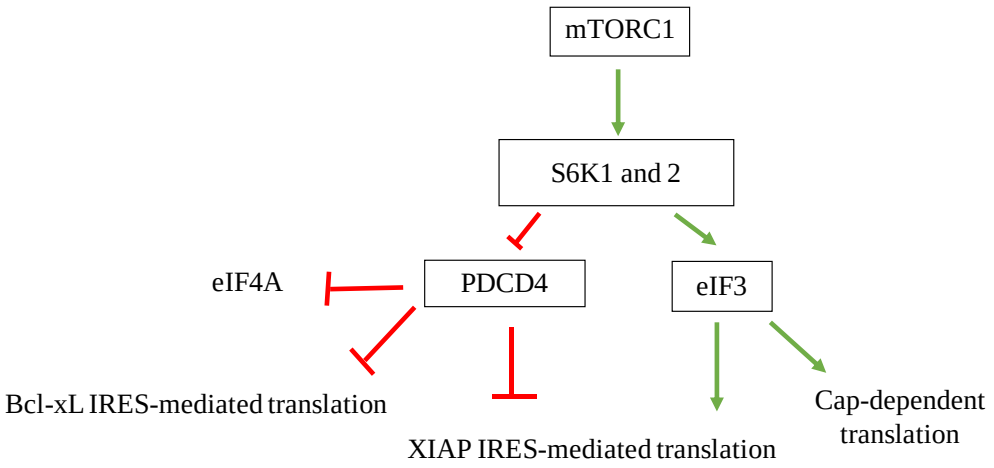


Figure 4.1: Schematic representation of the eIF3 and PDCD4 downstream targets. The mTORC1 activates S6K1 and 2, which then activates eIF3 and inhibits PDCD4. eIF3 enhances the cap-dependent translation and XIAP IRES-mediated translation. PDCD4 inhibits the XIAP IRES-mediated translation and general cap-dependent translation.

PDCD4 has been extensively studied for its tumor suppressor activity and more recently for its role in lipid metabolism and insulin resistance (Ding et al., 2016; Ferris et al., 2011; Lankat-Buttgereit & Goke, 2009; Vikhreva, Shepelev, Korobko, & Korobko, 2010). Besides PDCD4, eIF3 has also been shown to participate in mechanisms related to metabolism (Shah et al., 2016). Therefore, it is possible that the PDCD4-eIF3 interaction can have an effect beyond apoptosis-related mRNAs. It would be interesting to identify all the cellular mRNAs interacting with PDCD4 by UV-CLIP followed by sequencing. The sequencing results thus obtained can be compared with the list of cellular mRNAs shown to interact with eIF3 by Dr. Jamie Cate's research group (A. S. Lee et al., 2015). By doing so, we can identify the common pool of cellular mRNAs regulated by PDCD4 and eIF3. This will provide leads to establish the common role of the PDCD4-eIF3 interaction on mRNA expression.

4.2.2 Future-oriented outlook

eIF3 subunits and PDCD4 have been shown to promote and inhibit cell proliferation in glioblastoma (Hao, Liang, & Jiao, 2015; Sesen et al., 2014; Wang, Wang, Tang, & To, 2015). The roles of these proteins in different stages of cancer are not clear. Hence, it will be interesting to study the translatome profile of specific eIF3 subunits and PDCD4 in the different stages of cancer. To study the translatome profile, the specific eIF3 subunit or PDCD4 can be partially silenced. After partial silencing of the specific protein, mRNAs associated with the actively translating ribosomes can be isolated and sequenced. mRNAs thus identified in the partially silenced samples can be compared to the control to identify the set of mRNAs that are affected by the reduction of eIF3 subunit or PDCD4 protein levels. Comparison of these profiles across different stages of cancer will provide us information on the mRNA translation regulated by eIF3 subunits and PDCD4 in the different stages of cancer. This will add to our understanding of translation regulation during cancer progression and the knowledge can be employed in designing therapeutics. In recent times, eIFs that are a part of the eIF4F complex have been targeted for cancer therapy (Bhat et al., 2015; Pelletier et al., 2015). Similarly, eIF3 subunits and PDCD4 can also be looked into as targets for cancer therapy. To begin with, the preliminary information about eIF3 and PDCD4 obtained from glioblastoma cells has to be validated in a mouse model. To study the importance of eIF3 subunits and PDCD4 in glioblastoma progression, cancerous cells with compromised expression of eIF3 subunits and PDCD4 can be grafted into the mouse. The proliferation of the grafted cells can be monitored and compared with the grafted wild-type or control cells to pinpoint the significance of eIF3 and PDCD4 in glioblastoma. The eIF3 and PDCD4 are a target of the mTOR pathway and regulated by S6K1 and 2 (Laplante & Sabatini, 2012). Therefore, mTOR inhibitors or

S6K1 and 2 inhibitors can beused in combination with knockdown of eIF3 subunits and/or PDCD4 to monitor cancer progression.

Glioblastoma is one of the hard-to-treat cancers, therefore it is important to explore alternative strategies such as targeting the translation machinery or regulators of translation. Therefore, these studies will have an impact on our current understanding of translation control during cell death and metabolism that can be deployed in formulating new cancer therapy.

References

Andreev, D. E., O'Connor, P. B., Loughran, G., Dmitriev, S. E., Baranov, P. V., & Shatsky, I. N. (2017). Insights into the mechanisms of eukaryotic translation gained with ribosome profiling. *Nucleic Acids Res, 45*(2), 513-526. doi:10.1093/nar/gkw1190

Asangani, I. A., Rasheed, S. A., Nikolova, D. A., Leupold, J. H., Colburn, N. H., Post, S., & Allgayer, H. (2008). MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene, 27*(15), 2128-2136. doi:10.1038/sj.onc.1210856

Asano, K., Clayton, J., Shalev, A., & Hinnebusch, A. G. (2000). A multifactor complex of eukaryotic initiation factors, eIF1, eIF2, eIF3, eIF5, and initiator tRNA(Met) is an important translation initiation intermediate in vivo. *Genes Dev, 14*(19), 2534-2546.

Belsham, G. J., McInerney, G. M., & Ross-Smith, N. (2000). Foot-and-mouth disease virus 3C protease induces cleavage of translation initiation factors eIF4A and eIF4G within infected cells. *J Virol, 74*(1), 272-280.

Benchimol, S. (2001). p53-dependent pathways of apoptosis. *Cell Death Differ, 8*(11), 1049-1051. doi:10.1038/sj.cdd.4400918

Bhat, M., Robichaud, N., Hulea, L., Sonenberg, N., Pelletier, J., & Topisirovic, I. (2015). Targeting the translation machinery in cancer. *Nat Rev Drug Discov, 14*(4), 261-278. doi:10.1038/nrd4505

Biever, A., Valjent, E., & Puighermanal, E. (2015). Ribosomal Protein S6 Phosphorylation in the Nervous System: From Regulation to Function. *Front Mol Neurosci, 8*, 75. doi:10.3389/fnmol.2015.00075

Biyanee, A., Singh, P., & Klempnauer, K. H. (2015). Translation, Pdcd4 and eIF4A. *Oncoscience, 2*(9), 731-732. doi:10.18632/oncoscience.192

Bohm, M., Sawicka, K., Siebrasse, J. P., Brehmer-Fastnacht, A., Peters, R., & Klempnauer, K. H. (2003). The transformation suppressor protein Pdcd4 shuttles between nucleus and cytoplasm and binds RNA. *Oncogene, 22*(31), 4905-4910. doi:10.1038/sj.onc.1206710

Branzei, D., & Foiani, M. (2008). Regulation of DNA repair throughout the cell cycle. *Nat Rev Mol Cell Biol, 9*(4), 297-308. doi:10.1038/nrm2351

Carthy, C. M., Yanagawa, B., Luo, H., Granville, D. J., Yang, D., Cheung, P., . . . McManus, B. M. (2003). Bcl-2 and Bcl-xL overexpression inhibits cytochrome c release, activation of multiple caspases, and virus release following coxsackievirus B3 infection. *Virology, 313*(1), 147-157.

Cate, J. H. (2017). Human eIF3: from 'blobology' to biological insight. *Philos Trans R Soc Lond B Biol Sci, 372*(1716). doi:10.1098/rstb.2016.0176

Chai, J., Du, C., Wu, J. W., Kyin, S., Wang, X., & Shi, Y. (2000). Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature, 406*(6798), 855-862. doi:10.1038/35022514

Chang, J. H., Cho, Y. H., Sohn, S. Y., Choi, J. M., Kim, A., Kim, Y. C., . . . Cho, Y. (2009). Crystal structure of the eIF4A-PDCD4 complex. *Proc Natl Acad Sci U S A, 106*(9), 3148-3153. doi:10.1073/pnas.0808275106

Chen, R., Zou, Y., Mao, D., Sun, D., Gao, G., Shi, J., . . . Yu, L. (2014). The general amino acid control pathway regulates mTOR and autophagy during serum/glutamine starvation. *J Cell Biol, 206*(2), 173-182. doi:10.1083/jcb.201403009

Chen, Y., Knosel, T., Kristiansen, G., Pietas, A., Garber, M. E., Matsuhashi, S., . . . Petersen, I. (2003). Loss of PDCD4 expression in human lung cancer correlates with tumour progression and prognosis. *J Pathol, 200*(5), 640-646. doi:10.1002/path.1378

Chen, Z., Yuan, Y. C., Wang, Y., Liu, Z., Chan, H. J., & Chen, S. (2015). Down-regulation of programmed cell death 4 (PDCD4) is associated with aromatase inhibitor resistance and a poor prognosis in estrogen receptor-positive breast cancer. *Breast Cancer Res Treat, 152*(1), 29-39. doi:10.1007/s10549-015-3446-8

Cheneval, D., Kastelic, T., Fuerst, P., & Parker, C. N. (2010). A review of methods to monitor the modulation of mRNA stability: a novel approach to drug discovery and therapeutic intervention. *J Biomol Screen, 15*(6), 609-622. doi:10.1177/1087057110365897

Cheng, Z., Teo, G., Krueger, S., Rock, T. M., Koh, H. W., Choi, H., & Vogel, C. (2016). Differential dynamics of the mammalian mRNA and protein expression response to misfolding stress. *Mol Syst Biol, 12*(1), 855. doi:10.15252/msb.20156423

Choo, A. Y., Yoon, S. O., Kim, S. G., Roux, P. P., & Blenis, J. (2008). Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. *Proc Natl Acad Sci U S A, 105*(45), 17414-17419. doi:10.1073/pnas.0809136105

Clemens, M. J. (2001). Initiation factor eIF2 alpha phosphorylation in stress responses and apoptosis. *Prog Mol Subcell Biol, 27*, 57-89.

Creagh, E. M., Murphy, B. M., Duriez, P. J., Duckett, C. S., & Martin, S. J. (2004). Smac/Diablo antagonizes ubiquitin ligase activity of inhibitor of apoptosis proteins. *J Biol Chem, 279*(26), 26906-26914. doi:10.1074/jbc.M313859200

Csibi, A., Cornille, K., Leibovitch, M. P., Poupon, A., Tintignac, L. A., Sanchez, A. M., & Leibovitch, S. A. (2010). The translation regulatory subunit eIF3f controls the kinase-dependent mTOR signaling required for muscle differentiation and hypertrophy in mouse. *PLoS One, 5*(2), e8994. doi:10.1371/journal.pone.0008994

Dennis, M. D., Jefferson, L. S., & Kimball, S. R. (2012). Role of p70S6K1-mediated phosphorylation of eIF4B and PDCD4 proteins in the regulation of protein synthesis. *J Biol Chem, 287*(51), 42890-42899. doi:10.1074/jbc.M112.404822

Dever, T. E., & Green, R. (2012). The elongation, termination, and recycling phases of translation in eukaryotes. *Cold Spring Harb Perspect Biol, 4*(7), a013706. doi:10.1101/cshperspect.a013706

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

Ding, L., Gao, F., Zhang, M., Yan, W., Tang, R., Zhang, C., & Chen, Z. J. (2016). Higher PDCD4 expression is associated with obesity, insulin resistance, lipid metabolism disorders, and granulosa cell apoptosis in polycystic ovary syndrome. *Fertil Steril, 105*(5), 1330-1337 e1333. doi:10.1016/j.fertnstert.2016.01.020

Dong, Z., & Zhang, J. T. (2006). Initiation factor eIF3 and regulation of mRNA translation, cell growth, and cancer. *Crit Rev Oncol Hematol, 59*(3), 169-180. doi:10.1016/j.critrevonc.2006.03.005

Dorrello, N. V., Peschiaroli, A., Guardavaccaro, D., Colburn, N. H., Sherman, N. E., & Pagano, M. (2006). S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. *Science, 314*(5798), 467-471. doi:10.1126/science.1130276

Duan, G., & Walther, D. (2015). The roles of post-translational modifications in the context of protein interaction networks. *PLoS Comput Biol, 11*(2), e1004049. doi:10.1371/journal.pcbi.1004049

Edelmann, H. M., Kuhne, C., Petritsch, C., & Ballou, L. M. (1996). Cell cycle regulation of p70 S6 kinase and p42/p44 mitogen-activated protein kinases in Swiss mouse 3T3 fibroblasts. *J Biol Chem, 271*(2), 963-971.

Eggert, A., Grotzer, M. A., Zuzak, T. J., Ikegaki, N., Zhao, H., & Brodeur, G. M. (2002). Expression of Apo-3 and Apo-3L in primitive neuroectodermal tumours of the central and peripheral nervous system. *Eur J Cancer, 38*(1), 92-98.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol, 35*(4), 495-516. doi:10.1080/01926230701320337

Ewald, F., Ueffing, N., Brockmann, L., Hader, C., Telieps, T., Schuster, M., . . . Schmitz, I. (2011). The role of c-FLIP splice variants in urothelial tumours. *Cell Death Dis, 2*, e245. doi:10.1038/cddis.2011.131

Fang, J., Meng, Q., Vogt, P. K., Zhang, R., & Jiang, B. H. (2006). A downstream kinase of the mammalian target of rapamycin, p70S6K1, regulates human double minute 2 protein phosphorylation and stability. *J Cell Physiol, 209*(2), 261-265. doi:10.1002/jcp.20749

Fehler, O., Singh, P., Haas, A., Ulrich, D., Muller, J. P., Ohnheiser, J., & Klempnauer, K. H. (2014). An evolutionarily conserved interaction of tumor suppressor protein

Pdcd4 with the poly(A)-binding protein contributes to translation suppression by Pdcd4. *Nucleic Acids Res, 42*(17), 11107-11118. doi:10.1093/nar/gku800

Ferris, W. F., Marriott, C. E., Ali, T., Landy, C., Campbell, S. C., & Macfarlane, W. M. (2011). Tumor suppressor Pdcd4 is a major transcript that is upregulated during in vivo pancreatic islet neogenesis and is expressed in both beta-cell and ductal cell lines. *Pancreas, 40*(1), 61-66. doi:10.1097/MPA.0b013e3181f5f1ab

Fulda, S., & Debatin, K. M. (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene, 25*(34), 4798-4811. doi:10.1038/sj.onc.1209608

Fulda, S., Gorman, A. M., Hori, O., & Samali, A. (2010). Cellular stress responses: cell survival and cell death. *Int J Cell Biol, 2010*, 214074. doi:10.1155/2010/214074

Gaidos, G., Panaitiu, A. E., Guo, B., Pellegrini, M., & Mierke, D. F. (2015). Identification and Characterization of the Interaction Site between cFLIPL and Calmodulin. *PLoS One, 10*(11), e0141692. doi:10.1371/journal.pone.0141692

Galan, J. A., Geraghty, K. M., Lavoie, G., Kanshin, E., Tcherkezian, J., Calabrese, V., . . . Roux, P. P. (2014). Phosphoproteomic analysis identifies the tumor suppressor PDCD4 as a RSK substrate negatively regulated by 14-3-3. *Proc Natl Acad Sci U S A, 111*(29), E2918-2927. doi:10.1073/pnas.1405601111

Galban, S., & Duckett, C. S. (2010). XIAP as a ubiquitin ligase in cellular signaling. *Cell Death Differ, 17*(1), 54-60. doi:10.1038/cdd.2009.81

Gao, W., Li, Q., Zhu, R., & Jin, J. (2016). La Autoantigen Induces Ribosome Binding Protein 1 (RRBP1) Expression through Internal Ribosome Entry Site (IRES)-Mediated Translation during Cellular Stress Condition. *Int J Mol Sci, 17*(7). doi:10.3390/ijms17071174

Gillis, L. D., & Lewis, S. M. (2013). Decreased eIF3e/Int6 expression causes epithelial-to-mesenchymal transition in breast epithelial cells. *Oncogene, 32*(31), 3598-3605. doi:10.1038/onc.2012.371

Gingras, A. C., Svitkin, Y., Belsham, G. J., Pause, A., & Sonenberg, N. (1996). Activation of the translational suppressor 4E-BP1 following infection with encephalomyocarditis virus and poliovirus. *Proc Natl Acad Sci U S A, 93*(11), 5578-5583.

Graber, T. E., Baird, S. D., Kao, P. N., Mathews, M. B., & Holcik, M. (2010). NF45 functions as an IRES trans-acting factor that is required for translation of cIAP1 during the unfolded protein response. *Cell Death Differ, 17*(4), 719-729. doi:10.1038/cdd.2009.164

Grinberg, M., Sarig, R., Zaltsman, Y., Frumkin, D., Grammatikakis, N., Reuveny, E., & Gross, A. (2002). tBID Homooligomerizes in the mitochondrial membrane to induce apoptosis. *J Biol Chem, 277*(14), 12237-12245. doi:10.1074/jbc.M104893200

Gross, L., Vicens, Q., Einhorn, E., Noireterre, A., Schaeffer, L., Kuhn, L., . . . Martin, F. (2017). The IRES5'UTR of the dicistrovirus cricket paralysis virus is a type III IRES containing an essential pseudoknot structure. *Nucleic Acids Res, 45*(15), 8993-9004. doi:10.1093/nar/gkx622

Grover, R., Sharathchandra, A., Ponnuswamy, A., Khan, D., & Das, S. (2011). Effect of mutations on the p53 IRES RNA structure: implications for de-regulation of the synthesis of p53 isoforms. *RNA Biol, 8*(1), 132-142.

Grzmil, M., Rzymski, T., Milani, M., Harris, A. L., Capper, R. G., Saunders, N. J., . . . Norbury, C. J. (2010). An oncogenic role of eIF3e/INT6 in human breast cancer. *Oncogene, 29*(28), 4080-4089. doi:10.1038/onc.2010.152

Gu, L., Zhu, N., Zhang, H., Durden, D. L., Feng, Y., & Zhou, M. (2009). Regulation of XIAP translation and induction by MDM2 following irradiation. *Cancer Cell, 15*(5), 363-375. doi:10.1016/j.ccr.2009.03.002

Guicciardi, M. E., Mott, J. L., Bronk, S. F., Kurita, S., Fingas, C. D., & Gores, G. J. (2011). Cellular inhibitor of apoptosis 1 (cIAP-1) degradation by caspase 8 during TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. *Exp Cell Res, 317*(1), 107-116. doi:10.1016/j.yexcr.2010.10.005

Gutierrez-Fernandez, M. J., Higareda-Mendoza, A. E., Gomez-Correa, C. A., & Pardo-Galvan, M. A. (2015). The eukaryotic translation initiation factor 3f (eIF3f) interacts physically with the alpha 1B-adrenergic receptor and stimulates adrenoceptor activity. *BMC Biochem, 16*, 25. doi:10.1186/s12858-015-0054-5

Haghighat, A., Svitkin, Y., Novoa, I., Kuechler, E., Skern, T., & Sonenberg, N. (1996). The eIF4G-eIF4E complex is the target for direct cleavage by the rhinovirus 2A proteinase. *J Virol, 70*(12), 8444-8450.

Halbeisen, R. E., Galgano, A., Scherrer, T., & Gerber, A. P. (2008). Post-transcriptional gene regulation: from genome-wide studies to principles. *Cell Mol Life Sci, 65*(5), 798-813. doi:10.1007/s00018-007-7447-6

Hao, J., Liang, C., & Jiao, B. (2015). Eukaryotic translation initiation factor 3, subunit C is overexpressed and promotes cell proliferation in human glioma U-87 MG cells. *Oncol Lett, 9*(6), 2525-2533. doi:10.3892/ol.2015.3078

Hay, N., & Sonenberg, N. (2004). Upstream and downstream of mTOR. *Genes Dev, 18*(16), 1926-1945. doi:10.1101/gad.1212704

Hellen, C. U., & Sarnow, P. (2001). Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes Dev, 15*(13), 1593-1612. doi:10.1101/gad.891101

Hershey, J. W. (2015). The role of eIF3 and its individual subunits in cancer. *Biochim Biophys Acta, 1849*(7), 792-800. doi:10.1016/j.bbagrm.2014.10.005

Hershey, J. W., Sonenberg, N., & Mathews, M. B. (2012). Principles of translational control: an overview. *Cold Spring Harb Perspect Biol, 4*(12). doi:10.1101/cshperspect.a011528

Higareda-Mendoza, A. E., & Pardo-Galvan, M. A. (2010). Expression of human eukaryotic initiation factor 3f oscillates with cell cycle in A549 cells and is essential for cell viability. *Cell Div, 5*, 10. doi:10.1186/1747-1028-5-10

Hinnebusch, A. G. (2006). eIF3: a versatile scaffold for translation initiation complexes. *Trends Biochem Sci, 31*(10), 553-562. doi:10.1016/j.tibs.2006.08.005

Hinnebusch, A. G., & Lorsch, J. R. (2012). The mechanism of eukaryotic translation initiation: new insights and challenges. *Cold Spring Harb Perspect Biol, 4*(10). doi:10.1101/cshperspect.a011544

Holcik, M., & Korneluk, R. G. (2000). Functional characterization of the X-linked inhibitor of apoptosis (XIAP) internal ribosome entry site element: role of La autoantigen in XIAP translation. *Mol Cell Biol, 20*(13), 4648-4657.

Holz, M. K., Ballif, B. A., Gygi, S. P., & Blenis, J. (2005). mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell, 123*(4), 569-580. doi:10.1016/j.cell.2005.10.024

Jaafar, Z. A., Oguro, A., Nakamura, Y., & Kieft, J. S. (2016). Translation initiation by the hepatitis C virus IRES requires eIF1A and ribosomal complex remodeling. *Elife, 5*. doi:10.7554/eLife.21198

Jackson, R. J., Hellen, C. U., & Pestova, T. V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol, 11*(2), 113-127. doi:10.1038/nrm2838

Jackson, S. P., & Bartek, J. (2009). The DNA-damage response in human biology and disease. *Nature, 461*(7267), 1071-1078. doi:10.1038/nature08467

Jo, S. H., Kim, D. E., Clocchiatti, A., & Dotto, G. P. (2016). PDCD4 is a CSL associated protein with a transcription repressive function in cancer associated fibroblast activation. *Oncotarget, 7*(37), 58717-58727. doi:10.18632/oncotarget.11227

Kakumoto, K., Ikeda, J., Okada, M., Morii, E., & Oneyama, C. (2015). mLST8 Promotes mTOR-Mediated Tumor Progression. *PLoS One, 10*(4), e0119015. doi:10.1371/journal.pone.0119015

Karlsson, E., Magic, I., Bostner, J., Dyrager, C., Lysholm, F., Hallbeck, A. L., . . . Lundstrom, P. (2015). Revealing Different Roles of the mTOR-Targets S6K1 and S6K2 in Breast Cancer by Expression Profiling and Structural Analysis. *PLoS One, 10*(12), e0145013. doi:10.1371/journal.pone.0145013

Kasof, G. M., & Gomes, B. C. (2001). Livin, a novel inhibitor of apoptosis protein family member. *J Biol Chem, 276*(5), 3238-3246. doi:10.1074/jbc.M003670200

Kim, D., Akcakanat, A., Singh, G., Sharma, C., & Meric-Bernstam, F. (2009). Regulation and localization of ribosomal protein S6 kinase 1 isoforms. *Growth Factors, 27*(1), 12-21. doi:10.1080/08977190802556986

Kimball, S. R. (1999). Eukaryotic initiation factor eIF2. *Int J Biochem Cell Biol, 31*(1), 25-29.

Kireeva, M. L., Kashlev, M., & Burton, Z. F. (2013). RNA polymerase structure, function, regulation, dynamics, fidelity, and roles in gene expression. *Chem Rev, 113*(11), 8325-8330. doi:10.1021/cr400436m

Kluck, R. M., Esposti, M. D., Perkins, G., Renken, C., Kuwana, T., Bossy-Wetzel, E., . . . Newmeyer, D. D. (1999). The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. *J Cell Biol, 147*(4), 809-822.

Koff, J. L., Ramachandiran, S., & Bernal-Mizrachi, L. (2015). A time to kill: targeting apoptosis in cancer. *Int J Mol Sci, 16*(2), 2942-2955. doi:10.3390/ijms16022942

Komar, A. A., & Hatzoglou, M. (2011). Cellular IRES-mediated translation: the war of ITAFs in pathophysiological states. *Cell Cycle, 10*(2), 229-240. doi:10.4161/cc.10.2.14472

Krampe, B., & Al-Rubeai, M. (2010). Cell death in mammalian cell culture: molecular mechanisms and cell line engineering strategies. *Cytotechnology, 62*(3), 175-188. doi:10.1007/s10616-010-9274-0

Kuang, E., Fu, B., Liang, Q., Myoung, J., & Zhu, F. (2011). Phosphorylation of eukaryotic translation initiation factor 4B (EIF4B) by open reading frame 45/p90 ribosomal S6 kinase (ORF45/RSK) signaling axis facilitates protein translation during Kaposi sarcoma-associated herpesvirus (KSHV) lytic replication. *J Biol Chem, 286*(48), 41171-41182. doi:10.1074/jbc.M111.280982

Kumar, A., Ray, U., & Das, S. (2013). Human La protein interaction with GCAC near the initiator AUG enhances hepatitis C Virus RNA replication by promoting linkage between 5' and 3' untranslated regions. *J Virol, 87*(12), 6713-6726. doi:10.1128/JVI.00525-13

Kumar, N., Wethkamp, N., Waters, L. C., Carr, M. D., & Klempnauer, K. H. (2013). Tumor suppressor protein Pdcd4 interacts with Daxx and modulates the stability of Daxx and the Hipk2-dependent phosphorylation of p53 at serine 46. *Oncogenesis, 2*, e37. doi:10.1038/oncsis.2012.37

Labbe, K., McIntire, C. R., Doiron, K., Leblanc, P. M., & Saleh, M. (2011). Cellular inhibitors of apoptosis proteins cIAP1 and cIAP2 are required for efficient caspase-1 activation by the inflammasome. *Immunity, 35*(6), 897-907. doi:10.1016/j.immuni.2011.10.016

Lacerda, R., Menezes, J., & Romao, L. (2017). More than just scanning: the importance of cap-independent mRNA translation initiation for cellular stress response and cancer. *Cell Mol Life Sci, 74*(9), 1659-1680. doi:10.1007/s00018-016-2428-2

Lackner, D. H., Schmidt, M. W., Wu, S., Wolf, D. A., & Bahler, J. (2012). Regulation of transcriptome, translation, and proteome in response to environmental stress in fission yeast. *Genome Biol, 13*(4), R25. doi:10.1186/gb-2012-13-4-r25

Lankat-Buttgereit, B., & Goke, R. (2009). The tumour suppressor Pdcd4: recent advances in the elucidation of function and regulation. *Biol Cell, 101*(6), 309-317. doi:10.1042/BC20080191

Laplante, M., & Sabatini, D. M. (2009). mTOR signaling at a glance. *J Cell Sci, 122*(Pt 20), 3589-3594. doi:10.1242/jcs.051011

Laplante, M., & Sabatini, D. M. (2012). mTOR signaling in growth control and disease. *Cell, 149*(2), 274-293. doi:10.1016/j.cell.2012.03.017

Laplante, M., & Sabatini, D. M. (2013). Regulation of mTORC1 and its impact on gene expression at a glance. *J Cell Sci, 126*(Pt 8), 1713-1719. doi:10.1242/jcs.125773

Lee, A. S., Kranzusch, P. J., & Cate, J. H. (2015). eIF3 targets cell-proliferation messenger RNAs for translational activation or repression. *Nature, 522*(7554), 111-114. doi:10.1038/nature14267

Lee, A. S., Kranzusch, P. J., Doudna, J. A., & Cate, J. H. (2016). eIF3d is an mRNA cap-binding protein that is required for specialized translation initiation. *Nature, 536*(7614), 96-99. doi:10.1038/nature18954

Lee, J. Y., Kim, H. J., Rho, S. B., & Lee, S. H. (2016). eIF3f reduces tumor growth by directly interrupting clusterin with anti-apoptotic property in cancer cells. *Oncotarget, 7*(14), 18541-18557. doi:10.18632/oncotarget.8105

Lee, T. I., & Young, R. A. (2013). Transcriptional regulation and its misregulation in disease. *Cell, 152*(6), 1237-1251. doi:10.1016/j.cell.2013.02.014

Li, H., Zhu, H., Xu, C. J., & Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell, 94*(4), 491-501.

Li, J., & Yuan, J. (2008). Caspases in apoptosis and beyond. *Oncogene, 27*(48), 6194-6206. doi:10.1038/onc.2008.297

Liang, J., Cao, R., Wang, X., Zhang, Y., Wang, P., Gao, H., . . . Yang, W. (2017). Mitochondrial PKM2 regulates oxidative stress-induced apoptosis by stabilizing Bcl2. *Cell Res, 27*(3), 329-351. doi:10.1038/cr.2016.159

Lindqvist, L., Imataka, H., & Pelletier, J. (2008). Cap-dependent eukaryotic initiation factor-mRNA interactions probed by cross-linking. *RNA, 14*(5), 960-969. doi:10.1261/rna.971208

Liu, Y., Beyer, A., & Aebersold, R. (2016). On the Dependency of Cellular Protein Levels on mRNA Abundance. *Cell, 165*(3), 535-550. doi:10.1016/j.cell.2016.03.014

Liwak, U., Jordan, L. E., Von-Holt, S. D., Singh, P., Hanson, J. E., Lorimer, I. A., . . . Holcik, M. (2013). Loss of PDCD4 contributes to enhanced chemoresistance in Glioblastoma multiforme through de-repression of Bcl-xL translation. *Oncotarget, 4*(9), 1365-1372. doi:10.18632/oncotarget.1154

Liwak, U., Thakor, N., Jordan, L. E., Roy, R., Lewis, S. M., Pardo, O. E., . . . Holcik, M. (2012). Tumor suppressor PDCD4 represses internal ribosome entry site-mediated

translation of antiapoptotic proteins and is regulated by S6 kinase 2. *Mol Cell Biol, 32*(10), 1818-1829. doi:10.1128/MCB.06317-11

Ma, X. M., & Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol, 10*(5), 307-318. doi:10.1038/nrm2672

Maag, D., Fekete, C. A., Gryczynski, Z., & Lorsch, J. R. (2005). A conformational change in the eukaryotic translation preinitiation complex and release of eIF1 signal recognition of the start codon. *Mol Cell, 17*(2), 265-275. doi:10.1016/j.molcel.2004.11.051

Maduzia, L. L., Moreau, A., Poullet, N., Chaffre, S., & Zhang, Y. (2010). The role of eIF1 in translation initiation codon selection in Caenorhabditis elegans. *Genetics, 186*(4), 1187-1196. doi:10.1534/genetics.110.121541

Magnuson, B., Ekim, B., & Fingar, D. C. (2012). Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. *Biochem J, 441*(1), 1-21. doi:10.1042/BJ20110892

Marissen, W. E., Gradi, A., Sonenberg, N., & Lloyd, R. E. (2000). Cleavage of eukaryotic translation initiation factor 4GII correlates with translation inhibition during apoptosis. *Cell Death Differ, 7*(12), 1234-1243. doi:10.1038/sj.cdd.4400750

Martineau, Y.,Wang, X., Alain, T., Petroulakis, E., Shahbazian, D., Fabre, B., . . . Sonenberg, N. (2014). Control of Paip1-eukayrotic translation initiation factor 3 interaction by amino acids through S6 kinase. *Mol Cell Biol, 34*(6), 1046-1053. doi:10.1128/MCB.01079-13

Martinez-Salas, E., Francisco-Velilla, R., Fernandez-Chamorro, J., Lozano, G., & Diaz-Toledano, R. (2015). Picornavirus IRES elements: RNA structure and host protein interactions. *Virus Res, 206*, 62-73. doi:10.1016/j.virusres.2015.01.012

Martinez-Salas, E., Pacheco, A., Serrano, P., & Fernandez, N. (2008). New insights into internal ribosome entry site elements relevant for viral gene expression. *J Gen Virol, 89*(Pt 3), 611-626. doi:10.1099/vir.0.83426-0

McIlwain, D. R., Berger, T., & Mak, T. W. (2013). Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol, 5*(4), a008656. doi:10.1101/cshperspect.a008656

Mendoza, M. C., Er, E. E., & Blenis, J. (2011). The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends Biochem Sci, 36*(6), 320-328. doi:10.1016/j.tibs.2011.03.006

Miyamoto, S., Patel, P., & Hershey, J. W. (2005). Changes in ribosomal binding activity of eIF3 correlate with increased translation rates during activation of T lymphocytes. *J Biol Chem, 280*(31), 28251-28264. doi:10.1074/jbc.M414129200

Morris, D. R., & Geballe, A. P. (2000). Upstream open reading frames as regulators of mRNA translation. *Mol Cell Biol, 20*(23), 8635-8642.

Naeeni, A. R., Conte, M. R., & Bayfield, M. A. (2012). RNA chaperone activity of human La protein is mediated by variant RNA recognition motif. *J Biol Chem, 287*(8), 5472-5482. doi:10.1074/jbc.M111.276071

Nag, S., Qin, J., Srivenugopal, K. S., Wang, M., & Zhang, R. (2013). The MDM2-p53 pathway revisited. *J Biomed Res, 27*(4), 254-271. doi:10.7555/JBR.27.20130030

Nevins, T. A., Harder, Z. M., Korneluk, R. G., & Holcik, M. (2003). Distinct regulation of internal ribosome entry site-mediated translation following cellular stress is mediated by apoptotic fragments of eIF4G translation initiation factor family members eIF4GI and p97/DAP5/NAT1. *J Biol Chem, 278*(6), 3572-3579. doi:10.1074/jbc.M206781200

Nishi, H., Hashimoto, K., & Panchenko, A. R. (2011). Phosphorylation in protein-protein binding: effect on stability and function. *Structure, 19*(12), 1807-1815. doi:10.1016/j.str.2011.09.021

Novoa, I., & Carrasco, L. (1999). Cleavage of eukaryotic translation initiation factor 4G by exogenously added hybrid proteins containing poliovirus 2Apro in HeLa cells: effects on gene expression. *Mol Cell Biol, 19*(4), 2445-2454.

Oberoi-Khanuja, T. K., Murali, A., & Rajalingam, K. (2013). IAPs on the move: role of inhibitors of apoptosis proteins in cell migration. *Cell Death Dis, 4*, e784. doi:10.1038/cddis.2013.311

Owens, T. W., Gilmore, A. P., Streuli, C. H., & Foster, F. M. (2013). Inhibitor of Apoptosis Proteins: Promising Targets for Cancer Therapy. *J Carcinog Mutagen, Suppl 14*. doi:10.4172/2157-2518.S14-004

Pardo, O. E., & Seckl, M. J. (2013). S6K2: The Neglected S6 Kinase Family Member. *Front Oncol, 3*, 191. doi:10.3389/fonc.2013.00191

Parsyan, A., Svitkin, Y., Shahbazian, D., Gkogkas, C., Lasko, P., Merrick, W. C., & Sonenberg, N. (2011). mRNA helicases: the tacticians of translational control. *Nat Rev Mol Cell Biol, 12*(4), 235-245. doi:10.1038/nrm3083

Pelletier, J., Graff, J., Ruggero, D., & Sonenberg, N. (2015). Targeting the eIF4F translation initiation complex: a critical nexus for cancer development. *Cancer Res, 75*(2), 250-263. doi:10.1158/0008-5472.CAN-14-2789

Pelletier, J., & Sonenberg, N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature, 334*(6180), 320-325. doi:10.1038/334320a0

Porta, C., Paglino, C., & Mosca, A. (2014). Targeting PI3K/Akt/mTOR Signaling in Cancer. *Front Oncol, 4*, 64. doi:10.3389/fonc.2014.00064

Prabakaran, S., Lippens, G., Steen, H., & Gunawardena, J. (2012). Post-translational modification: nature's escape from genetic imprisonment and the basis for dynamic information encoding. *Wiley Interdiscip Rev Syst Biol Med, 4*(6), 565-583. doi:10.1002/wsbm.1185

Pugh, C. W., Gleadle, J., & Maxwell, P. H. (2001). Hypoxia and oxidative stress in breast cancer. Hypoxia signalling pathways. *Breast Cancer Res, 3*(5), 313-317.

Ray, P. S., Grover, R., & Das, S. (2006). Two internal ribosome entry sites mediate the translation of p53 isoforms. *EMBO Rep, 7*(4), 404-410. doi:10.1038/sj.embor.7400623

Ren, Y. G., Wagner, K. W., Knee, D. A., Aza-Blanc, P., Nasoff, M., & Deveraux, Q. L. (2004). Differential regulation of the TRAIL death receptors DR4 and DR5 by the signal recognition particle. *Mol Biol Cell, 15*(11), 5064-5074. doi:10.1091/mbc.E04-03-0184

Rojas, M., Arias, C. F., & Lopez, S. (2010). Protein kinase R is responsible for the phosphorylation of eIF2alpha in rotavirus infection. *J Virol, 84*(20), 10457-10466. doi:10.1128/JVI.00625-10

Roy, R., Durie, D., Li, H., Liu, B. Q., Skehel, J. M., Mauri, F., . . . Pardo, O. E. (2014). hnRNPA1 couples nuclear export and translation of specific mRNAs downstream of FGF-2/S6K2 signalling. *Nucleic Acids Res, 42*(20), 12483-12497. doi:10.1093/nar/gku953

Safa, A. R. (2013). Roles of c-FLIP in Apoptosis, Necroptosis, and Autophagy. *J Carcinog Mutagen, Suppl 6*. doi:10.4172/2157-2518.S6-003

Safa, A. R., & Pollok, K. E. (2011). Targeting the Anti-Apoptotic Protein c-FLIP for Cancer Therapy. *Cancers (Basel), 3*(2), 1639-1671. doi:10.3390/cancers3021639

Sarnow, P. (1989). Translation of glucose-regulated protein 78/immunoglobulin heavy-chain binding protein mRNA is increased in poliovirus-infected cells at a time when cap-dependent translation of cellular mRNAs is inhibited. *Proc Natl Acad Sci U S A, 86*(15), 5795-5799.

Schimmer, A. D. (2004). Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. *Cancer Res, 64*(20), 7183-7190. doi:10.1158/0008-5472.CAN-04-1918

Schmid, T., Jansen, A. P., Baker, A. R., Hegamyer, G., Hagan, J. P., & Colburn, N. H. (2008). Translation inhibitor Pdcd4 is targeted for degradation during tumor promotion. *Cancer Res, 68*(5), 1254-1260. doi:10.1158/0008-5472.CAN-07-1719

Scoles, D. R., Yong, W. H., Qin, Y., Wawrowsky, K., & Pulst, S. M. (2006). Schwannomin inhibits tumorigenesis through direct interaction with the eukaryotic initiation factor subunit c (eIF3c). *Hum Mol Genet, 15*(7), 1059-1070. doi:10.1093/hmg/ddl021

Sesen, J., Cammas, A., Scotland, S. J., Elefterion, B., Lemarie, A., Millevoi, S., . . . Skuli, N. (2014). Int6/eIF3e is essential for proliferation and survival of human glioblastoma cells. *Int J Mol Sci, 15*(2), 2172-2190. doi:10.3390/ijms15022172

Shah, M., Su, D., Scheliga, J. S., Pluskal, T., Boronat, S., Motamedchaboki, K., . . . Wolf, D. A. (2016). A Transcript-Specific eIF3 Complex Mediates Global Translational

Control of Energy Metabolism. *Cell Rep, 16*(7), 1891-1902. doi:10.1016/j.celrep.2016.07.006

Shahbazian, D., Roux, P. P., Mieulet, V., Cohen, M. S., Raught, B., Taunton, J., . . . Sonenberg, N. (2006). The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. *EMBO J, 25*(12), 2781-2791. doi:10.1038/sj.emboj.7601166

Sharma, D. K., Bressler, K., Patel, H., Balasingam, N., & Thakor, N. (2016). Role of Eukaryotic Initiation Factors during Cellular Stress and Cancer Progression. *J Nucleic Acids, 2016*, 8235121. doi:10.1155/2016/8235121

Sherrill, K. W., Byrd, M. P., Van Eden, M. E., & Lloyd, R. E. (2004). BCL-2 translation is mediated via internal ribosome entry during cell stress. *J Biol Chem, 279*(28), 29066-29074. doi:10.1074/jbc.M402727200

Shi, J., Hershey, J. W., & Nelson, M. A. (2009). Phosphorylation of the eukaryotic initiation factor 3f by cyclin-dependent kinase 11 during apoptosis. *FEBS Lett, 583*(6), 971-977. doi:10.1016/j.febslet.2009.02.028

Shi, Y. (2004). Caspase activation, inhibition, and reactivation: a mechanistic view. *Protein Sci, 13*(8), 1979-1987. doi:10.1110/ps.04789804

Showkat, M., Beigh, M. A., & Andrabi, K. I. (2014). mTOR Signaling in Protein Translation Regulation: Implications in Cancer Genesis and Therapeutic Interventions. *Mol Biol Int, 2014*, 686984. doi:10.1155/2014/686984

Silke, J., & Meier, P. (2013). Inhibitor of apoptosis (IAP) proteins-modulators of cell death and inflammation. *Cold Spring Harb Perspect Biol, 5*(2). doi:10.1101/cshperspect.a008730

Song, M. S., Salmena, L., & Pandolfi, P. P. (2012). The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol, 13*(5), 283-296. doi:10.1038/nrm3330

Song, N., Wang, Y., Gu, X. D., Chen, Z. Y., & Shi, L. B. (2013). Effect of siRNA-mediated knockdown of eIF3c gene on survival of colon cancer cells. *J Zhejiang Univ Sci B, 14*(6), 451-459. doi:10.1631/jzus.B1200230

Spilka, R., Ernst, C., Mehta, A. K., & Haybaeck, J. (2013). Eukaryotic translation initiation factors in cancer development and progression. *Cancer Lett, 340*(1), 9-21. doi:10.1016/j.canlet.2013.06.019

Spriggs, K. A., Cobbold, L. C., Jopling, C. L., Cooper, R. E., Wilson, L. A., Stoneley, M., . . . Willis, A. E. (2009). Canonical initiation factor requirements of the Myc family of internal ribosome entry segments. *Mol Cell Biol, 29*(6), 1565-1574. doi:10.1128/MCB.01283-08

Sun, C., Todorovic, A., Querol-Audi, J., Bai, Y., Villa, N., Snyder, M., . . . Cate, J. H. (2011). Functional reconstitution of human eukaryotic translation initiation factor 3 (eIF3). *Proc Natl Acad Sci U S A, 108*(51), 20473-20478. doi:10.1073/pnas.1116821108

Sutton, V. R., Davis, J. E., Cancilla, M., Johnstone, R. W., Ruefli, A. A., Sedelies, K., . . . Trapani, J. A. (2000). Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. *J Exp Med, 192*(10), 1403-1414.

Suzuki, C., Garces, R. G., Edmonds, K. A., Hiller, S., Hyberts, S. G., Marintchev, A., & Wagner, G. (2008). PDCD4 inhibits translation initiation by binding to eIF4A using both its MA3 domains. *Proc Natl Acad Sci U S A, 105*(9), 3274-3279. doi:10.1073/pnas.0712235105

Sweeney, T. R., Abaeva, I. S., Pestova, T. V., & Hellen, C. U. (2014). The mechanism of translation initiation on Type 1 picornavirus IRESs. *EMBO J, 33*(1), 76-92. doi:10.1002/embj.201386124

Tavares, M. R., Pavan, I. C., Amaral, C. L., Meneguello, L., Luchessi, A. D., & Simabuco, F. M. (2015). The S6K protein family in health and disease. *Life Sci, 131*, 1-10. doi:10.1016/j.lfs.2015.03.001

Taylor, R. C., Cullen, S. P., & Martin, S. J. (2008). Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol, 9*(3), 231-241. doi:10.1038/nrm2312

Terenin, I. M., Andreev, D. E., Dmitriev, S. E., & Shatsky, I. N. (2013). A novel mechanism of eukaryotic translation initiation that is neither m7G-cap-, nor IRES-dependent. *Nucleic Acids Res, 41*(3), 1807-1816. doi:10.1093/nar/gks1282

Thakor, N., Smith, M. D., Roberts, L., Faye, M. D., Patel, H., Wieden, H. J., . . . Holcik, M. (2017). Cellular mRNA recruits the ribosome via eIF3-PABP bridge to initiate internal translation. *RNA Biol, 14*(5), 553-567. doi:10.1080/15476286.2015.1137419

Thompson, S. R. (2012). So you want to know if your message has an IRES? *Wiley Interdiscip Rev RNA, 3*(5), 697-705. doi:10.1002/wrna.1129

Tsai, B. P., Jimenez, J., Lim, S., Fitzgerald, K. D., Zhang, M., Chuah, C. T., . . . Waterman, M. L. (2014). A novel Bcr-Abl-mTOR-eIF4A axis regulates IRES-mediated translation of LEF-1. *Open Biol, 4*(11), 140180. doi:10.1098/rsob.140180

Tsujimoto, Y. (1998). Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes Cells, 3*(11), 697-707.

van Gorp, A. G., van der Vos, K. E., Brenkman, A. B., Bremer, A., van den Broek, N., Zwartkruis, F., . . . Coffer, P. J. (2009). AGC kinases regulate phosphorylation and activation of eukaryotic translation initiation factor 4B. *Oncogene, 28*(1), 95-106. doi:10.1038/onc.2008.367

Vikhreva, P. N., & Korobko, I. V. (2014). Expression of Pdcd4 tumor suppressor in human melanoma cells. *Anticancer Res, 34*(5), 2315-2318.

Vikhreva, P. N., Shepelev, M. V., Korobko, E. V., & Korobko, I. V. (2010). [Pdcd4 tumor suppressor: properties, functions, and their application to oncology]. *Mol Gen Mikrobiol Virusol*(2), 3-11.

Walters, B., & Thompson, S. R. (2016). Cap-Independent Translational Control of Carcinogenesis. *Front Oncol, 6*, 128. doi:10.3389/fonc.2016.00128

Wang, G., Wang, J. J., Tang, H. M., & To, S. S. (2015). Targeting strategies on miRNA-21 and PDCD4 for glioblastoma. *Arch Biochem Biophys, 580*, 64-74. doi:10.1016/j.abb.2015.07.001

Wei, N., Liu, S. S., Chan, K. K., & Ngan, H. Y. (2012). Tumour suppressive function and modulation of programmed cell death 4 (PDCD4) in ovarian cancer. *PLoS One, 7*(1), e30311. doi:10.1371/journal.pone.0030311

Weingarten-Gabbay, S., Elias-Kirma, S., Nir, R., Gritsenko, A. A., Stern-Ginossar, N., Yakhini, Z., . . . Segal, E. (2016). Comparative genetics. Systematic discovery of cap-independent translation sequences in human and viral genomes. *Science, 351*(6270). doi:10.1126/science.aad4939

Wen, F., Zhou, R., Shen, A., Choi, A., Uribe, D., & Shi, J. (2012). The tumor suppressive role of eIF3f and its function in translation inhibition and rRNA degradation. *PLoS One, 7*(3), e34194. doi:10.1371/journal.pone.0034194

Wigington, C. P., Jung, J., Rye, E. A., Belauret, S. L., Philpot, A. M., Feng, Y., . . . Corbett, A. H. (2015). Post-transcriptional regulation of programmed cell death 4 (PDCD4) mRNA by the RNA-binding proteins human antigen R (HuR) and T-cell intracellular antigen 1 (TIA1). *J Biol Chem, 290*(6), 3468-3487. doi:10.1074/jbc.M114.631937

Xu, C., Bailly-Maitre, B., & Reed, J. C. (2005). Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest, 115*(10), 2656-2664. doi:10.1172/JCI26373

Yang, H. S., Cho, M. H., Zakowicz, H., Hegamyer, G., Sonenberg, N., & Colburn, N. H. (2004). A novel function of the MA-3 domains in transformation and translation suppressor Pdcd4 is essential for its binding to eukaryotic translation initiation factor 4A. *Mol Cell Biol, 24*(9), 3894-3906.

Yang, H. S., Jansen, A. P., Komar, A. A., Zheng, X., Merrick, W. C., Costes, S., . . . Colburn, N. H. (2003). The transformation suppressor Pdcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation. *Mol Cell Biol, 23*(1), 26-37.

Yang, R., Wek, S. A., & Wek, R. C. (2000). Glucose limitation induces GCN4 translation by activation of Gcn2 protein kinase. *Mol Cell Biol, 20*(8), 2706-2717.

Yu, X., Zheng, B., & Chai, R. (2014). Lentivirus-mediated knockdown of eukaryotic translation initiation factor 3 subunit D inhibits proliferation of HCT116 colon cancer cells. *Biosci Rep, 34*(6), e00161. doi:10.1042/BSR20140078

Zarogoulidis, P., Lampaki, S., Turner, J. F., Huang, H., Kakolyris, S., Syrigos, K., & Zarogoulidis, K. (2014). mTOR pathway: A current, up-to-date mini-review (Review). *Oncol Lett, 8*(6), 2367-2370. doi:10.3892/ol.2014.2608

Zhang, X., Gee, H., Rose, B., Lee, C. S., Clark, J., Elliott, M., . . . Tran, N. (2016). Regulation of the tumour suppressor PDCD4 by miR-499 and miR-21 in oropharyngeal cancers. *BMC Cancer, 16*, 86. doi:10.1186/s12885-016-2109-4

Zhang, X., Zou, T., Rao, J. N., Liu, L., Xiao, L., Wang, P. Y., . . . Wang, J. Y. (2009). Stabilization of XIAP mRNA through the RNA binding protein HuR regulated by cellular polyamines. *Nucleic Acids Res, 37*(22), 7623-7637. doi:10.1093/nar/gkp755

Zhen, Y., Li, D., Li, W., Yao, W., Wu, A., Huang, J., . . . Wu, B. (2016). Reduced PDCD4 Expression Promotes Cell Growth Through PI3K/Akt Signaling in Non-Small Cell Lung Cancer. *Oncol Res, 23*(1-2), 61-68. doi:10.3727/096504015X14478843952861

Zhen, Y., Liu, Z., Yang, H., Yu, X., Wu, Q., Hua, S., . . . Fang, W. (2013). Tumor suppressor PDCD4 modulates miR-184-mediated direct suppression of C-MYC and BCL2 blocking cell growth and survival in nasopharyngeal carcinoma. *Cell Death Dis, 4*, e872. doi:10.1038/cddis.2013.376