Regulation of the cellular inhibitor of apoptosis 1 (cIAP1) translation by IRES trans-acting factors and impact on cancer

by **Mame Daro Faye,**

M.Sc. Biochemistry

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

Abstract

Apoptosis is the mechanism by which complex multicellular organisms induce the programmed death of damaged cells, thus maintaining tissue homeostasis. One of the main hallmarks of cancer, apoptosis is tightly regulated by pro- and anti-apoptotic factors whose equilibrium will decide of the fate of the cell. Among these factors, the cellular inhibitor of apoptosis cIAP1 is a key regulator of nuclear factor-κB dependent signaling and of caspase-8 mediated apoptosis. cIAP1 expression is controlled primarily at the translational level through an internal ribosome entry site (IRES) that facilitates the recruitment of the ribosome to the translation initiation start independently of the 5' cap. We have previously identified four putative IRES trans-acting factors (ITAFs) that bind specifically to the cIAP1 IRES, namely NF45, NF90, IGF2BP1 and RH1. My research project characterised NF45 as an ITAF that positively regulates the IRES-mediated translation of cIAP1 and of the X-linked inhibitor of apoptosis, XIAP. This regulation is important for maintaining Survivin and Cyclin E protein levels and insuring proper cell division. Furthermore, I showed that IGF2BP1 is another ITAF that is overexpressed in rhabdomyosarcoma cancer (RMS) and positively regulates cIAP1 translation, thus leading to apoptotic resistance in these cells. Importantly, the use of Smac mimetics, chemical compounds that cause cIAP1 proteasomal degradation, induces TNFα-mediated apoptosis of RMS cells and leads to growth inhibition of RMS xenograft tumors as well as significantly improved survival. Finally, I show that certain modulators of innate immunity synergize with Smac mimetics to improve the killing of RMS cancer cells. Hence, cIAP1 translation regulation by NF45 and IGF2BP1 is highly important for maintaining proper functioning of the cell and dysregulation of these ITAFs can lead to carcinogenesis.

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List of Abbreviations

ALL	Acute lymphoblastic leukemia
APAF1	Apoptotic protease activating factor 1
ARE	AU-rich element
Bcl-2	B-cell CLL/lymphoma 2
Bcl-xL	Bcl-2-like 1, extra large
β-GAL	β-Galactosidase
BH	Bcl-2 homology domain
BIR	Baculovirus IAP repeat
BIR	Baculovirus IAP repeat-containing protein 2
BSA	Bovine serum albumin
c-Myc	Cellular-myelocytomatosis oncogene
CAT	Chloramphenicol acetyl transferase
Cdk	Cyclin-dependent kinase
cDNA	complementary DNA
cIAP1	cellular inhibitor of apoptosis protein 1
cIAP2	cellular inhibitor of apoptosis protein 2
CLL	Chronic lymphocytic leukaemia
CRD-BP	Coding region determinant-binding protein
CrPV	Cricket paralysis virus
DAP5/p97	Death-associated protein 5/protein 97
DIABLO	Direct IAP binding protein with low pI
DISC	Death inducing signaling complex
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediamine tetraacetic acid
eIF	Eukaryotic initiation factor
ELG1	Enhanced level of genome instability 1
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephelomyocarditis virus
ER	Endoplasmic reticulum
FADD	Fas-associated death domain protein
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor -2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glioblastoma mutiforme
GCN2	General control non-derepressible-2
GST	Glutathione s- transferase
HCV	Hepatitis C virus
HIAP2	Human inhibitor of apoptosis protein 2
HEK293	Human embryonic kidney cells
HIV-2	Human immunodeficiency virus type 2
hnRNP A1	Heterogeneous ribonucleoprotein A1

hnRNP C1/C2	Heterogeneous ribonucleoprotein C1/C2
HRI	Haem-regulated inhibitor
HRV	Human rhinovirus
HuR	Human antigen R
IAP	Inhibitor of apoptosis protein
IGF2	Insulin-like growth factor 2
IGF2BP1	Insulin-like growth factor 2 mRNA-binding protein
IKK	Inhibitor of κB kinase
IMP1	IGF2 mRNA binding protein 1
IPTG	Isopropythiol-β-galactoside
IR	Ionizing radiation
IRES	Internal ribosome entry site
ITAF	IRES trans-acting factor
KOC	K homology domain containing protein overexpressed in cancer
La	La autoantigen
LB	Luria-Bertani
m7G	7-methylguanosine
MAPK	Mitogen-activated protein kinase
MIHB	Mammalian IAP homolog B
miRNA	MicroRNA
mRNA	Messenger RNA
mRNP	Messenger ribonucleoprotein
MYT2	Myelin transcription factor 2
NF-κB	Nuclear factor-κB
NFκBIβ	Nuclear factor-κB inhibitor β
NF45	Nuclear factor 45
NF90	Nuclear factor 90
NIK	NF-kB inactivating kinase
NRF	NF-kB repressing factor
Omi/HtrA2	OMI/High temperature requirement A2
ONPG	Ortho-nitrophenyl-β-galactoside
ORF	Open reading frame
PABP	PolyA-binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDCD4	Programmed cell death gene 4
PERK	PKR-like endoplasmic reticulum kinase
PKC	Protein kinase C
PKR	Protein kinase activated by double-stranded RNA
PMSF	Phenylmethylsulfonyl fluoride

PTB	Polypyrimidine tract binding protein
qRT-PCR	Quantitative reverse transcription - polymerase chain reaction
RING	Really interesting new gene
RIP	Receptor interacting protein
RIPA	Radioimmunoprecipitation assay
RISC	RNA induced silencing complex
RPL13A	Ribosomal protein large subunit 13A
RRM	RNA recognition motif
rRNA	Ribosomal RNA
S6K1	Ribosomal protein S6 kinase 1
S6K2	Ribosomal protein S6 kinase 2
SCLC	Small cell lung cancer
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
Smac	Second mitochondria-derived activator of caspase
SNM1	Sensitivity to nitrogen mustard 1
TNF-α	Tumour necrosis factor- α
TNFR	Tumour necrosis factor receptor
TRAF	Tumour necrosis factor receptor associated factor
TRAIL	TNF-α-related apoptosis-inducing ligand
UNR	Upstream of N-Ras
uORF	Upstream open reading frame
UPR	Unfolded protein response
UTR	Untranslated region
UPR	Unfolded protein response
UVR	Ultraviolet irradiation
VCIP	Vascular endothelial growth factor and type I collagen inducible protein
X-DC	X-linked dyskeratosis congenita
XAF1	XIAP associated factor 1
XIAP	X-linked inhibitor of apoptosis protein
ZBP1	Zipcode binding protein

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CHAPTER 1

General Introduction

1.1 Preamble

This introductory chapter provides a broad overview of the literature on the translation control in apoptosis and in cancer as well as the current knowledge on the expression regulation of the cellular inhibitor of apoptosis 1 (cIAP1). Section 1.2 introduces the reader to the concept of RNA metabolism and its importance in carcinogenesis. Sections 1.3 consists of an introduction to the apoptosis pathway and the proteins regulating it. Section 1.4 describes cIAP1 roles in cell survival and how its expression is regulated, with a specific focus on cIAP1 IRES-mediated translation and regulation by IRES trans-acting factors (ITAFs). Section 1.5 presents the hypothesis and specific objectives of this thesis.

1.2 The importance of RNA metabolism in cancer

More than a century ago, it was proposed that cancer arises from chromosomal derangements that pushed cells to divide uncontrollably.[1] Many years later, this concept was confirmed by the discovery of cancer causing gene mutations[2, 3] and ever since, the efforts of the molecular biology scientific community have focused on discovering more mutated genes and how they relate to cancer. This was facilitated by the launch of the Human Genome Project in 1990, which had for goal to identify and map all human genes and was declared nearly completed in 2003.[4] This concerted effort of the scientific community led to the discovery of many tumor suppressors and oncogenes whose dysfunction caused cancer; and to many advances in cancer therapeutics. However, it was quickly realised that the causal relationship between a gene mutation and its consequence on the protein output and subsequently on cancer development was more complex than the simple dogma of molecular biology first proposed by Francis Crick in 1956, according to which one gene gives rise to a single RNA which gives rise to a single protein.[5] Indeed, genome and exome sequencing have revealed the existence of gene mutations that lead to aberrant alternative splicing and generation of different protein isoforms with altered functions in cancer.[6, 7] With the discovery of microRNAs and long non-coding RNAs, it also became evident that RNA can not only control RNA stability and translation[8] but also DNA transcription.[9] Therefore, the unidirectional dogma of genetic flow only from DNA to RNA to protein did not apply anymore. It was also quickly realised that the cell responded to stresses, as is the case in cancer, by regulating protein output not only through modifications of its transcription program but for a quicker and more efficient response to the stress, mainly through post-transcriptional modes of regulation such as mRNA export, localization, stability and

translation, to name just a few.[10, 11] Hence, a new field of studying these modes of genetic regulation, the field of ‘‘RNA metabolism’’, was born and its importance in regulating carcinogenesis is now being recognised.

In particular, the regulation of protein translation plays an important role in cancer initiation and progression. Indeed, it was discovered years ago that cancer cells require increased rates of protein synthesis to sustain increased proliferation and this correlated with an increase in ribosome biogenesis,[12] thus explaining why transformed cells have enlarged nucleoli, the site of ribosome biogenesis.[13] Furthermore, genetic alterations in the components of the translation machinery have been linked to spontaneous cancers and in particular, mutations in components of the ribosome lead to ‘‘ribosomopathies’’ that are associated with increased cancer susceptibility to a subset of cancers, as is the case in X-linked dyskeratosis congenita (X-DC).[14] But maybe more important is the fact that some of the major signalling pathways that are involved in carcinogenesis, such as the Myc and PI3K pathways, alter the levels and activities of components of the translation machinery in order to control gene expression at the protein translation level, all to the benefit of the cancer cells.[15] Finally, cancer cells are often resistant to acute cellular stresses such as nutrient deprivation, DNA damage and hypoxia which usually would lead to an inhibition of global protein synthesis and cell death, and they do so by reprogramming their translation program in order to sustain the synthesis of pro-survival and anti-apoptotic proteins that will allow them to survive.[16] Hence, protein translation impinges on the regulation of different hallmarks of cancer such as proliferation, resistance to cell death, angiogenesis and metastasis.

1.3 Apoptosis

Among the hallmarks of cancer, apoptosis is probably one of the most tightly controlled. Indeed, several proteins involved in the apoptotic response are selectively regulated in conditions of stress in order to decide of the fate of the cell, either survival or death. Here, I will provide a brief overview of the apoptosis pathway and of apoptosis regulating proteins.

1.3.1 Brief overview of the apoptosis pathway

Apoptosis is the mechanism by which metazoan organisms induce the programmed death of damaged cells, thus maintaining tissue homeostasis. Apoptosis is also important for tissue remodeling and repair during development and differentiation, by inducing the ‘‘suicide’’ of excess or damaged cells with high specificity.[17] Regulation of apoptosis is a critical process in metazoan as insufficient apoptosis can lead to cancers or autoimmune disease whereas excess apoptosis can lead to degenerative disease or immunodeficiency.[18]

The main effectors of the apoptosis pathways are caspases, a family of 11 cysteine proteases in humans, that cleave proteins in the cell specifically at cysteine aspartyl residues and can be grouped in initiating caspases or effector ones.[19] Activation of caspases results in the cleavage of key protein components necessary for important cellular processes such as structural proteins of the cystoskeleton and DNA repair proteins. Caspases can also activate other degradating enzymes such as DNAses that will in turn cleave the DNA in the nucleus. All of these events underlie the specific morphological changes observed during apoptosis: cell shrinkage, DNA fragmentation and membrane blebbing.[20] The apoptotic cells are then

cleared by macrophages in a tidy process that avoids inflammation of the neighboring tissues.[20]

Caspases can be activated by two main apoptotic pathways, namely the intrinsic and the extrinsic apoptotic pathways, depending on the nature and origin of the signalling trigger. Extracellular ligand binding to death receptors such as the tumor necrosis factor α receptors (TNFR), Fas ligand or TRAIL receptor, triggers oligomerization of these death receptors and the formation of a death inducing signalling complex (DISC). In the case of TNFα binding to the TNFR1 receptor, the DISC complex is composed of TNFRSF1A-associated via death domain (TRADD), TNFR-associated factor 2 (TRAF2), TRAF5 and the protein kinase RIPK1 proteins among other accessory proteins.[21] Release of the DISC complex leads to recruitment of the Fas-associated death domain (FADD) through its interaction with TRADD and subsequent recruitment of caspase 8.[21, 22] Caspase 8 is then auto-activated through induced proximity and can activate the effector caspases 3 and 7 which will in turn proceed with the execution phase of the apoptotic program described (Figure 1.1).The intrinsic or mitochondrial apoptotic pathway is activated by internal triggers such as DNA damage or oncogene activation.[11, 22] These triggers activate the mitochondrial protein Bid into tBid, which induces conformational changes into the B-cell CLL/lymphoma 2 (Bcl2) family proteins Bak and Bax. This leads to the insertion of Bak and Bax into the outer mitochondrial membrane, permeabilisation of the membrane and release of Cytochrome c into the cytosol. Cytochrome c then binds the apoptotic protease activating factor 1 (APAF1) and pro-caspase 9, inducing formation of the apoptosome. Pro-caspase 9 is processed into active caspase 9 within the apoptosome and caspase 9 subsequently cleaves and activates the effector caspases 3 and 7 (Figure 1.1).[11, 23] Bak and Bax permeabilisation of the outer

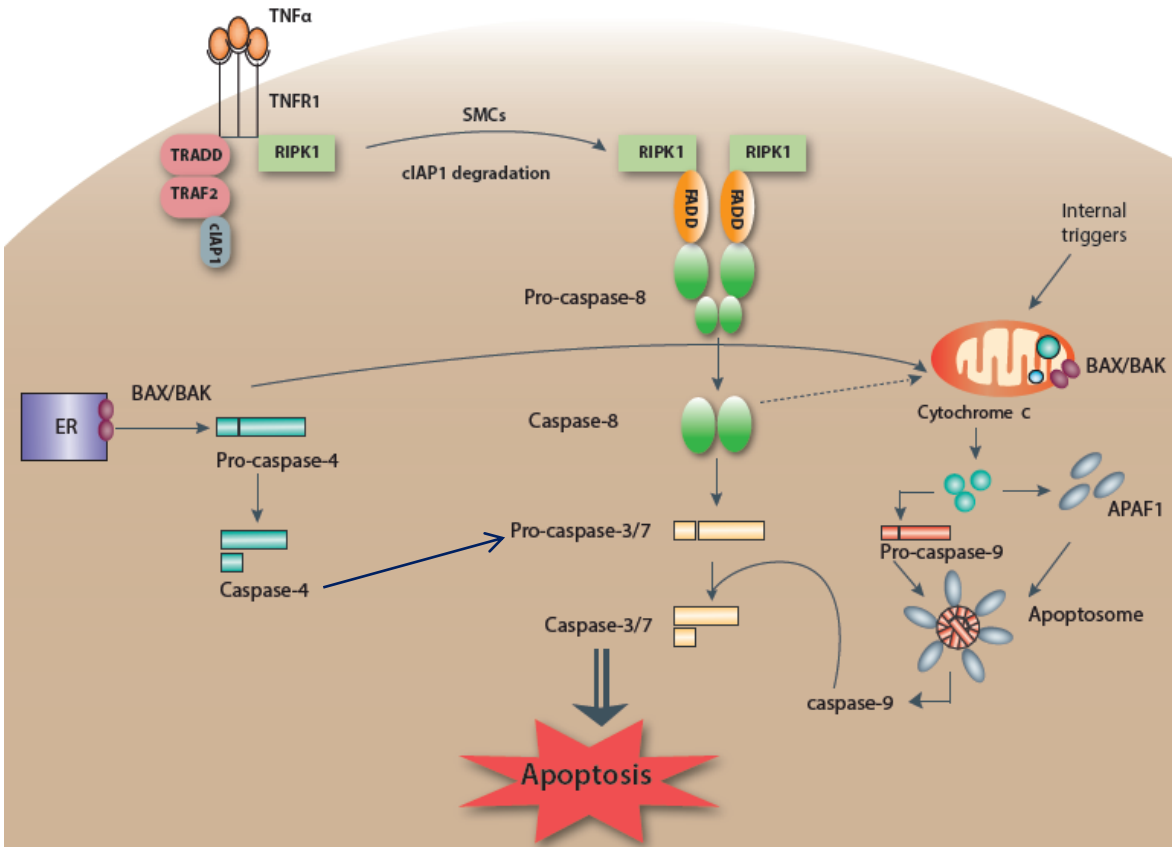
TNFα
TNFR1
TRADD
TRAF2
cIAP1
RIPK1
SMCs
cIAP1 degradation
RIPK1
RIPK1
FADD
FADD
Pro-caspase-8
Internal triggers
BAX/BAK
Cytochrome c
ER
BAX/BAK
Pro-caspase-4
Caspase-8
APAF1
Pro-caspase-9
Pro-caspase-3/7
Caspase-4
Caspase-3/7
Apoptosome
caspase-9
Apoptosis

Figure 1.1: Description of the apoptosis pathways

Schematic depicting the different ways to apoptosis. In the extrinsic pathway, binding of a death ligand such as TNFα to its receptor (TNFR1 in this case) leads to the formation the DISC complex in the absence of cIAP1, the platform for pro-caspase 8 activation. Active caspase 8 then cleaves the effector caspases 3 and 7, leading to their activation and apoptosis. In the intrinsic pathway, BAX/BAK-mediated permeabilisation of the mitochondrial membrane leads to cytochrome c release, formation of the apoptosome that activates caspases 9, and subsequent activation for caspases 3 and 7. Finally, in the ER-specific intrinsic pathway, specific triggers leading to endoplasmic reticulum stress (ER stress) can activate caspase 4 leading to apoptosis. Figure adapted from [11]

mitochondrial membrane also induces the release of other apoptosis regulating proteins such as mitochondria-derived activator of caspase (Smac)/direct IAP binding protein with low pI (DIABLO)[24, 25] and Omi/HtrA2[26] which bind to and inhibit anti-apoptotic inhibitor of apoptosis proteins (IAP), thus further promoting caspase activation. The apoptosis inducing factor (AIF) is also released upon mitochondrial membrane permeabilisation and participates in DNA fragmentation and chromatin condensation.[27] Endoplasmic reticulum (ER) stress also results in the activation of a third apoptotic pathway that is ER-specific. This pathway leads to the activation of caspase-4 in humans and subsequently effector caspases 3 and 7.[11] Importantly, the extrinsic apoptosis pathway can feed into the intrinsic one through activation of Bid by caspase 8 and the same is observed during ER stress as well, thus leading to amplification of the apoptotic signals which will ultimately result in the activation of the effector caspases 3 and 7 (Figure 1.1).[11]

1.3.2 Regulation of the apoptosis pathways

Because apoptosis is such an important process for tissue homeostasis, it is tightly regulated by pro- and anti-apoptotic factors that will decide of the fate of a given cell following an insult or developmental cue. There are two main families of proteins that are involved in the inhibition of apoptosis, namely the Inhibitor of Apoptosis proteins (IAP) family and the Bcl2 family of proteins.

The IAP family is composed of eight members in mammals: NAIP (BIRC1), cIAP1 (BIRC2), cIAP2 (BIRC3), XIAP (BIRC4), Survivin (BIRC5), Bruce (BIRC6), livin (ML-IAP, BIRC7) and ILP2 (BIRC8).[28, 29] IAP proteins are characterised by the presence of a baculovirus IAP repeat (BIR) domain, in one, two or three copies depending of the IAP.[29]

This domain mediates protein-protein interactions and is essential for the anti-apoptotic function of the IAPs.[30, 31] Most IAPs at the exception of NAIP, Survivin, and Bruce, also contain a C-terminal Really Interesting New Gene (RING) domain that gives them Ubiquitin ligase activity[32] and some IAPs contain an Ubiquitin associated (UBA) domain that interacts with ubiquitinated proteins.[33, 34] In addition, the cellular IAPs cIAP1 and cIAP2 contain a caspase recruitment domain (CARD) whose function is not well characterized[23] but that was recently shown to participate in cIAP1 autoregulation by inhibiting its E3 ligase activity.[35] Interestingly, of all IAPs, only XIAP has the potential to directly inhibit caspase activity *in vivo*,[36] either by binding to the active site of caspase 3 and 7[31] or by preventing the homodimerisation and activation of caspase 9.[37] Although cIAP1 and cIAP2 are inefficient in directly inhibiting caspase activity, they play an important role in cell survival by regulating the NF-κB pathway and by inhibiting caspase 8 activation through the extrinsinc apoptosis pathway (discussed below). Survivin also plays an important role in inhibiting apoptosis, mainly through its association with XIAP to protect the latter from proteasomal degradation.[38]

The Bcl2 family contains both pro- and anti-apoptotic proteins that are defined by the presence of the Bcl-2 homology domains (BH1 to BH4). The anti-apoptotic proteins of the family include Bcl-2, Bcl-xL, Bcl-W, Mcl-1, Bcl2A1, and Bcl-B which all contain the four types of BH domains. The pro-apoptotic proteins consist of members of the Bax family (Bax, Bak, Bok) that contain BH1, BH2 and BH3 domains, and the BH3-only members (Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma, Blk, BNIP3, Spike).[39] Anti-apoptotic Bcl2 members function by binding and sequestering BH3-only proteins, as well as Bak and Bax,

thus preventing mitochondrial membrane permeabilisation, cytochrome c release and caspase 9 activation.[40]

Interestingly, the expression of several of the IAP and Bcl2 family of proteins is regulated at the translation level in order to rapidly respond to an apoptotic signal. Examples of such regulation are discussed in Chapter 2.

1.4 Cellular inhibitor of apoptosis 1 (cIAP1)

1.4.1 Important functions in cell survival

cIAP1 (also known as BIRC2, HIAP2 or MIHB) was first identified through its interaction with the TNFR associated factors TRAF1 and TRAF2.[41] cIAP1 and its homolog cIAP2 are key regulators of cell survival and nuclear factor-κB (NF-κB) dependent signaling in mammalian cells.[23, 29, 42] Indeed, although cIAP1 has been shown to directly interact with effector caspases and weakly block apoptosis in *vitro*,[43] its most important contribution in modulating cell survival is through its ubiquitin-dependent regulation of both the canonical (RelA/p50) and non-canonical NF-κB (RelB/p52) pathways (reviewed in [23]). Binding of TNFα to the TNFR1 receptor triggers the formation of a complex referred to as complex I and composed of TRADD, TRAF2, TRAF5, RIPK1, cIAP1 and cIAP2.[21] Through its RING domain, cIAP1 functions as an E3 ligase that ubiquitinates components of complex I such as RIPK1, thus promoting the recruitment of the linear ubiquitin chain assembly complex (LUBAC) as well as the kinases TAK1–TAB2–TAB3 and IKKγ–IKKα–IKKβ.[44] Once recruited, LUBAC conjugates M1-linked Ubiquitin chains on IKKγ to further stabilize complex I.[45] This leads to phosphorylation of the NFκB inhibitor β (NFκBIβ) by IKKβ and its ubiquitin-dependent proteasomal degradation, thus allowing NFκB (RelA/p50) to

translocate to the nucleus where it drives the expression of pro-survival target genes.[23] cIAP1 is thus essential in driving TNFR1-mediated activation of the canonical NF- κB pathway (Figure 1.2).[46, 47] Importantly, by doing so, cIAP1 prevents at the same time the RIPK1-dependent activation of caspase 8,[48] further favoring cell survival (Figure 1.2). Indeed, only in the absence of cIAP1 and cIAP2 can the RIPK1-containing DISC complex be available to form the activating platform for caspase 8 as described above.[49, 50] Furthermore, under ligand-induced activation of other TNFR family members such as CD40, recruitment of cIAP1 at the receptor prevents the ubiquitin-dependent degradation of the NF-κB inducing kinase (NIK), leading activation of the non-canonical NF-κB pathway (Figure 1.2).[51] Indeed, accumulation of NIK causes its auto-activation and phosphorylation of IKKα which in turn phosphorylates NFκB2 (p100), leading to proteasome-dependent partial degradation into its active p52 form and translocation of the non-canonical RelB/p52 to the nucleus.[52]

The important role played by cIAP1 is not only in inhibiting apoptosis but also in favoring cell survival by ubiquitin-dependent signaling events that leads to activation of the NF-κB pathway. Therefore, overexpression as well as loss of cIAP1 can result in deregulated NFκB activation, tumour cell survival and chemoresistance depending on the cellular context.[53] Indeed, loss of the IAPs favours the development of multiple myolema whereas genomic amplification of 11q22, which contains the cIAP1 and cIAP2, happens at high frequency in medulloblastomas,[54] glioblastomas,[55] oral squamous cell carcinomas,[56] lung[57] and pancreatic cancers.[58] Furthermore, cIAP1 expression is required to sustain the rapid growth of MYC-driven liver tumours and osteosarcomas with spontaneous amplification of

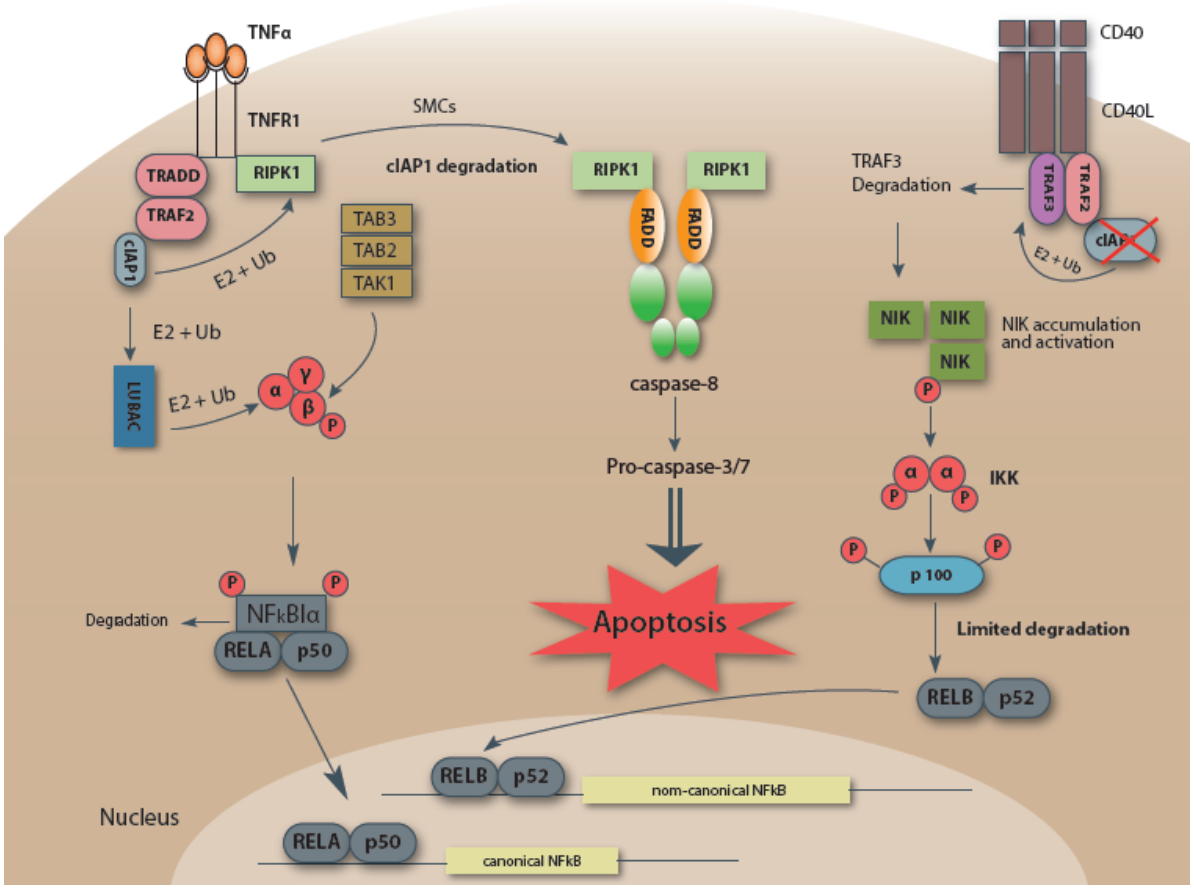
TNFα
TNFR1
TRADD
TRAF2
cIAP1
RIPK1
E2 + Ub
E2 + Ub
LUBAC
E2 + Ub
α
γ
β
P
TAB3
TAB2
TAK1
SMCs
cIAP1 degradation
RIPK1
RIPK1
FADD
FADD
caspase-8
Pro-caspase-3/7
Apoptosis
CD40
CD40L
TRAF3
Degradation
TRAF3
TRAF2
cIAP
E2 + Ub
NIK
NIK
NIK
NIK accumulation
and activation
P
α
α
IKK
P
P
P
P
p 100
Limited degradation
RELB
p52
P
P
Degradation
NFkBIα
RELA
p50
RELB
p52
nom-canonical NFkB
Nucleus
RELA
p50
canonical NFkB

Figure 1.2: cIAP1 roles in regulating the NFκB pathways and caspase-8 mediated apoptosis

Schematic illustrating the role of cIAP1 in regulating the canonical NFκB pathway, the non-canonical NFκB and caspase-8 mediated apoptosis. For simplicity, not all the protein factors involved in these processes are shown (see text for details). Figure adapted from [23]

the cIAP1 and cIAP2 gene.[59, 60] All of this provides a strong rationale for studying how cIAP1 expression is regulated.

1.4.2 Regulation of cIAP1 expression

Given cIAP1 important role in mediating cell survival, its expression is tightly regulated. cIAP1 expression is regulated at the transcriptional level by the NFκB transcription factor,[48] thus providing a positive feedback modulation of NFκB pathway. At the post-translational level, cIAP1 protein stability is regulated by auto-ubiquitination, leading to its proteasomal degradation.[49] In addition to binding to the BIR domains of cIAP1, cIAP2 and XIAP to block their access to caspases, the mitochondrial protein Smac/DIABLO can also induce the auto-ubiquitination of cIAP1 and cIAP2, further enhancing their proteasomal degradation.[49, 50] These important properties of Smac/DIABLO led to the development of a family of small pharmaceutical compounds that mimic the N-terminal IBM tetrapeptide (AVPI) of Smac, termed Smac mimetic compounds (SMCs). Smac-induced depletion of cIAP1 results in the activation of the non-canonical NFκB pathway, NFκB mediated autocrine TNFα production and caspase 8 dependent apoptosis.[47] Hence, Smac mimetics are efficient in inducing the apoptosis of TNFα producing cancer cells and are currently in phase I and II clinical trials (reviewed in [23, 42]).

cIAP1 expression is also regulated post-transcriptionally at the mRNA stability level through binding of the heterogenous ribonucleoprotein A1 (hnRNPA1) to an AU-rich sequence in the cIAP1 3'UTR and destabilization of the transcript in conditions of UV stress.[61] Interestingly, the cIAP1 5'UTR is very long (1.2 kilobases), highly structured and contains several upstream open reading frame (uORF), thus making the transcript refractory

to translation initiation from the main open reading frame under normal cellular conditions.[62] However, our laboratory and others have now established that cIAP1 expression is regulated in conditions of stress through an internal ribosome entry site (IRES) mode of translation initiation.[63-66] IRESes are RNA sequence elements found in the 5'UTR of a small number of viral and cellular mRNAs that facilitate the recruitment of the ribosome to the translation initiation start, independently of the 5' cap.[11, 67] Many of the cellular mRNAs containing an IRES encode proteins important to cell proliferation and apoptosis and the IRES enables them to be translated during conditions of cellular stress to decide of the fate of the cell.[16, 68] For example, the cIAP1 IRES activity is enhanced in response to drug-induced endoplasmic reticulum stress,[63, 65] etoposide or sodium arsenite treatments as well as viral infection.[64, 66] The mechanisms by which cellular IRESes mediate ribosome recruitment are not well established. However, it seems that cellular IRESes require protein factors, named IRES trans-acting factors (ITAFs) that help in the recruitment of the ribosome by acting either as scaffold proteins or RNA chaperones.[69] ITAFs involved in the regulation of cIAP1 IRES-mediated translation, as well as their involvement in carcinogenesis, are reviewed in details in Chapter III.

Interestingly, despite their redundancy in modulating the NFκB pathway, cIAP1 and cIAP2 differ in their expression regulation. Indeed, cIAP2 expression is only known to be regulated at the transcription level by the NFκB transcription factor and at the protein stability level by cIAP1-mediated or autoubiquitination dependent proteasomal degradation.[48, 70] Therefore, the complex regulation of cIAP1 expression might reflect a preferential role in the regulation the NFκB pathway and caspase 8 mediated apoptosis, as well as specialized roles in other cellular contexts.

1.5 Hypothesis and research objectives

In an effort to better understand the regulation of cIAP1 IRES-mediated translation, our laboratory have performed RNA chromatography coupled with mass spectrometry analysis to search for potential ITAFs that help in the recruitment of the ribosome at the cIAP1 IRES. This led to the identification of four trans-acting protein factors that interact specifically with the cIAP1 IRES, namely RNA Helicase A (RHA), nuclear factor 45 (NF45), nuclear factor 90 (NF90) and insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1).[65] Among these protein factors, NF45, a member of the nuclear factor associated of activated T cells (NFAT) family of transcription factors, has been validated as a *bona fide* ITAF that enhances cIAP1 IRES-mediated translation.[65] Hence, my PhD research project aimed at further characterising the cIAP1 IRES function, with an emphasis on the NF45 and IGF2BP1 ITAFs. And given the role of these two proteins in promoting carcinogenesis, I hypothesized that:

''NF45 and IGF2BP1 are novel IRES trans-acting factors that regulate cIAP1 IRES-dependent translation in the context of cancer''

The specific objectives of my thesis research project were:

1. Establish the roles of NF45 and IGF2BP1 as modulators of cIAP1 IRES-dependent translation.
2. Characterize the mechanism(s) by which these ITAFs modulate cIAP1 translation.
3. Investigate the relevance of cIAP1 translation modulation in the context of carcinogenesis.

The role of NF45 in modulating the cIAP1 IRES, as well as other AU-rich IRESes such as the XIAP IRES, and the consequence of this regulation on Hela cells ploidy is presented in chapter 4 of this dissertation. Data presented in chapter 5 establishes IGF2BP1 as a *bona fide* ITAF for the cIAP1 IRES and uncovers an important role for IGF2BP1 and cIAP1 in mediating the apoptotic resistance of rhabdomyosarcoma cancer cells. Chapter 6 contains unpublished data that provides a rationale for combining cIAP1 protein depletion through the use of Smac mimetic compounds and induction of TNFα expression by modulators of innate immunity for the treatment of rhabdomyosarcoma cancer. Finally, Appendix A is a published methods paper that describes polysome profiling, a technique that was used throughout my thesis to determine the state of cIAP1 translation regulation by NF45 and IGF2BP1.

1.6 References

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CHAPTER 2

Translation control in apoptosis

2.1 Preamble

This chapter consists of a review article entitled "Translation control in apoptosis" published in the journal *Experimental Oncology* (Volume 34, October 2012). It provides a comprehensive review of the different mechanisms of translation control in response to apoptotic stress. Section 2.3 is an introduction to the different mechanisms of translation initiation. Section 2.4 is about global translation control during apoptosis, with a few examples of initiation factors modifications following an apoptotic trigger. Section 2.5 talks about selective translation via IRES, with a specific focus on the cIAP1, XIAP and p53 IRES. Finally, Section 2.6 gives an introduction to the role of microRNAs in translation regulation.

Author list:

Mame Daro Faye, Urszula Liwak and Martin Holcik

Author contributions

MDF and UL wrote the manuscript. MH made the figures, contributed ideas and provided editorial support.

MDF's contribution: MDF wrote sections 2.4, 2.5.1, and 2.5.3

2.2 Abstract

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

2.3 Mechanisms of Translation Initiation

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

The principal method of translation initiation occurs by means of a cap-dependent scanning mode, which is the primary source of *de novo* synthesized cellular proteins under normal growth conditions (Figure 2.1). This process requires the involvement of many

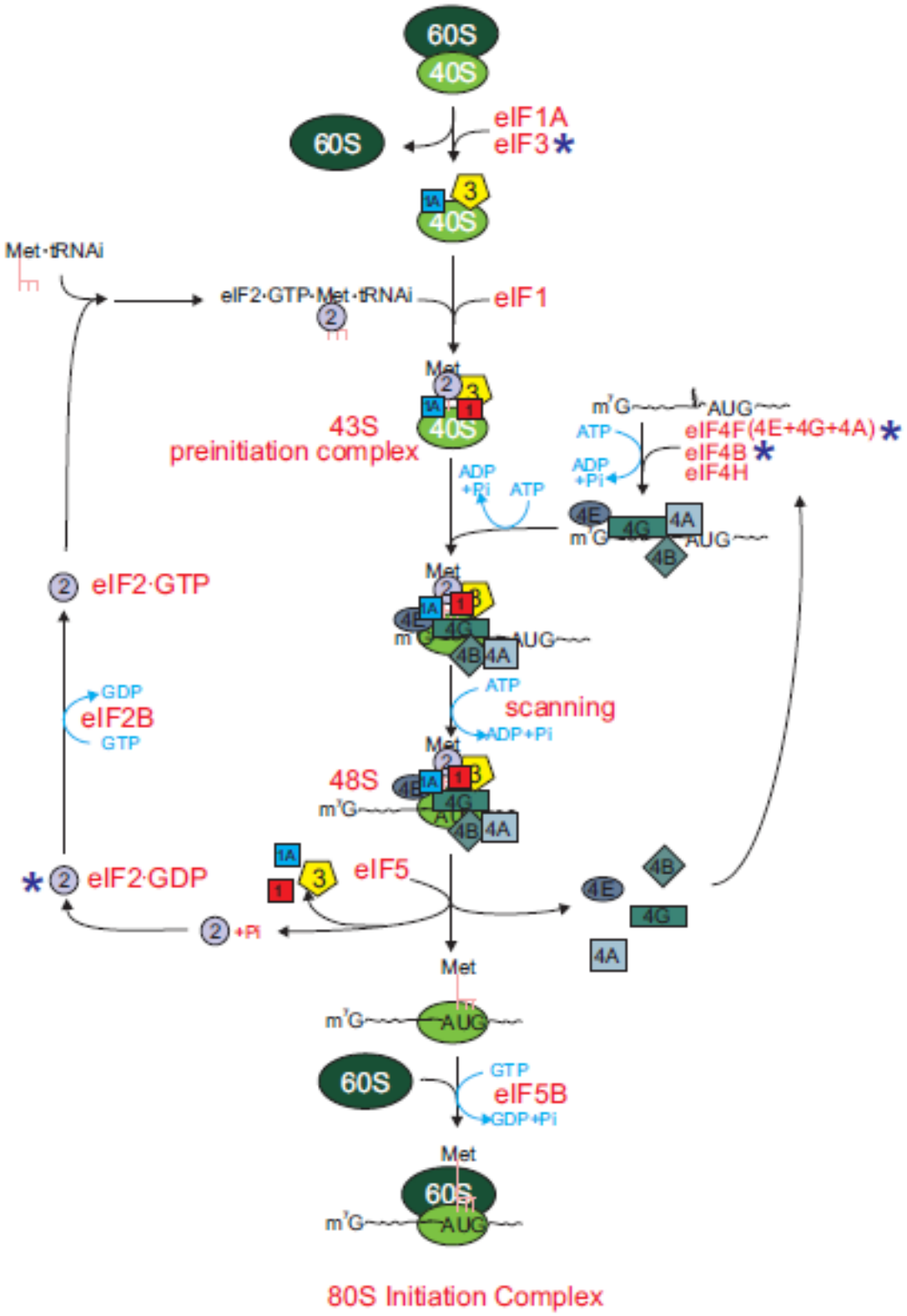
60S
40S
eIF1A
eIF3*
60S
3
1A
40S
Met-tRNAi
eIF2·GTP·Met-tRNAi
2
eIF1
Met
2
3
1A
1
40S
43S
preinitiation complex
m7G
AUG
ATP
ADP
+Pi
eIF4F(4E+4G+4A)*
eIF4B*
eIF4H
ADP
+Pi
ATP
4E
4A
m7G
4G
AUG
4B
Met
2
eIF2·GTP
1A
1
4E
4G
AUG
m7G
4B
4A
ATP
scanning
ADP+Pi
GDP
eIF2B
GTP
Met
2
48S
1A
1
4E
4G
m7G
4B
4A
1A
3
1
eIF5
*
2
eIF2·GDP
2
+Pi
4B
4E
4G
4A
Met
m7G
AUG
60S
GTP
eIF5B
GDP+Pi
Met
60S
m7G
AUG
80S Initiation Complex

Figure 2.1: Schematic diagram outlining the key points of regulation during translation initiation. For simplicity, not all initiation factors are shown. Initiation factors that are described in this review are indicated with asterisks. (Adapted from[122]).

eukaryotic initiation factors (eIFs), which themselves can be regulated to control rates of protein synthesis, as will be discussed below. In brief, the cap-dependent process involves the recognition of the 5' m^7G cap structure, invariably present on all mature cellular messenger RNAs (mRNAs), by the eIF4F complex, comprised of the cap binding protein eIF4E, the scaffold protein eIF4G, and the RNA helicase eIF4A. Separately, the formation of the 43S pre-initiation complex occurs through the association of the 40S ribosomal subunit with eIF3, eIF1A, and eIF2 bound to the initiator methionyl transfer RNA (Met-$tRNA_i^{Met}$). The 43S pre-initiation complex is then recruited to the mRNA through the interaction between eIF3 and eIF4G and is believed to subsequently scan the mRNA until it locates the initiation codon, typically AUG, in an appropriate context. Subsequently, joining of the 60S ribosomal subunit occurs, which forms the translationally competent 80S ribosome, while the eIFs are released and recycled for the next round of initiation. Furthermore, poly(A)-binding protein (PABP) associates with the poly(A) tail on the 3' terminus of mRNA and is thought to interact with eIF4G causing circularization of the mRNA to enhance translation as well as to protect the mRNA from degradation (see Figure 2.1 for details, reviewed in [1]).

The process of translation consumes a significant amount of cellular energy (estimated to be as much as 50%, depending on the organism [2]). It is therefore not surprising that exposure of cells to majority of environmental stressors such as hypoxia, irradiation, or nutrient deprivation leads to modifications of the eIFs involved in the regulation of cap-dependent translation, ultimately resulting in attenuation of global protein synthesis. In addition to saving cellular energy, the attenuation of translation prevents synthesis of unwanted proteins that could obstruct the cellular stress response. Under these conditions, cells are able to cope with the stress or, if the damage to the cell is beyond repair, to initiate

apoptosis. To facilitate the decision making process, some proteins, in particular those required for the stress response, are selectively translated even though cap-dependent translation is attenuated. It is the relative levels of these pro- and anti-apoptotic proteins that are important in tipping the balance in favour of survival or cell death. The question of how is a cell able to translate proteins when the required eIFs for cap-dependent translation are not available is at the centre of investigations in many laboratories, and a subject of this review.

One important mechanism that has acquired recent attention is the internal ribosome entry site (IRES) mediated translation initiation process that utilizes specialized RNA elements to selectively recruit ribosomes to mRNA without a need for the cap structure.[3] IRES elements are found in the 5' untranslated region (UTR) of mRNAs and were initially discovered in RNAs of picornaviruses. [4] Although the RNAs of these viruses do not contain a m^7G cap, they are still effectively translated. In addition, many viruses encode proteases that cleave several canonical eIFs in order to block translation of host proteins. For example, upon infection of cells with polio virus, the virus-encoded protease 2A specifically cleaves eIF4G thus inactivating the eIF4F complex and effectively preventing ribosome recruitment to capped cellular mRNAs. This ensures that the host cell's translational machinery is now available for virus protein translation [5]. Importantly, the polio virus IRES element is able to utilize the cleaved eIF4F complex and recruit the ribosome for efficient translation of its own proteins. In other viruses, such as the hepatitis C virus (HCV), the presence of eIF4F is not required at all and the IRES is able to recruit the ribosome in its absence.[6] Thus, even with the loss of some eIFs, the viral IRES elements are able to recruit the ribosome for efficient translation. These observations led researchers to study cellular mRNAs to

determine if a similar mechanism(s) exists. In recent years, it has been proposed that an estimated 10% of all cellular mRNAs may contain IRES elements. Interestingly, many of these mRNAs encode proteins involved in processes such as cell proliferation and apoptosis, and are critical in determining the survival of a cell under physiological and pathophysiological stress conditions.[3] For example, IRESs have been identified in mRNAs encoding XIAP, cIAP1, Bcl-xL, Bcl-2, Bag-1, Apaf-1, p53, c-myc, DAP5, all proteins that are critically involved in the regulation of cell survival.

Although the mechanism of IRES-mediated translation is still poorly understood, it has become evident that not all cellular IRES elements act in a similar manner. That is, most cellular IRES elements require binding of some of the canonical initiation factors as well as for other protein factors termed ITAFs (IRES-*trans* acting factors) that modulate the IRES activity.[3] Most of the ITAFs identified thus far are RNA binding proteins that fulfill a variety of functions including involvement in mRNA splicing (for a review on splicing in apoptosis see [7]), export, stress granule formation, as well as important roles in translation initiation. The binding of ITAFs can either enhance or repress IRES activity; it is thought that the positive regulators act either as RNA chaperons that aid in the formation of the proper IRES structure, or directly recruit the ribosome. The precise mechanism of how the repressive ITAFs function is not clear. Interestingly, many ITAFs shuttle between the nucleus and cytoplasm and this shuttling is regulated by posttranslational modifications such as phosphorylation in response to a variety of triggers. Therefore, the cytoplasmic availability of positive or negative regulators can determine the extent of IRES translation (see below).

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

Induction of apoptosis is accompanied by a pronounced down-regulation of protein synthesis.[8] This inhibition in global translation rates is characterized by a decrease in polysome chains, suggesting that at least some regulation occurs at the translation initiation step.[9] Indeed, there is extensive evidence that apoptosis triggered by different stimuli leads to modifications in a defined set of canonical initiation factors that ultimately results in the inhibition of translation initiation (reviewed in [8, 10]). These modifications generally consist of changes in phosphorylation status (e.g. eIF2α, eIF4E, eIF3, eIF4E binding proteins (4E-BPs)) or protein cleavage by caspases or viral proteases (e.g. eIF4G, eIF4B, eIF3 (Table 2.1; Figure 2.2).

2.4.1 eIF2

eIF2 plays a central role in translation initiation by bringing the initiator Met-tRNA to the 40S ribosomal subunit for the formation of the 43S pre-initiation complex. eIF2 is composed of three subunits (α, β and γ), of which the γ subunit is bound by GTP that is later hydrolyzed during translation initiation.[1] GDP to GTP exchange is necessary for regenerating active eIF2 and this process is catalyzed by eIF2B (Figure 2.1). However, in response to different stress stimuli, the α subunit of eIF2 is phosphorylated at serine 51 (Ser51), thus increasing its affinity for eIF2B and trapping the two in an inactive complex.[11] As a result, pre-initiation complex formation and global mRNA translation are inhibited. eIF2α phosphorylation is mediated by four different kinases that are activated by various stress triggers (reviewed in [1]): HRI (haem-regulated inhibitor) which is activated by iron

Table 2.1: Modification of translation initiation factors during apoptosis

Table showing selected eukaryotic translation initiation factors that are modified during apoptosis induced by different triggers. The type of modifications and their consequence for translation and cell survival, along with key references are shown on the right.

Translation initiation factors	Modifications	Effects	Apoptotic triggers	References
eIF2	Phosphorylation of eIF2α subunit at Ser51	Inhibition of GDP to GTP exchange on eIF2: • inhibition of global translation and apoptosis • translation of specific transcripts and cell survival	Iron deficiency, heavy metals, osmotic or oxidative stress, heat shock, double stranded RNA, amino acid starvation, unfolded protein response (UPR)	[1, 11]
eIF4E	De-phosphorylation	Global translation inhibition.	Stimuli that activate protein phosphatase 2A	[20]
4E-BPs	De-phosphorylation	Competition with eIF4G on eIF4E. Global translation inhibition and apoptosis	DNA damage, TRAIL, staurosporine, rapamycin	[12, 16, 21, 22]
4E-BPs	Cleavage by caspases at Asp-24	Cleaved form strongly binding eIF4E and inhibition of cap-dependent translation	Staurosporine, etoposide, p53 activation	[21, 22]
eIF4GI and eIF4GII	Cleavage by caspase 3 at Asp-532 and Asp-1176 of eIF4GI. Cleavage by caspase 3 at Asp-560, 851, 978, 1162 and 1407 of eIF4GII.	Cleaved forms (except for M-FAG from eIF4GI) cannot bridge eIF4E, 4A and eIF3 together. Global translation and attenuation of anti-apoptotic response	TNFα, TRAIL, cisplatin, etoposide, cycloheximide, MG132, serum deprivation, Fas receptor activation	[3, 9, 16]
p97/DAP5/NAT1	Cleavage by caspase 3 at Asp-790	p86 fragment with eIF4A and eIF3 binding sites but no eIF4E site. Inhibition of cap-dependent translation but stimulation of specific IRES-dependent translation	UPR, Fas receptor activation	[18, 26–28]
eIF4B	Cleavage by caspase 3 at Asp-45	Fragment is still able to interact with eIF4F and eIF3 but lacks the region mediating PABP binding	Cycloheximide, Fas receptor activation	[16, 31–33]
eIF3j (p35)	Cleavage by caspase 3 at Asp-242	Reduced affinity of the eIF3 complex for the 40S ribosomal subunit. Inhibition of global protein translation	Cycloheximide, Fas receptor activation	[16, 29]
eIF3f (p47)	Phosphorylation	Enhanced association with the core subunits of eIF3. Inhibition of global protein translation	Staurosporine	[30]

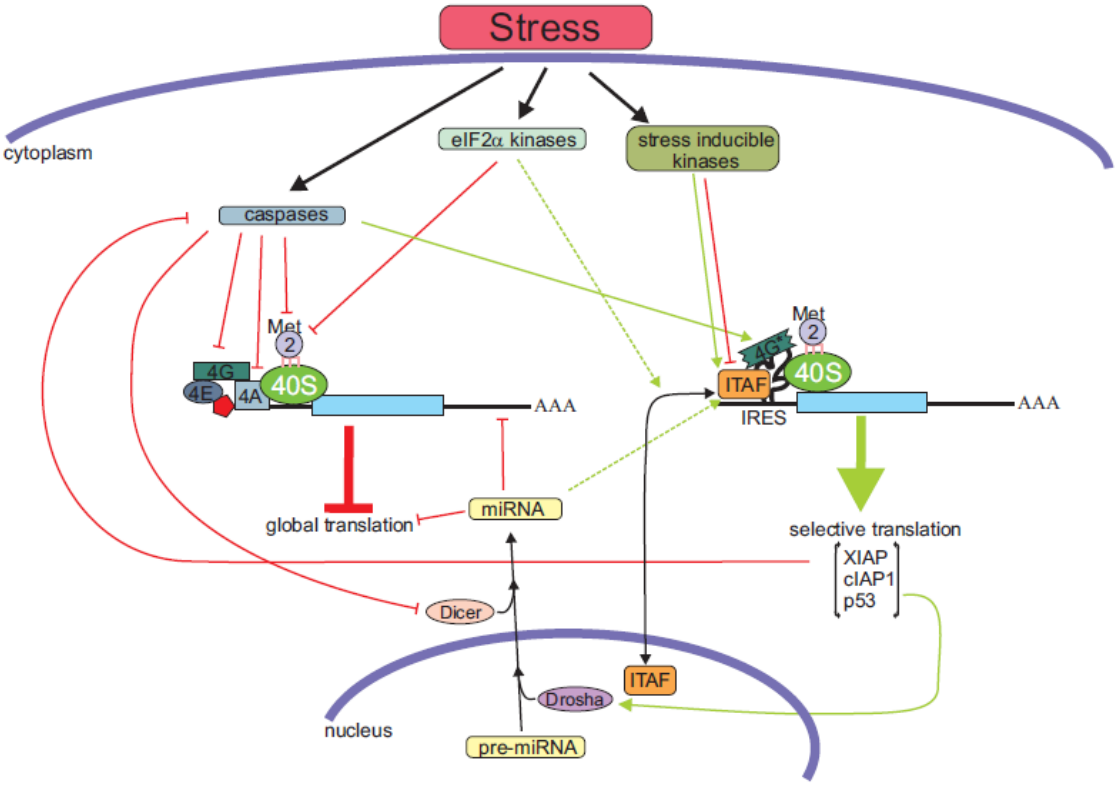
Stress
cytoplasm
eIF2α kinases
stress inducible kinases
caspases
Met
2
4G
4E
4A
40S
AAA
ITAF
IRES
global translation
miRNA
selective translation
XIAP
cIAP1
p53
Dicer
Drosha
nucleus
pre-miRNA

Figure 2.2: A model of the interconnectedness of translation and apoptosis. Only factors pertinent to this review are shown. The left side of the model shows regulation of cap-dependent translation; the right side depicts IRES-mediated translation. Green lines indicate positive, while red line negative interactions. Dotted line depicts indirect effect.

deficiency, heavy metals, osmotic or oxidative stress and heat shock; PKR (protein kinase activated by double-stranded RNA) which is activated by double stranded RNA from viral infections or interferon-induced apoptosis; GCN2 (general control non-derepressible-2) which is activated by amino acid starvation; and PERK (PKR-like endoplasmic reticulum kinase) which is activated during the unfolded protein response (UPR).

The link between eIF2□ phosphorylation and apoptosis is not straightforward, and eIF2α phosphorylation can either be a cause or consequence of the cell's commitment to apoptosis. For instance, in MCF-7 breast cancer cells treated with TNFα or TRAIL (TNFα-related apoptosis-inducing ligand) eIF2α phosphorylation by PKR is dependent on caspase 8 activity and happens several hours before the onset of apparent apoptosis.[12, 13] In contrast, in mouse embryonic fibroblasts treated with TNFα or deprived of serum, eIF2α phosphorylation is necessary to induce caspase 3 activation and subsequently apoptosis.[14] Interestingly, forced expression of a phosphomimetic S51D mutant of eIF2α is sufficient to activate caspase 3 and induce apoptosis in the absence of any other triggers, whereas expression of a non-phosphorylatable S51A mutant protects cells from TNF□ or serum deprivation. It has also been reported that eIF2α itself can be cleaved in apoptotic cells, mainly by caspase 3, but also by caspases 6, 8 and 10.[15, 16] The functional relevance of this cleavage product is not fully understood but it has been shown that its GTP exchange rate was higher, independent of eIF2B, and that it may contribute to translation inhibition.[15]

In contrast to inhibiting global translation, eIF2α phosphorylation can up- or downregulate selective translation. For example, under hypertonic stress, eIF2α phosphorylation was shown to induce cytoplasmic accumulation of heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), a known ITAF, which in turn inhibited IRES-mediated

translation of the anti-apoptotic proteins XIAP and Bcl-xL, thus sensitizing the cells to apoptosis.[17] Interestingly, in the context of the adaptive UPR, eIF2α phosphorylation can be a signal that promotes cell survival rather than apoptosis. In this context, eIF2α phosphorylation by PERK leads to the selective translation of transcription factors, such as ATF4 that controls the expression of pro-survival and anti-apoptotic proteins such as cIAP1.[3, 18]

2.4.2 eIF4E

The cap-binding protein eIF4E is another initiation factor whose availability is regulated by phosphorylation during apoptosis. eIF4E is phosphorylated at Ser209 by the MAPK integrating kinases Mnk1 and Mnk2 in response to different stimuli such as treatment of cells with growth factors, anisomycin or UV, that activate the ERK and p38 MAPK pathways.[19] eIF4E phosphorylation reduces its affinity for the 5' cap structure, thus stimulating translation initiation. Conversely, eIF4E dephosphorylation by protein phosphatase A leads to inhibition of global translation. However, the eIF4E binding to the 5'cap is regulated mainly through the phosphorylation status of eIF4E binding proteins (4E-BPs). 4E-BP1, 2, and 3 are proteins that share structural similarities to the fragment of eIF4G responsible for binding to eIF4E. 4E-BPs are phosphorylated in normal growth conditions by the mTOR signaling pathway (reviewed in [20]). However, during apoptosis induced by DNA damage, TRAIL, the protein kinase inhibitor staurosporine, or the mTOR inhibitor rapamycin, there is a decrease in 4E-BPs phosphorylation.[12, 16, 21, 22] Hypo-phosphorylated 4E-BPs have a higher affinity for eIF4E and as a consequence, they competitively prevent eIF4G from binding to eIF4E, thus reducing the availability of the

eIF4F complex and resulting in inhibition of translation (reviewed in [1]). Similar to eIF2α phosphorylation, loss of 4E-BPs phosphorylation occurs during the early phase of the apoptosis cascade,[12, 16] leading to global translation inhibition and commitment to cell death. For example, ectopic expression of a non-phosphorylatable mutant of 4E-BP1 sensitizes multiple myeloma cells to dexamethasone-induced apoptosis.[23] Furthermore, apoptotic triggers such as staurosporine, etoposide or activation of p53 can lead to caspase-mediated cleavage of 4E-BP,[21, 22] producing cleaved form that binds strongly to eIF4E and inhibits cap-dependent translation.[24]

2.4.3 eIF4G

The availability and function of the initiation factors eIF4G1 and eIF4GII is regulated during apoptosis primarily through cleavage by caspases. Upon treatment of cells with apoptotic triggers such as TNFα, TRAIL, cisplatin or etoposide, eIF4GI and eIF4GII are cleaved by caspase-3 at two different sites. This gives rise to three cleavage products named Fragments of Apoptotic cleavage of eIF4G (N-FAG, M-FAG and C-FAG.[9, 16, 25] The middle fragment, M-FAG, retains its ability to interact with eIF4A, eIF4E and eIF3, and supports cap-dependent translation during the early phase of apoptosis. However, M-FAG is degraded with prolonged exposure to stress resulting in the inhibition of cap-dependent translation and attenuation of the anti-apoptotic response. Interestingly, cleavage of the eIF4G-related protein factor p97/DAP5/NAT1 by caspases releases a p86 isoform that stimulates the IRES-mediated translation of apoptosis regulating factors such as XIAP, cIAP1, c-myc, APAF1 and p97/DAP5 itself.[18, 26-28] Hence, cleavage of eIF4G and p97/DAP5

proteins regulates the fate of the cell by tipping the balance between the translation of pro- and anti-apoptotic factors.

2.4.4 eIF3

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

2.4.5 eIF4B

The initiation co-factor eIF4B which stimulates eIF4A helicase activity and ribosome binding to the mRNA, is also modified during apoptosis. eIF4B is cleaved in its C-terminal region by caspase 3 both *in vitro* and in BJAB cells treated with anti-Fas or cycloheximide.[16] However, eIF4B can also be cleaved in a caspase-3 independent manner in apoptotic MCF-7 cells that lack caspase 3.[12] The N-terminal fragment of eIF4B is still able to interact with eIF4F and eIF3[16]; however, it lacks the region that mediates its interaction with PABP.[31] The effects of eIF4B truncation on translation and apoptosis are not well characterized. However, a substantial amount of work has been done on elucidating eIF4B's role in cell survival and

proliferation (reviewed in [32]). For instance, eIF4B depletion from HeLa cells using RNA interference was shown to selectively reduce the translation of genes involved in cell proliferation (such as cdc25C, c-myc, and ornithine decarboxylase) and survival (such as Bcl-2 and XIAP). Moreover, eIF4B depletion caused a decrease in HeLa cells proliferation rates, enhanced apoptosis and sensitized these cells to camptothecin-induced cell death.[33]

In general, modifications of translation initiation factors, whether they are a cause or a consequence of the initiation of the apoptotic cascade, are aimed at inhibiting global protein synthesis. This general inhibition of translation contributes to the shutdown of all cellular processes, and is believed to conserve cellular energy and prevent the synthesis of protein factors that could stall the apoptotic process. However, in cases of adaptive stress, translation can be reprogrammed such that the translation of specific mRNA transcripts continues and influences the fate of the cell (Figure 2.2).

2.5 Selective translation via IRES

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

2.5.1 Cellular Inhibitor of Apoptosis 1 (cIAP1)

The inhibitor of apoptosis (IAP) family of proteins is comprised of eight members in mammals that regulate many key cellular processes including signaling, cell division, and apoptosis, and are the subject of another review in this special issue (D. Vucic, this issue).

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

Interestingly, activation of the cIAP1 IRES in the context of ER stress is dependent on caspase activation and is accompanied by cleavage of eIF4GI and its homolog p97/DAP5/NAT1 during the early phase of the UPR.[18] As mentioned above, the

p97/DAP5/NAT1 cleavage product, p86/DAP5 functions as an ITAF that stimulates the activity of several IRES elements including cIAP.[18, 26-28] Indeed, ectopic overexpression of a p86/DAP5 fragment, but not the full length p97/DAP5 protein, in both HEK293T and rabbit reticulocyte lysates was able to specifically drive cIAP1 IRES activity and increase cIAP1 endogenous protein levels.[18] It was later shown that both p97/DAP5/NAT1 and p86/DAP5 bind to the cIAP1 IRES, possibly through association with other accessory proteins.[18, 41]

The structure of the cIAP1 IRES and the proteins that specifically interact with this IRES were characterized recently. One of these proteins, NF45, enhances cIAP1 IRES-dependent translation and mediates cIAP1 induction in response to thapsigargin-induced ER stress and UPR.[39] NF45 is an NFAT-related transcription factor that was first identified to regulate interleukin-2 transcription, together with its binding partner NF90.[42] NF45 and NF90 are involved in several cellular processes such as transcription,[43] viral replication [44] and microRNA processing [45] and our study confirmed its role in IRES-mediated translation.[39] More recently, NF45 has been implicated in mitotic control in HeLa cells since depletion of NF45/NF90 complexes by RNA interference in these cells leads to the generation of large multinucleated cells, a result of impaired cytokinesis and cell growth due to defects in DNA break repair.[46] In line with this new role for NF45, we have recently discovered that NF45 preferentially regulates a cohort of AU-rich IRES-containing mRNAs including cIAP1 and XIAP, which are responsible for the multinucleated phenotype of NF45-deficient cells (MDF and MH unpublished observations). Loss of NF45 results in reduced IRES-mediated translation of XIAP and cIAP1 mRNAs. Interestingly, the resulting decrease in XIAP expression causes an increase in Survivin protein levels, likely due to Survivin protein stabilization.[47] Survivin, another member of the IAP family, plays an

important role in microtubule spindle checkpoint regulation and its aberrant expression leads to cytokinesis defects,[48] thus explaining the multinucleated phenotype of NF45-deficient cells. Similarly, through its control of cIAP1 translation NF45 regulates cyclin E expression. Nuclear cIAP1 was shown to transcriptionally regulate cyclin E,[49] and we found that either NF45 or cIAP1 depletion caused a decrease in cyclin E expression that is rescued by NF45 re-expression. Coordinated changes in cyclin E and Survivin expression in NF45-depleted cells would lead to a block in cell cycle, mitotic catastrophe and defects in cytokinesis thus explaining the senescence-like phenotype of these cells. These observations uncovered a novel role for NF45 in controlling ploidy and highlight the importance of IRES-mediated translation in the regulation of mitosis, cell growth and apoptosis.

2.5.2 X-linked inhibitor of apoptosis protein (XIAP)

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

Studies into the regulation of XIAP expression led to the discovery of an IRES element located in its 5' UTR region which mediates XIAP protein translation under conditions of cellular stress such as γ-irradiation or nutrient deprivation, thus providing the cell with protection against apoptosis.[51] Interestingly, XIAP protein is encoded by two mRNA splice variants that differ only in their 5'UTR regions.[52] The more abundant, shorter

transcript produces the majority of XIAP protein under normal growth conditions by cap-dependent translation. However, during cellular stress, the longer transcript that contains the IRES element supports efficient translation even though global cap-dependent translation is attenuated.[52] The secondary structure of the XIAP IRES and its associated ITAFs has been determined.[53] Some of these, such as La autoantigen,[54] hnRNP C1/C2,[55] and HuR[56] have been shown to enhance XIAP IRES translation, whereas others, such as hnRNP A1,[57] PTB[53] and PDCD4[58] have been shown to repress XIAP IRES translation.

Interestingly, cytoplasmic localization of XIAP ITAFs appears to play a key role in the regulation of XIAP translation in response to stress. For example, osmotic shock causes an accumulation of hnRNP A1 in the cytoplasm by activating the mitogen-activated protein kinase kinase$_{3/6}$-p38 signaling pathway resulting in phosphorylation of hnRNP A1, thus preventing its import into the nucleus.[59] Once in the cytoplasm, hnRNP A1 binds with the XIAP IRES and inhibits protein expression.[57] Another example of an ITAF being regulated at the level of localization was shown by Gu *et al*.[60] in acute lymphoblastic leukemia (ALL) cells treated with ionizing radiation (IR). They observed that IR treatment resulted in the misregulation of the oncogene MDM2. MDM2 overexpression is observed in many cancers and correlates with poor patient outcome because it binds to and inhibits the activity of the tumour suppressor p53.[60] It is known that the phosphorylation status of MDM2 dictates its localization such that survival signals promote nuclear localization and cell proliferation whereas cellular stress results in dephosphorylation of MDM2 and subsequent retention in the cytoplasm. However, the cytoplasmic function of MDM2 was not well understood. Upon treatment with IR, dephosphorylated MDM2 is retained in the cytoplasm and is no longer associated with its main target, p53.[60] Instead, cytoplasmic MDM2 is able to directly and

specifically bind to the XIAP IRES. It is interesting to note that many cancers express elevated levels of a mutated form of MDM2 that does not contain the N-terminal p53 binding domain[61] and it is this remaining C-terminal portion of MDM2 that is responsible for interacting with the XIAP IRES and upregulating its IRES-mediated translation. Importantly, the MDM2-mediated increase in XIAP expression leads to enhanced resistance to IR-induced apoptosis.

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

2.5.3 Tumour suppressor p53

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

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

the p53 5'UTR was able to direct *in vitro* translation of the p53 mRNA in the absence of a cap structure[75], further confirming the existence of a p53 IRES.

Interestingly, Ray *et al.*[75] proposed a model in which two different IRES structures control the translation of two different p53 isoforms, namely the full-length p53 protein (FL-p53) and the ΔN-p53 (p40/47) isoform. The ΔN-p53 protein is translated from an alternative initiation codon situated within the coding sequence, 40 nucleotides downstream of the FL-p53 translation start site.[76, 77] It has been suggested that ΔN-p53 acts as a dominant-negative form that antagonizes p53-mediated transcription and growth regulation.[76] However it appears that ΔN-p53 functions are much more complex since the protein does not contain an MDM2 binding site, is able to oligomerize with FL-p53 to induce different transcription patterns,[77] and induces apoptosis when expressed in p53-null cells.[76] They further showed that expression of the two p53 isoforms is regulated in a cell-cycle dependent manner, *via* an IRES mechanism of translation.[75] In fact, the IRES driving FL-p53 protein expression is more active during the G2/M transition when p53 activity is required the most, whereas the IRES driving ΔN-p53 expression is active during the G1/S transition.[75] These findings are more consistent with ΔN-p53 being an antagonist of p53 activity where at the G1/S phase it would drive the expression of genes necessary for transition through the cell cycle. Differential regulation of FL-p53 and ΔN-p53 via translational control may also have an effect on cell sensitivity to apoptosis. For instance, doxorubicin-induced DNA damage and tunicamycin-induced ER stress give rise to different patterns of p53 isoform expression where H1299 lung carcinoma cells overexpressing the ΔN-p53 are less sensitive to doxorubicin treatment and more sensitive to tunicamycin.[74] Thus, translational control via the IRES is an important mechanism by which p53 can integrate and respond to the different

apoptotic or proliferative cues the cell is exposed to. Another layer of complexity is brought about by the different ITAFs that can bind to the p53 IRES and modulate its activity in response to stress. The ribosomal protein L26,[72] PTB[78] and hnRNP C1/C2[79] were all shown to enhance p53 expression, whereas nucleolin was shown to repress it.[72] The La autoantigen, hnRNP U and p53 itself might also be potential p53 IRES trans-acting factors.[72] PTB binds specifically to the p53 IRES structure and a reduction in PTB protein levels by RNA interference leads to a decrease in IRES activity and blunting of p53 isoforms induction in the presence of doxorubicin.[78] Furthermore, treatment of A549 human lung carcinoma cells with doxorubicin causes PTB translocation from the nucleus to the cytoplasm, corresponding with an increase in p53 expression. Interestingly PTB cytoplasmic levels are maximal at the G_2/M phase and low at the G_1/M transition,[80] suggesting that PTB might be the factor contributing to increased p53 translation during the G_2/M checkpoint.[78] These results further support the notion that cell stressors can alter the expression level, cellular localization or status of different ITAFs to modulate the output of p53 protein available to respond to the stress and decide the fate of the cell.

Most of the work done on p53 in the context of carcinogenesis was aimed at characterizing the effects of p53 coding region mutations on the transcriptional activities of the protein. However, it has become apparent that mutations can also occur within p53 5'UTR and may have relevance to the pathology of cancer.[81] Indeed, a cancer-derived triple silent mutation at positions 185, 188 and 191 that was previously shown to alter MDM2 binding to the p53 mRNA,[81] as well as a single silent mutation at position 200 of the p53 5'UTR[81] were found to alter p53 IRES activity, alter the profile of ITAFs binding to the IRES and blunt the IRES induction in response to doxorubicin.[82] Thus, it is possible that

mutations within the p53 IRES may lead to carcinogenesis by decreasing p53 induction and protective activity in response to DNA damage. The recent characterization of the p53 IRES structure[82] may help in identifying more cancer-derived mutations that are relevant to p53 function and that could be used as predictors of response to certain cancer treatments.

Another aspect that may be relevant to the pathology of cancer was the recent finding that p53 IRES-dependent translation is impaired during oncogene-induced senescence (OIS) in DKC1^{m} cells.[83] The DKC1 gene encodes the dyskerin protein which is responsible for modifying uridines in ribosomal RNA into pseudouridines, and mutations in DKC1 have been linked to the development of X-linked dyskeratosis congenita (X-DC). X-DC patients have increased susceptibility to cancer, as reflected by the fact that more than 50% of DKC1^{m} mice develop tumours of different origin.[84] Interestingly, Yoon *et al*.[85] showed that DKC1-mutated cells are impaired in IRES-mediated translation, providing one of the first *in vivo* links between IRES-mediated translation and the onset of oncogenesis. In addition, during OIS, in which p53 translation is normally induced to counteract the oncogenic insult,[86] p53 IRES-mediated translation is impaired both in DKC1^{m} mice cells and in X-DC patient derived cells. This results in a significant decrease in p53 protein induction and of its target genes *p21* and *MDM2* in response to etoposide treatment or γ-irradiation which correlated with a reduction in the number of apoptotic cells as compared to wild-type.[83] These results were corroborated by an independent group that showed that DKC1 knock-down in both MCF-7 breast cancer cells and in primary breast cancer cells caused a decrease in p53 IRES-mediated translation, which led to a decrease in p53 transcriptional activity and apoptosis upon doxorubicin treatment.[87] Together, these findings show that defects in p53

IRES-mediated translation are relevant not only to the OIS process but also to carcinogenesis, especially in the context of the X-DC pathology.

2.6 MicroRNA mediated regulation

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

miRNAs are transcribed from the genome by RNA polymerase II or III as long, double-stranded hairpin transcripts containing a 5'cap and 3' poly-A tail, termed primary miRNA (pri-miRNA).[89] Pri-miRNAs are further processed into a smaller double stranded structure in the nucleus by the RNase III-like enzyme Drosha and DGCR8 to produce the precursor miRNA (pre-miRNA)[90] that are subsequently are exported into the cytoplasm by exportin-5 where they are further processed by the RNase III enzyme Dicer, yielding an approximately 22 nucleotide long, double-stranded product.[91] Only one of the miRNA strands is then incorporated into the RNA-induced silencing complex (RISC) containing the argonaute (AGO) protein, while the other strand is degraded.[89, 92] Upon recognition of their target mRNA via near-perfect complementarity, miRNAs can direct the degradation of the target mRNA by the 5'-to-3' mRNA decay pathway which involves deadenylation by the CAF1-CCR4-NOT deadenylase complex, followed by decapping by DCP2, and ultimately degradation by the exonuclease XRN1.[93, 94] As well, miRNAs that bind with near perfect

complementarity can direct endonucleolytic cleavage of their target mRNAs through the catalytically active Argonaute protein present in the RISC complex.[95] On the other hand, miRNAs can also bind their target mRNAs via imperfect complementarity resulting in a loss of protein product but no change in mRNA levels suggesting that an inhibition of translation occurs rather than degradation of mRNA. Early studies showed that these inhibited mRNAs were found associated with polysomes indicating that repression occurred at a post-initiation stage, likely during translation elongation.[96, 97] However, in recent years, it has been suggested that miRNAs inhibit translation initiation by interfering with the eIF4F and the poly-A binding complexes. Further evidence for miRNAs affecting the initiation step of translation was provided by Humphreys *et al*.[98] who showed that a construct containing the IRES element of cricket paralysis virus (CrPV) lacking a cap and poly-A structure was unaffected by miRNAs, suggesting that the initiation step was the target of miRNA regulation, since regulation of elongation or termination should still occur in the presence of the CrPV IRES. These data strengthen the link between miRNA function and translational control which is important not only during times of cellular stress, but also during the cell's decision to undergo apoptosis. As mentioned above, many mRNAs that are involved in the cellular stress response contain IRES elements, which not only allow the mRNA to be translated during attenuation of cap-dependent translation, but may also aid in protecting the mRNA from miRNA induced silencing. Furthermore, cytoplasmic processing bodies (P-bodies), which are involved in processes such as translation inhibition and mRNA degradation, have been suggested to be involved in retaining repressed mRNAs in the RISC complex thus preventing translation.[99-101] Interestingly, certain stressors can cause release of some repressed mRNAs from the P-bodies where they are able to re-enter into polysomes for

efficient translation.[102] For example, in Huh7 hepatoma cells, the cationic amino acid transporter (CAT-1) mRNA is found in the P-bodies. However, upon exposure to amino acid starvation, the RNA binding protein HuR is relocalized from the nucleus to the cytoplasm where it binds the CAT-1 mRNA and releases it from the P-bodies. This allows translation of the mRNA to respond to the cellular stress. It is likely that this mechanism of release under stress occurs for other mRNAs in response to specific stressors and the combination of these mechanisms allows the cell to quickly respond to its changing environment by translating pro- or anti-apoptotic proteins that are crucial for deciding the fate of the cell. Although the exact mechanism of miRNA-mediated inhibition is still under debate, the result of inhibiting protein translation remains unchanged.

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

ultimately leading to tumour growth and survival. (For a detailed review on miRNAs see [105, 106]).

miRNA 21 is the most consistently up-regulated miRNA across many cancer types. Chan *et al*.[107] discovered that reducing miR-21 levels in glioblastoma cells increased apoptosis, which correlated with a decrease in tumour growth. Of the many targets of miR21,[108] PDCD4 is an important target that is frequently down-regulated in a variety of cancers.[109, 110] The tumour suppressive function of PDCD4 stems from its ability to bind to and inhibit eIF4A, thus blocking cap-dependent translation and attenuating cell growth.[111-113] Upon loss of PDCD4, the cell loses this ability to regulate protein translation, leading to enhanced cell proliferation and increased tumour formation. However, we and others have shown recently that PDCD4 plays a more specific role in translation by regulating translation of a specific set of targets (as described above[58, 114]). For example, PDCD4 can bind to and negatively regulate the expression of p53 under normal growth conditions. However, upon induction of DNA damage by ultraviolet irradiation (UV), PDCD4 is degraded, thus allowing for an up-regulation in p53 levels.[115] Similarly, PDCD4-dependent repression of XIAP and Bcl-xL in response to FGF 2 has been described above. This suggest that an increase in miR-21 leading to a loss in PDCD4 may not only result in an increase in overall translation through de-repression of eIF4A, but also to a specific increase in expression of anti-apoptotic proteins, thus leading to enhanced resistance to apoptosis-inducing chemotherapeutics.[58]

The Bcl-2 family of proteins has also been identified as being regulated directly by miRNAs. For example, the miR-15-16 cluster of miRNAs can induce apoptosis by inhibiting Bcl-2, an anti-apoptotic factor involved in maintaining mitochondrial membrane

homeostasis. As is common for many miRNAs, this cluster is down regulated in many cancers. For example, the miR-15-16 cluster is deleted in B-cell chronic lymphocytic leukaemia (CLL[116]), pituitary adenoma[117], and prostate carcinoma.[118] This down-regulation of miRNAs contributes to the increased expression of Bcl-2 that is often observed in many cancers, and promotes chemoresistance by inhibiting the release of mitochondrial cytochrome c required for activation of caspase 9.

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

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

2.7 Conclusions

Regulation of translation can be both the consequence and the cause of apoptosis. We have chosen examples to illustrate how this process is highly dynamic and is crucial for the cell's ability to respond to environmental cues (Figure 2.2). We have highlighted the critical points of control prior to and during the onset of apoptosis with the hope of convincing the reader that the ability to translate specific proteins in response to stress is essential to decide the fate of the cell. Both the IRES and miRNA-mediated control of translation initiation are emerging as key mechanisms that regulate selective translation. IRES mediated translation allows for a selective translation of a subset of mRNAs in times of attenuation of global cap-dependent translation by bypassing the requirement for canonical initiation factors that are subject to inhibitory modification during apoptosis. In contrast, miRNA-mediated control of translation may, in addition to regulating the expression of specific target mRNAs, protect IRES containing mRNAs from degradation. In combination, these examples demonstrate how misregulation of translation initiation plays a crucial part in tumourigenesis and chemoresistance through enhanced resistance to apoptosis.

2.8 Acknowledgements

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CHAPTER 3

The Role of IRES Trans-Acting Factors in Apoptosis and Cancer

3.1 Preamble

This chapter consists of a review paper entitled ‘‘The role of IRES trans-acting factors in apoptosis and cancer’’ and submitted for publication in the journal *Biophysica Biochemica Acta – Gene Regulatory Mechanisms*. It provides a comprehensive review of emerging IRES trans-acting factors and their key roles in RNA metabolism, with a specific focus on IRES-mediated translation and its implication on carcinogenesis. In particular the roles of four IRES trans-acting factors - IGF2BP1 (Section 2.4), NF45 (Section 2.5), PDCD4 (Section 2.6) and DAP/p97 (Section 2.7) – in promoting different hallmarks of cancer such as proliferation, resistance to apoptosis and metastasis, are discussed.

Author list

Mame Daro Faye and Martin Holcik

Author contributions

MDF wrote the manuscript. MH made the figures, contributed ideas and provided editorial support.

3.2 Abstract

Regulation of protein expression through RNA metabolism is a key aspect of cellular homeostasis. Upon specific cellular stresses, distinct transcripts are selectively controlled to modify protein output in order to quickly and appropriately respond to the stress. Reprogramming of the translation machinery is one node of this strict control that typically consists of an attenuation of the global, cap-dependent translation and accompanying switch to alternative mechanisms of translation initiation, such as the internal ribosome entry site (IRES)-mediated initiation. In cancer, many aspects of the RNA metabolism are frequently misregulated to provide cancer cells with the growth and survival advantage. This includes changes in the expression and function of RNA binding proteins termed IRES trans-acting factors (ITAFs) that are central to IRES translation. In this review, we will examine select emerging as well as established ITAFs with important roles in cancer initiation and progression, and in particular their role in IRES-mediated translation.

3.3 Introduction

Protein synthesis is a critical regulatory process for maintaining cellular homeostasis as it can rapidly and reversibly modify the protein output in response to different cues [1]. Therefore, and also because translation is energetically very demanding (consuming in excess of 50% of the cell's energy), it is tightly regulated. In fact, misregulation of the translation machinery can lead to several disease states, including carcinogenesis. Regulation of translation occurs at all steps of the process, namely the initiation, elongation and termination; however, it is generally accepted that translation initiation is the rate limiting step, and the most tightly regulated one [2]. Mammalian translation initiation starts with the

recognition of mRNA by the translation machinery through binding of the m7G cap by the cap-binding protein, eukaryotic initiation factor 4E (eIF4E). In turn, eIF4E is bound by the scaffolding protein eIF4G and the RNA helicase eIF4A to form the eIF4F complex. This complex is subsequently bound by additional initiation factors, including eIF3 which recruits the small ribosomal subunit associated with Met-tRNA-bound eIF2 (43S ribosome complex) to the 5' end of the mRNA. It is believed that the resultant 48S ribosomal complex scans along the mRNA until the correct initiation codon (most often AUG) in the proper context is recognized and it is then joined by the 60S ribosomal subunit to form the 80S translating ribosome that proceeds with polypeptide chain elongation.

Carcinogenesis has traditionally been linked to genetic alterations and defects in the transcription program of cells. However, it is now becoming apparent that alterations in the rate of protein synthesis, or in the composition of the translation machinery are also strongly linked to cancer cell properties. For instance, it is now well established that increased protein synthesis and ribosome biogenesis is required to maintain increased cancer cell proliferation [3]. In contrast, induction of apoptosis (such as that triggered by the use of chemotherapeutic drugs) is accompanied by substantial and rapid down-regulation of protein synthesis [4]. These changes in global translation rates are mainly due to changes in the activity or abundance of the canonical translation initiation factors mentioned above, although some evidence suggests that degradation of mRNAs also plays an important role in this process [5]. Modifications of the translation initiation factors and of ribosomes in cancer have been extensively reviewed ([6-8] and in this issue), hence they will not be covered in much detail in this article. Nonetheless, it is important to note that the cells' ability to regulate translation is frequently focused on two key control points which are shared by virtually all mRNAs –

components of the eIF4F complex and eIF2a (Figure 3.1). Indeed, stress-induced phosphorylation of eIF2a is one way by which global protein synthesis is inhibited and decreased eIF2a phosphorylation is often linked to carcinogenesis [9]; however, some studies suggest that increased eIF2a phosphorylation might be associated with tumour progression [10, 11]. To reconcile these differences, it was proposed that the role of eIF2a in cancer probably depends on the stage of the disease where stress-induced eIF2a phosphorylation in the first stages of cancer leads to decreased translation rates and facilitate selective tumour cells survival whereas in later stages, there is an uncoupling of eIF2a kinases activity with translation repression, thus favouring tumour progression (reviewed in [6]). Overexpression of the cap-binding protein eIF4E is observed in several cancers and is often associated with worse clinical outcome and decreased survival [12]. Concomitantly, increased eIF4B binding proteins (4E-BPs) phosphorylation, which leads to increased eIF4E availability, is also observed in several cancers and is associated with tumour progression as well as decreased survival [13]. Caspase activity during stress-induced apoptosis can also lead to the proteolytic cleavage of initiation factors and general inhibition of translation, although tumour cells frequently find other ways to survive these conditions (reviewed in [8]).

Although inactivation of translation initiation factors by means of phosphorylation or proteolytic cleavage generally leads to translation inhibition, it can also promote assembly of modified or alternative initiation complexes that are believed to promote selective translation of mRNAs required during carcinogenesis and tumour progression. For instance, eIF4E overexpression is thought to preferentially activate the translation of mRNAs with highly

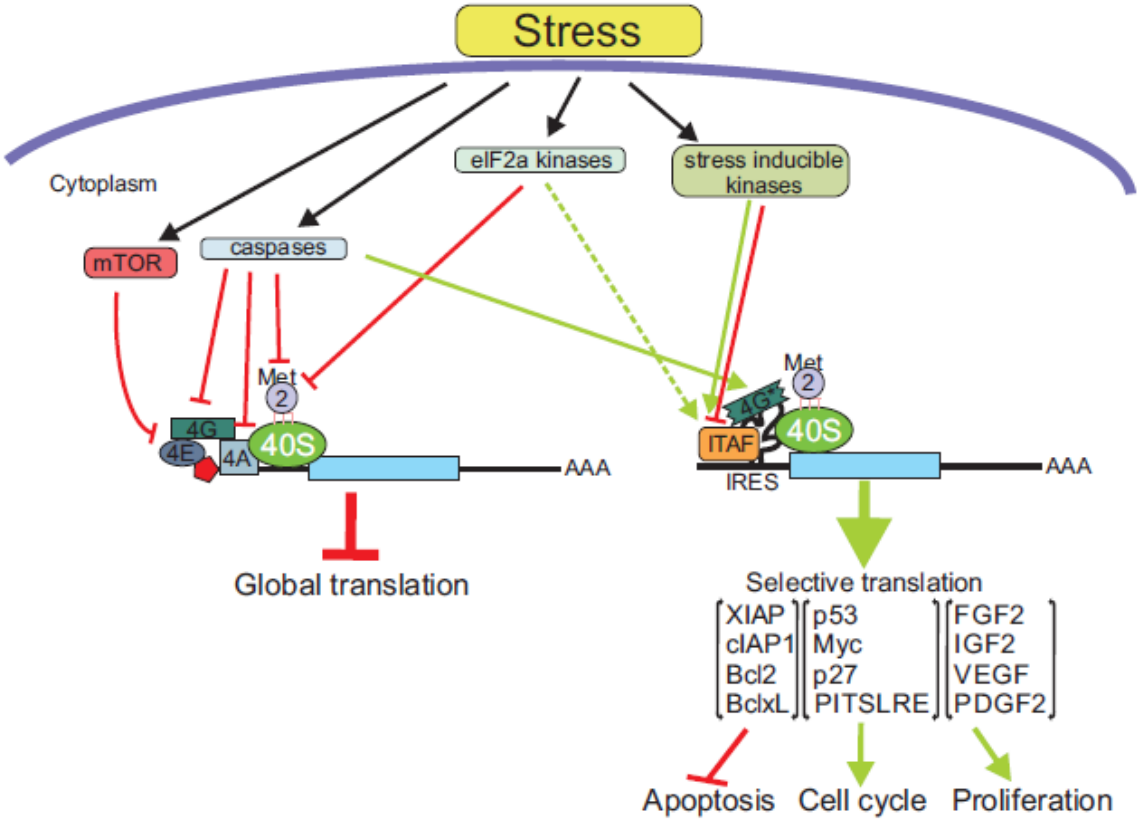
Stress
Cytoplasm
eIF2a kinases
stress inducible kinases
mTOR
caspases
Met
2
4G
4E
4A
40S
AAA
Global translation
4G*
ITAF
IRES
Selective translation
XIAP
cIAP1
Bcl2
BclxL
p53
Myc
p27
PITSLRE
FGF2
IGF2
VEGF
PDGF2
Apoptosis
Cell cycle
Proliferation

Figure 3.1. A schematic diagram illustrating regulation of translation during stress. The left side of the model depicts regulation of general (cap-dependent) translation; the right side depicts selective (IRES-mediated) translation. Green lines indicate positive, while red lines negative impact. Dotted line depicts indirect effect. For simplicity, not all factors are shown. (Adapted from [8]).

structured 5'UTRs, some of which are involved in cancer progression, such as c-Myc, VEGF-A and FGF2 [14]. Existence of an alternative translation initiation was also suspected by the fact that under conditions when cap-dependent translation is severely compromised (e.g. nutrient deprivation, hypoxia, irradiation and viral infection), a subset of mRNAs is still efficiently translated e.g. [15] and this alternative mode of translation control is required for effective stress response [16, 17]. It was therefore proposed that selective translation by alternative mode of translation initiation is a key mechanism which is required for cellular survival under stress and is used by cells to fine-tune their stress response, including during carcinogenesis [2, 6, 7]. One such alternative mode of translation initiation is the Internal Ribosome Site Entry (IRES)-mediated translation. IRESes are discreet regulatory elements present in the 5'UTR of select cellular mRNAs that can recruit the ribosome independently of the 5'cap [18]. It is estimated that about 3% of mRNAs in the cell could be translated by an IRES-dependent mechanism [15, 19]. Interestingly, many proteins encoded by mRNAs with an IRES play important roles in cell survival (cIAP1, XIAP, Bcl-2, Bcl-xL, Apaf-1, Bag1), proliferation (Myc, FGF2, IGF2, PDGF2), cell cycle (p53, p27, PITSLRE) and angiogenesis (VEGF-A, HIF-1α), all processes that are important in cancer initiation and progression and that will be discussed herein. However, it needs to be emphasized that not all cellular IRES have been validated and that the existence of cellular IRESes is still hotly debated [20]. Notwithstanding this criticism, many cellular IRESes were shown to be *bona fide* regulatory elements that drive translation of their respective proteins during cellular stress when global protein synthesis is compromised (Figure 3.1).

Precisely how IRES-mediated translation initiation operates and is regulated is still not fully understood; however, it is known that most cellular IRESes require binding of some

canonical initiation factors to initiate translation [21], but also interaction with other protein factors that have been termed IRES trans-acting factors (ITAFs) [2]. ITAFs are RNA-binding proteins that act to facilitate or block ribosome's recruitment to the IRES, thus enhancing or inhibiting translation of these mRNAs [22, 23]. Interestingly, apart from their regulation of translation, many ITAFS are involved in other aspects of RNA metabolism that are important in carcinogenesis such as mRNA splicing, export and stability. In the following review we will describe the emerging as well as established ITAFs with important roles in cancer initiation and progression, and in particular their role in IRES-mediated translation.

3.4 Insulin-like Growth Factor 2 mRNA Binding Protein 1 (IGF2BP1)

IGF2BP1 is a member of the VICKZ family of proteins named after its founding members from different organisms (Vg1RBP/Vera, IMP1-3, CRD-BP, KOC and ZBP1) [24]. IGF2BP1 and its human paralogs, IGF2BP2 and IGF2BP3 (or IMP1-3), were first identified as RNA binding proteins that interact with the human IGF2-leader 3 5'UTR and negatively regulate its translation [25]. IGF2BP1 also regulates the translation and stability of several other transcripts involved in carcinogenesis or tumor progression such as cIAP1 [26], MK5 [27], c-Myc [28], MDR1 [29], PTEN [27], CD44 [30], GLI1 [31] and CTNNB1 [32]. Interestingly, IGF2BP1 is an oncofetal protein that is expressed during embryogenesis and is silenced in adult tissues [33] but becomes *de novo* expressed in many human cancers (reviewed in [24]). Importantly, this re-expression has been correlated with enhanced metastasis and poor prognosis in several cancers [24, 34] pointing to the involvement of IGF2BPs in carcinogenesis, although the precise mechanisms are not fully known (Figure 3.2).

3.4.1 Regulation of cell proliferation

Recently, a role emerged for the IGF2BP proteins in modulating translation, especifically through an IRES-dependent mechanism. For example, IGF2BP2 was shown to promote IGF2-leader 3 mRNA translation through its IRES, upon phosphorylation by mTOR [35]. A recent study also showed that mTOR complex 2 phosphorylates IGF2BP1, thus enhancing its binding to the IGF2-leader 3 5'UTR and promoting IGF2 IRES-mediated translation [36]. The importance of regulation of IGF2 translation by IGF2BP1 for cell proliferation and embryonal growth was demonstrated *in vivo* as IGF2BP1-null mice are 25% smaller than their wild-type littermates at birth [33], and defects in IGF2BP1-null mouse embryonic fibroblast (MEFs) proliferation can be fully restored by IGF2 treatment, or IGF2BP1 re-expression [36]. It is therefore not surprising that IGF2BP1overexpression leads to increased proliferation of cancer cells *in vitro* and an increase in tumour growth *in vivo*. For example, ectopic expression of IGF2BP1 in the colorectal cancer cell (CRC) lines SW480 and LoVo promoted xenograft tumor growth, whereas intestine-conditional knock-out of IGF2BP1 led to a significant decrease in the number of tumours in the ApcMin/+ mouse model of intestinal cancer [37]. Importantly, IGF2BP1 was the most upregulated mRNA binding protein in 60 human hepatocellular carcinomas (HCC) tumour samples compared to normal liver tissue, and depletion of IGF2BP1 from several liver cancer cells drastically decreased cell proliferation *in vitro* and the growth of xenograft tumours[38].

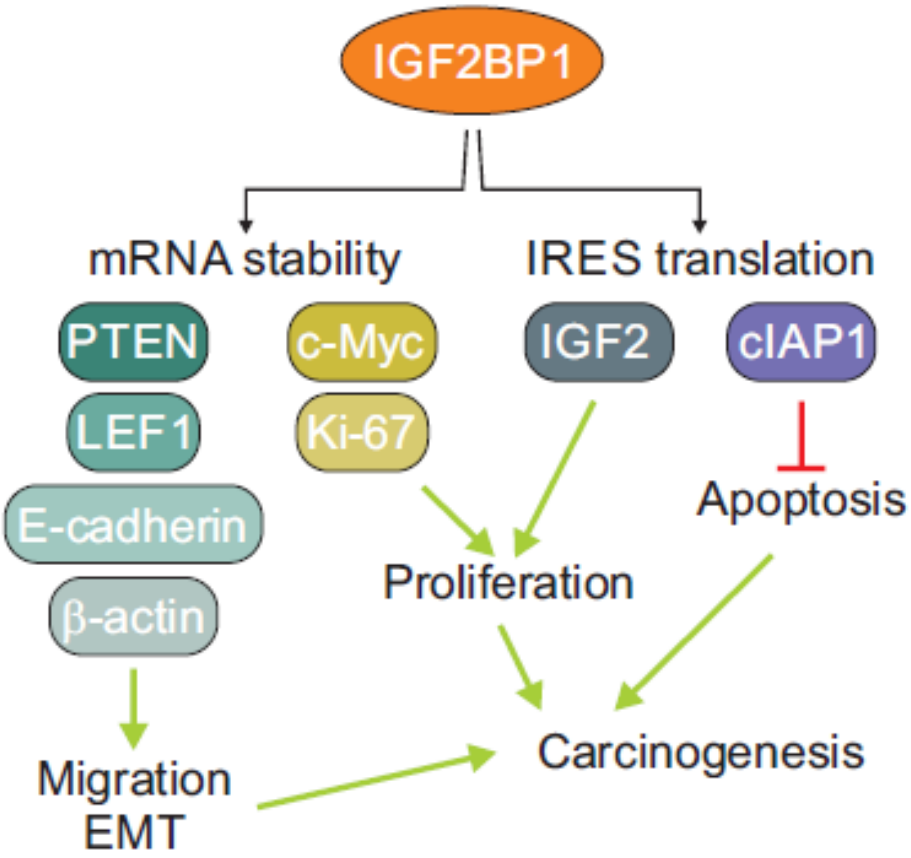
IGF2BP1
mRNA stability
IRES translation
PTEN
c-Myc
IGF2
cIAP1
LEF1
Ki-67
E-cadherin
Apoptosis
Proliferation
β-actin
Migration
EMT
Carcinogenesis

Figure 3.2: Pleotropic effect of elevated IGF2BP1 levels on carcinogenesis.

Re-expression of oncofetal protein IGF2BP1 that is seen in many cancers enhances both the mRNA stability and IRES-mediated translation of key regulators of apoptosis, proliferation and epithelial-to-mesenchymal transition, ultimately driving carcinogenesis. Green lines indicate positive, while red lines negative impact.

The role of IGF2BP1 in enhancing proliferation of cancer cells can be partially attributed to the promotion of the c-Myc mRNA stability and translation. IGF2BP1 was shown to be part of an mRNPs complex that binds to the coding-region instability determinant (CRD) region of c-Myc mRNA, and prevents its degradation by endonucleases upon ribosome stalling in a rare codon stretch at the beginning of the CRD [29, 39]. Accordingly, there is growing correlative and functional evidence linking IGF2BP1 regulation of c-Myc expression to cancer cell growth and survival, specifically in ovarian [40], breast [41], colorectal [42] and HCC [38] cancers. Interestingly, c-Myc is translated through an IRES-dependent as well as cap-dependent mechanisms [43], and one of the ITAFs that positively regulates c-Myc IRES translation, YB-1, is also part of the mRNPs complex that interacts with IGF2BP1 to promote c-Myc stability via the CRD [28]. Hence, it would be interesting to explore whether IGF2BP1 also acts as an ITAF for the c-Myc IRES, or if it participates in YB-1 recruitment to the IRES. Of note, a mutated form of the c-Myc IRES which enhances IRES activity and results in an increased expression of c-Myc was identified in a cohort of multiple myeloma patients [44]. It was later determined that YB-1 and PTB, another c-Myc ITAFs, bind more strongly and synergistically to the mutated c-Myc IRES [45]. Interestingly, there is a strong correlation between PTB, YB-1 and c-Myc expression in multiple myeloma-derived cell lines, suggesting that these ITAFs contribute to high levels of c-Myc in myeloma cancer cells [45].

3.4.2 Regulation of apoptosis

Recently, our group identified IGF2BP1 as one of the protein factors that bind to the cIAP1 IRES [46], and confirmed that IGF2BP1 is a *bona fide* ITAF that enhances cIAP1

IRES-mediated translation [26]. Interestingly, IGF2BP1 is highly overexpressed in primary human rhabdomyosarcoma (RMS) tumours when compared to healthy muscle tissues, and in several established RMS cancer cell lines, and the high levels of IGF2BP1 drive cIAP1 translation. Given the critical role cIAP1 plays in the regulation of cell survival *via* the NF-κB pathway and caspase-8 mediated cell death (reviewed in [47, 48]), we postulated that the IGF2BP1-dependent overexpression of cIAP1 in RMS would confer an increased apoptotic resistance on these cells. Indeed, a subset of RMS cancer cells is highly resistant to TNFα or TRAIL induced apoptosis [49], and IGF2BP1 knock-down greatly sensitizes these cells to death ligands induced apoptosis. Furthermore, cIAP1 re-expression in IGF2BP1-depleted cells was sufficient to block TNFα-induced apoptosis, confirming that cIAP1 is the main factor mediating apoptotic resistance of RMS cancer cells. Importantly, treatment of RMS cells with Smac mimetics (SMCs), a class of chemical compounds that cause auto-ubiquitination and proteasome degradation of the cIAPs [50], greatly sensitized RMS to TNFα or TRAIL induced apoptosis *in vitro* and significantly extended survival of mice bearing RMS xenografts, thus highlighting the importance of IGF2BP1 and its target cIAP1 in mediating apoptotic resistance in RMS [26].

IGF2BP1 knock-down also sensitized both primary and metastatic melanoma cell lines to the chemotherapeutic agents dacarbazine, temozolomide, vinblastine, and etoposide by increasing apoptosis and decreasing cell growth [51]. This was attributed in part to IGF2BP1 regulation of the microphthalmia-associated transcription factor (MITF) expression as reintroduction of MITF in melanoma cells partially compensated for the IGF2BP1 loss. Acquired resistance of ovarian cancer cells to Taxanes was also attributed to IGF2BP1 stabilization of the multidrug resistance 1 (MDR1) transcript [29, 52]. Depletion of

IGF2BP1 from liver cancer cells also induced apoptosis and reduced the growth of HepG2 xenograft tumours [38]. Mechanistically, this was attributed to IGF2BP1 positive regulation of c-Myc and Ki-67 mRNA stability. Interestingly, IGF2BP1 depletion from HepG2 significantly decreased the progression and size of xenograft tumours but not their number, suggesting that IGF2BP1 is important in the progression but not the initiation of this type of tumour [38]. IGF2BP1 was also shown to promote the survival of colon cancer cells through its promotion of c-Myc and K-Ras expression. In this study, depletion of IGF2BP1 induced caspase-3 and PARP-mediated apoptosis through de-repression of the p53-inducible pro-apoptotic protein CYFIP2 and downstream inhibition of K-Ras expression [42]. Hence, there is growing evidence of IGF2BP1 promoting the apoptotic resistance of cancer cells by either stimulating the expression of anti-apoptotic or suppressing expression of pro-apoptotic genes. However, mechanistic linking of IGF2BP1 to the inhibition of apoptosis in tumour models is scarce, and more work needs to be done to identify IGF2BP1 regulated transcripts that are responsible for this important aspect of IGF2BP1 oncogenicity *in vivo*.

3.4.3 Regulation of metastasis and the epithelial to mesenchymal transition (EMT)

There is also growing evidence for IGF2BP1 involvement in the regulation of cell migration and metastasis. IGF2BP1 regulates the translation and stability of several mRNAs important in these processes including β-actin [53], c-Myc [28, 39, 40], CD44 [30] and E-cadherin [37]. IGF2BP1 and its chicken ortholog, ZBP1, have been extensively characterized for their binding to a 54-nucleotide element named the zipcode within the 3'UTR of β-actin, and for the modulation of localized translation of β-actin at the lamellipodia of fibroblasts and neurons [53, 54]. IGF2BP1 has also been shown to modulate lamellipodia formation and

promote directed cell migration in glioblastoma, osteosarcoma, ovarian, bladder and CRC derived cell lines. In CRC xenograft models, IGF2BP1 was shown to promote the dissemination of tumour cells into the blood, an early metastatic event [37], and transgenic IGF2BP1 expression in the mammary tissues of lactating mice also induced the formation of primary tumours and metastasis in 95% of the mice [55]. However, there exists conflicting data describing an inhibitory role for IGF2BP1 in metastasis, as other xenograft studies show that forced expression of IGF2BP1 in the breast cancer cell line MTLn3 reduced their metastatic potential [56], and that silencing of IGF2BP1 is associated with increased invasion, partly due to a decrease in E-cadherin accumulation at the adhesion sites of T47D and MDA231 breast cancer cells [57, 58]. A recent mechanistic study may, however, offer a possible explanation for these discrepancies [59]. IGF2BP1 regulation of cell adhesion and migration is two-fold. First, IGF2BP1 acts as a key regulator of actin dynamics through regulation of β-actin localized translation [53], but also by regulating the availability of monomers for actin polymerisation. IGF2BP1 binds to the MAPK4 3'UTR and inhibits its translation, thus leading to decreased activation of the MAPK-activated protein kinase MK5 and subsequent hypo-phosphorylation of HSP27 [27]. Because phosphorylated HSP27 is an actin sequestering factor [60], this in turn leads to increased availability of monomeric actin to support enhanced actin dynamics, correlating with increased cell matrix adhesion and velocity of cell migration [59]. On the other hand, IGF2BP1 also promotes PTEN mRNA stability and expression, thus leading to enhanced cell polarization and directed migration in a RAC-1 dependent manner [27]. Hence, in cancer cells, IGF2BP1 is fine-tuning the balance between having enough cell adhesion to insure a "firm grip" for migration to occur or having too much cell adhesion that becomes detrimental to migration.

There is also evidence that IGF2BP1 regulates the epithelial to mesenchymal transition (EMT), a key process for cancer cells to acquire metastatic potential, by promoting the expression of the transcription factors LEF1 and SNAI2/Slug. IGF2BP1 knock-down in HEK293 and U2OS cells led to destabilisation of the LEF1 transcript, decreased SNAI2/Slug and fibronectin expression, increased β-actin protein levels as well as increased recruitment of β-catenin and E-cadherin to cell junctions, thus pushing these cells towards a more epithelial-like phenotype [61]. Accordingly, IGF2BP1 promoted cancer cell dissemination through a loss of epithelial properties and increased $CD24^{+}CD44^{+}$ tumor-initiating cell phenotype in a colorectal xenograft model [37]. Furthermore, in an *in vivo* model of colonic wound healing, IGF2BP1 was shown to be required for the full induction of the prostaglandin-2 mRNA in colonic mesenchymal stem cells, a step that is necessary for these cells migratory potential and optimal healing of colonic lesions [62]. However, it appears that IGF2BP1 alone does not have the potential to initiate the EMT process, but rather sustains it, as forced expression of IGF2BP1 in MCF-7 and MDCK epithelial-like cells was not sufficient to either induce mesenchymal-like properties or increase their migratory potential [61].

It is evident that IGF2BP1 is emerging as an important oncogenic factor, in particular with respect to its regulation of the translation and stability of several mRNA involved in the control of apoptosis, cell proliferation and metastasis. However, more work needs to be done to address the reported discrepancies in IGF2BP1 functions, especially with respect to the regulation of cell migration, and to confirm IGF2BP1 role in cancer through appropriate animal models and clinical studies.

3.5 Nuclear Factors 45 and 90 (NF45 and NF90)

NF45 was first identified as a nuclear factor of activated T cells (NFAT)-related transcription factor that regulates interleukin-2 transcription, together with its binding partner

NF90 [63]. NF45 is usually found in complex with other members of the Nuclear Factor Associated with dsRNA (NFAR) family (in particular NF90 and its isoforms), and this association mutually safeguards their protein stability [64]. Although originally characterized as DNA-binding proteins, NF45 and NF90 do not interact directly with DNA and their function in transcription and DNA metabolism is presumably mediated through interaction with other DNA binding proteins [65]. However, both NF45 and NF90 can bind double-stranded, as well as structured singled-stranded RNAs, and most of their described function is in the regulation of translation [46, 66, 67], RNA stability [68], RNA export [69], microRNA biogenesis [70, 71] and viral gene expression [72, 73]. Interestingly, NF45 was found to be overexpressed in HCC when compared to normal liver tissue [74], as well as in childhood germ cell tumours [75]. NF45 was also identified as one of the three novel "Trend-of-Disease-Progression (ToP)" genes involved in HCC and was proposed as a potential marker for early detection of progression from normal colon to adenoma or inflammatory bowel disease, to full colorectal carcinoma disease [76]. Similarly, NF90 was shown to be significantly overexpressed in lung adenocarcinoma tumors and metastases compared to adjacent normal tissues [77], and in epithelial ovarian tumours compared to normal tissue [78], thus suggesting an involvement for NF45 and NF90 in carcinogenesis and cancer progression.

3.5.1 Regulation of mitosis, cytokinesis and DNA damage response

We have previously identified NF45 as one of the proteins that interacts with the cIAP1 IRES and confirmed that NF45 is an ITAF that enhances IRES-mediated translation of the cIAP1 mRNA [46]. Translation of the cIAP1 mRNA is negligible during normal growth conditions due to the presence of multiple upstream open reading frames (uORFs) in its 5'UTR [79], but becomes activated *via* an IRES mechanism during conditions of stress, such as the unfolded protein response (UPR) [80]. Interestingly, while NF45 is required for the IRES-mediated induction of cIAP1 expression during the UPR, its binding partner, NF90, is not required for this function [46]. Further characterization of NF45 as an ITAF uncovered that NF45 preferentially binds to AU-rich 5'UTR regions [46], and that an AU-richness of an IRES (> 60% AU content) is an excellent predictor for its dependence on NF45 [67]. A screen for different NF45-dependent cellular IRESes identified three additional IRES mRNAs (XIAP1, NRF and ELG) whose translation is enhanced by NF45. Importantly, in NF45-deficient HeLa cells, the decrease in cIAP1 translation results in a decrease in cyclin E expression, whereas reduced XIAP translation leads to an increase in Survivin protein levels, thus contributing to the mitotic defects and severe multinucleated phenotype observed in NF45-depleted cells [67] (and see below).

The depletion of NF45 in HeLa cells results in flattening of the cells, increased F-actin expression, increased cell size, multinucleation and slow growth [64]. The slow growth was suggested to be due to defects in DNA synthesis [64], but is also consistent with a decrease in cyclin E expression due to reduced cIAP1 IRES-mediated translation [67]. Of note, NF45 binding partner NF90 was shown to repress the translation of senescence-associated secretory phenotype (SASP) mRNAs such as MCP-1, GROa, IL-6 and IL-8 [81] and this may

in part explain the senescence phenotype also observed in the NF45 deficient HeLa cell lines [64]. In addition, a time-lapse microscopy study of nuclear division indicated that upon NF45 knock-down, multinucleated cells arose from the incomplete cytokinesis of daughter cells followed by the fusion of several binucleated cells [82]. This is consistent with a reduction in XIAP IRES-mediated translation and consequent increase in Survivin protein stability observed upon NF45 knock-down [67], as Survivin is a key regulator of mitosis whose aberrant expression has been linked to multinucleation [83]. Interestingly, NF90 depletion also results in multinucleation, [64] and the NF45/NF90 complex was shown to regulate DNA damage repair trough the association with the DNA-dependent protein kinase (DNA-PK), [82, 84]. Indeed, depletion of the NF90/NF45 complex leads to a defect in non-homologous end joining repair (NHEJ) comparable to that of DNA-PK depleted cells and increased γ-Histone 2A.X foci, indicative of an increase in double-stranded DNA breaks. Importantly, NF90/NF45 depleted cells are more sensitive to ionising radiation, consistent with defects in their DNA damage repair pathways [82]. Hence, NF45 and NF90 are critical modulators of translation and DNA damage repair pathways whose deregulation can lead to aberrant mitosis and DNA damage, all processes that are conducive to carcinogenesis (Figure 3.3).

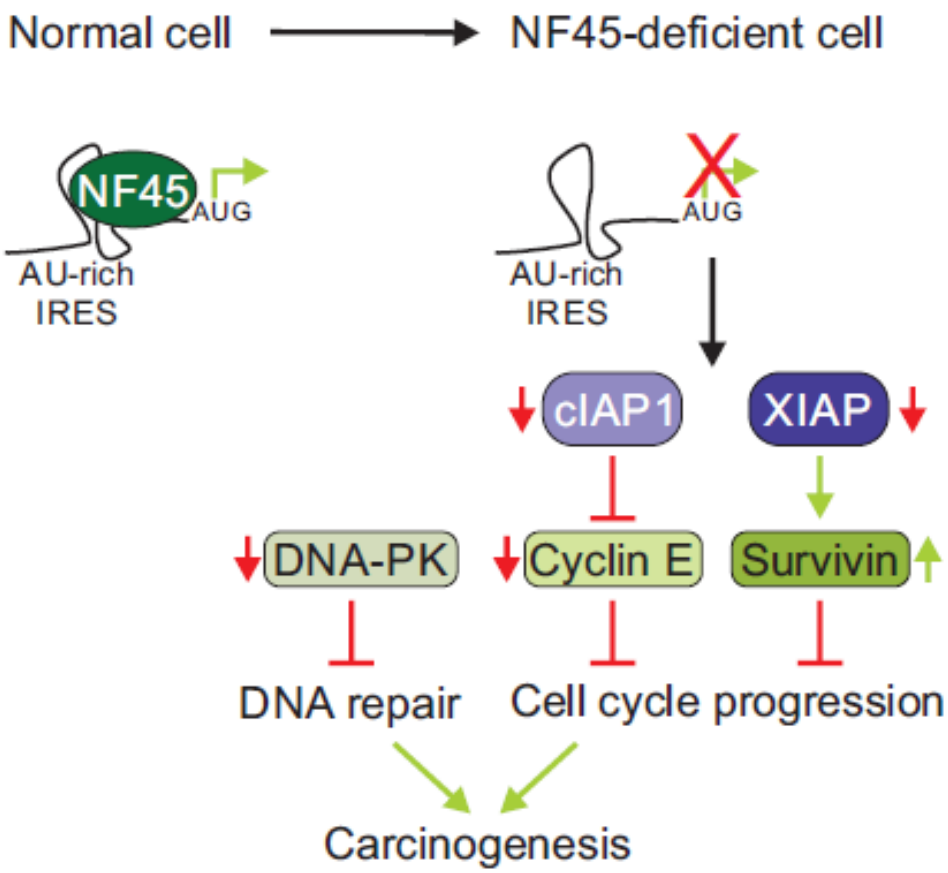
Normal cell
NF45-deficient cell
NF45
AUG
AU-rich
IRES
AUG
AU-rich
IRES
cIAP1
XIAP
DNA-PK
Cyclin E
Survivin
DNA repair
Cell cycle progression
Carcinogenesis

Figure 3.3: NF45 regulates genomic stability and cytokinesis. In normal cells NF45 drives expression of a cohort of mRNAs required for cellular homeostasis (left). Depletion of NF45 (right) results in an attenuation of IRES-mediated translation from AU-rich IRES such as cIAP1 and XIAP, which subsequently leads to a decrease in Cyclin E, and an increase in Survivin expression, ultimately resulting in an aberrant progression through the cell cycle and accumulation of polyploid cells. Concomitantly, NF45 deficiency results in a loss of DNA-PK activity and a consequent inhibition of the DNA repair pathways. Green lines indicate positive, while red lines negative impact.

3.5.2 Regulation of hypoxia and apoptosis

NF90 was also shown to bind to the hypoxia stability region (HSR) of the vascular endothelial growth factor (VEGF) 3'UTR and to modulate its mRNA stability and translation during conditions of hypoxia [68]. NF90 knock-down (and consequent NF45 depletion) limited the induction of VEGF mRNA and protein expression during hypoxia, but in a HIF-1-independent manner. Furthermore, NF90 stable depletion from MDA-MB-435 breast cancer cells lead to a decrease in VEGF mRNA stability and polysome association under hypoxic conditions. Importantly, NF90-depleted cells showed reduced growth and angiogenic potential in a xenograft tumor model [68], highlighting the importance of NF90 and possibly NF45 in these tumorigenic processes. The NF90/NF45 complex was also shown to contribute to the apoptotic resistance of human papilloma virus (HPV)-transformed cervical carcinoma cells through their regulation of p53 and p21 expression [85]. Indeed, NF90 or NF45 knock-down greatly increased p53 and p21 protein levels in HPV-derived HeLa and SiHa, but not in other cancerous cell type or HPV-negative cervical cancer cells. This is because NF90/NF45 regulation of p53 and subsequently p21 expression is linked to their regulation of the HPV genome, specifically of the E6 RNA transcription. NF90/NF45 depletion inhibits transcription from the HPV early promoter and subsequently of the E6 RNA [85]. This in turn leads to increased p53 protein stability and subsequent increase in p21 transcription, as the HPV E6 and E6AP proteins accelerate p53 proteasome-mediated turnover [86, 87]. Importantly, NF90/NF45 depleted HeLa cells were more sensitive to camptothecin-induced apoptosis due to p53 derepression [85].

3.5.3 Regulation of microRNA processing

NF45 and NF90 are also part of a large protein complex formed by the microprocessor complex Drosha/DGCR8 and other RNA-binding proteins, including RNA helicases and heterogeneous nuclear ribonucleoproteins (hnRNPs) [88]. Although the NF90/NF45 complex does not interact directly with Drosha, it has been shown to negatively regulate microRNA (miRNA) processing by binding directly to some pri-miRNAs and preventing their processing by the microprocessor complex [70]. The NF90/NF45 complex had higher affinity for pri-let-7a miRNA species and NF90 depletion led to a decrease in pri-let-7a, an increase in mature let-7a miRNA and consequently, an anti-proliferative effect on HEK293 cells [70]. Interestingly, TRAIL Receptor 2 (TRAIL-R2) was found to interact with both the Drosha/DGCR8 and the NF90/NF45 complexes, and TRAIL-R2 knockdown in pancreatic cancer cell lines increased Drosha-mediated processing of let-7 pri-mRNAs and inhibition of cell proliferation, possibly through reduced levels of the let-7 target HMGA2 [71]. Importantly, TRAIL-R2 knock-down in pancreatic ductal adenocarcinoma cells slowed their growth in a xenograft tumor model. Similarly, NF90 knock-down inhibited the progression of MDA-MB-231 breast cancer xenografts through increased processing of the urokinase-type plasminogen activator targeting miRNA [89].

All these studies indicate that NF45 and NF90 are RNA-binding factors that, through their post-transcriptional regulation of several key mRNAs, are critical to the normal processes of mitosis, cell proliferation and apoptosis, and that when overexpressed may lead to carcinogenesis. However additional work needs to be done to address the distinct mechanisms by which NF45 and NF90 regulate separately, or in complex, these cellular processes and the consequences of this regulation *in vivo*.

3.6 Programmed Cell Death 4 (PDCD4)

PDCD4 was first identified as a gene whose expression is increased during programmed cell death, hence its name [90]. Since then, PDCD4 has emerged as a well-documented tumour suppressor that inhibits carcinogenesis and tumour progression, mainly through the regulation of translation (reviewed in [91] and herein). First indications of PDCD4 function as a tumour suppressor came from the work in the JB6 epidermal cell line, where low levels of PDCD4 promoted neoplastic transformation upon stimulation with carcinogens, whereas transformation-resistant cells expressed high levels of PDCD4. Importantly, antisense knock-down of PDCD4 reverted the latter cells to a transformation-sensitive phenotype, thus indicating that PDCD4 is a potent inhibitor of tumour initiation [92]. PDCD4 mRNA and protein expression are downregulated in a variety of tumours and in most instances correlate with tumour progression and invasiveness (reviewed in [91]). The mechanisms of PDCD4 downregulation in tumours are still not fully explored, however two main ones have been characterized: at the mRNA level, PDCD4 expression is controlled by miR-21, one of the major oncogenic microRNAs whose expression is misregulated during carcinogenesis. miR-21 upregulation has been reported in numerous cancers and has been linked to tumour growth, apoptotic resistance, invasion and metastasis [93, 94]. PDCD4 is one of the most important functional targets of miR-21 and there exists an inverse correlation between miR-21 and PDCD4 expression in many cancers [95-97]. At the protein level, PDCD4 can be phosphorylated by Akt and S6 kinases, leading to its proteasomal degradation [98, 99].

3.6.1 Regulation of cell cycle

PDCD4 was proposed to be an inhibitor of global translation through its interaction with eIF4A and inhibition of eIF4A helicase activity [100]. Mutational and structural analyses showed that the two MA-3 domains of PDCD4 are necessary and sufficient for eIF4A binding and translation repression [101, 102]. Hence it was suggested that PDCD4 would inhibit the expression of mRNAs with highly structured 5'UTRs that require eIF4A activity for their efficient translation [103]; however, only few such mRNAs have been confirmed to be regulated by PDCD4. Among these, and with high relevance to carcinogenesis is the tumour suppressor p53 [104]. PDCD4 inhibits translation of p53, and this inhibition is dependent on PDCD4-eIF4A interaction which is mediated by the p53 5'UTR. Interestingly, treatment of HepG2 cells with DNA-damaging agents such as UV light or etoposide decreased PDCD4 protein levels, thus allowing for p53 protein induction. These observations led to a proposed mechanism in which PDCD4 suppresses p53 mRNA translation in unstressed cells resulting in low steady-state p53 protein levels, whereas in stressed cells PDCD4 becomes downregulated, allowing for p53 protein induction and response to the initiating stress [104]. Importantly, PDCD4 was also shown to regulate the activity of p53 by interfering with its CBP (cAMP-responsive-element binding protein) –dependent acetylation and PDCD4 knock-down protected UV-irradiated cells from apoptosis in a p53-dependent manner [105]. The proto-oncogene c-myb is also regulated by PDCD4 at the translational level, although unlike p53, PDCD4 inhibits c-myb translation independently of the 5'UTR and *via* a responsive element located in the coding region [106]. Recently, it has been reported that PDCD4 binds a similar element in the proto-oncogene A-myb coding region, and that PDCD4 inhibits c-myb and A-myb expression by interfering with translation elongation [107].

Interestingly, the regulation of A-myb translation by PDCD4 is independent of PDCD4 interaction with eIF4A [107].

3.6.2 Regulation of apoptosis

PDCD4 also contributes to the apoptotic resistance of cancer cells through the regulation of IRES-mediated translation of anti-apoptotic proteins XIAP and Bcl-xL. We have shown recently that in normally growing cells PDCD4 binds to these IRESes, inhibits formation of the 48S initiation complex and represses XIAP and Bcl-xL translation; consequently, removal of PDCD4 results in increased XIAP and Bcl-xL translation [108]. Interestingly, treatment of small cell lung cancer cells with FGF2 protects them from etoposide induced cell death by upregulating XIAP and Bcl-xL [109]. Stimulation of cells with FGF2 leads to activation of S6K2 which subsequently phosphorylates PDCD4, leading to its proteosomal degradation and consequent increase in XIAP and Bcl-xL IRES-mediated translation [108] (Figure 3.4). Importantly, the loss of PDCD4 seen in glioblastoma tumours correlates with increased expression of Bcl-xL, and the resultant chemoresistance can be reverted by direct inhibition of Bcl-xL [110].

3.6.3 Regulation of EMT and metastasis

PDCD4 has also been implicated in the regulation of EMT, invasion and metastasis. PDCD4 overexpression was reported to inhibit invasion of colon [111], breast [112] and ovarian cancer cells [113], whereas PDCD4 knock-down in colon cancer cells led to a fibroblast-like phenotype and promoted invasion [114]. PDCD4 knockdown in colon cancer HT29 and GEO cells caused a decrease in E-cadherin expression as well as β-catenin

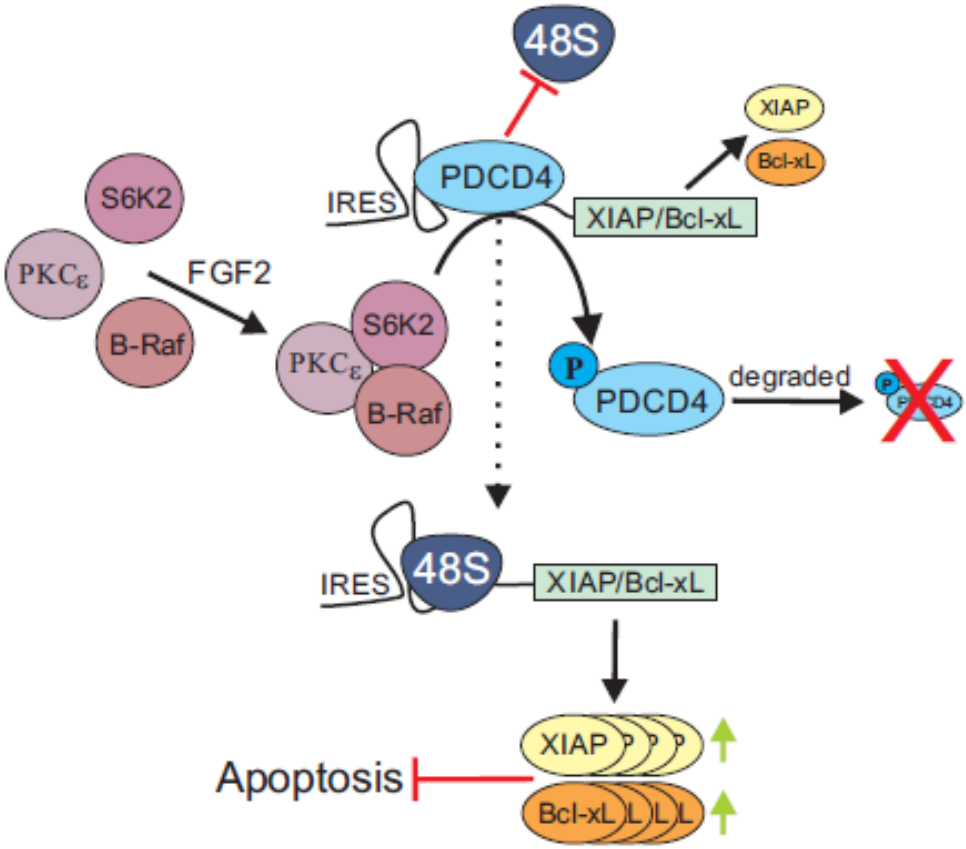
48S
XIAP
Bcl-xL
IRES
PDCD4
XIAP/Bcl-xL
S6K2
PKCε
FGF2
B-Raf
S6K2
PKCε
B-Raf
P
PDCD4
degraded
IRES
48S
XIAP/Bcl-xL
XIAP
Bcl-xL
Apoptosis

Figure 3.4: PDCD4 controls cell survival downstream of FGF-2 and S6K2. Normally, IRES-bound PDCD4 blocks access of the ribosome to the specific mRNAs, thus inhibiting efficient translation from these mRNAs (top). FGF-2 triggers phosphorylation of PDCD4 by S6K2, and subsequent degradation of PDCD4 through the proteosome (middle) leads to the derepression of the IRES-mediated translation of cellular mRNAs (such as XIAP and Bcl-xL) that mediate FGF-2-S6K2 pro-survival signaling, which results in enhanced apoptotic resistance and survival of cancer cells (bottom). Green lines indicate positive, while red lines negative impact.

translocation to the nucleus, two hallmarks of EMT [115]. Down-regulation of E-cadherin expression upon PDCD4 knock-down was partly due to an increase in the expression of E-cadherin inhibitor SNAI1 (Snail), one of the key transcription factors involved in EMT [114, 116]. Recently, it was confirmed that PDCD4 knock-down causes EMT and promotes metastasis *in vivo*, PDCD4 knock-down in an orthotopic GEO colon cancer mouse model led to a decrease in E-cadherin and α-catenin and an increase in N-cadherin and β-catenin expression, indicative of EMT [117]. Snai1 expression in these tumors was also increased and inversely correlated with E-cadherin, confirming the previous *in vitro* data. PDCD4 knock-down also led to an increase in c-Myc nucleic staining presumably due to the increase in β-catenin expression, thus contributing to the invasion and migratory potential of colon cancers [117]. Furthermore, all mice implanted with PDCD4-shRNA-depleted GEO cells developed lymph node and liver metastases whereas none of the mice implanted with control-shRNA or parental cells did [117]. Altogether, these studies demonstrate that PDCD4 is an effective repressor of EMT, invasion and metastasis. However, how PDCD4 regulates Snail and β-catenin expression, or if it regulates the expression of other transcripts involved in metastasis is still not known and should be the focus of future studies. Of note, Snail mRNA contains a highly structured 5'UTR [118], and was shown to be regulated at the translation level by eIF3e during EMT in breast epithelial cells [119]; it would therefore be interesting to examine whether PDCD4 regulates translation of the Snail mRNA.

3.7 Death-associated protein DAP5/p97

The death-associated protein DAP5/p97 (also known as NAT1 and eIF4G2) is a member of the eIF4G family of eukaryotic translation initiation factors. In normal growth conditions, DAP5/p97 functions as an inhibitor of cap-dependent translation [120], although it has been also shown to facilitate global translation after growth factor stimulation [121]. Furthermore, in stressed cells, DAP5/p97 is processed by caspases, resulting in the removal of the 11 kDa C-terminus and generating 86 kDa variant (DAP5/p86) which retains the eIF4A and eIF3 binding sites but is not capable of eIF4E binding [122]. Consequently, DAP5/p86 drives selective, IRES-mediated translation of cell death-regulating proteins (see below) (Figure 3.5) [80, 123, 124]. Hence, DAP5/p97 is a caspase-activated translation initiation factor, and its proteolytic processing functions as an apoptotic translation switch that regulates cell fate by tipping the balance between the translation of pro- and anti- apoptotic proteins (reviewed in [2, 18]). In addition, DAP5/p97 promotes its own IRES-mediated translation both in caspase-dependent and caspase-independent manner, suggesting the existence of a positive feedback loop that ensures elevated levels of DAP5/p97 to support IRES-mediated translation of select mRNAs during conditions of reduced global protein synthesis [124]. Importantly, DAP5/p97 is abundantly expressed in proliferating cells and its knock-down inhibits global protein translation and cell proliferation[125]. In addition, and consistent with the role of DAP5/p97 as an activator of translation of select mRNAs in particular as part of the cellular stress response, DAP5/p97 knockdown is not compatible with cell survival [125, 126].

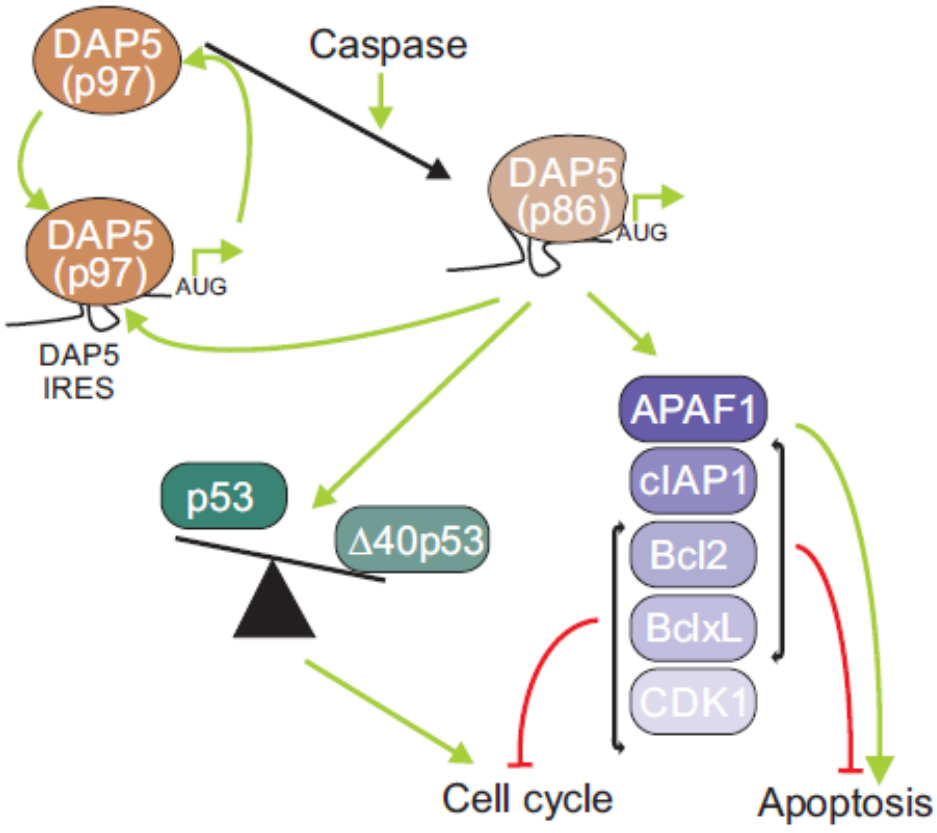
DAP5
(p97)
Caspase
DAP5
(p86)
AUG
DAP5
(p97)
AUG
DAP5
IRES
APAF1
cIAP1
Bcl2
BclxL
CDK1
p53
Δ40p53
Cell cycle
Apoptosis

Figure 3.5: DAP5 is a caspase-controlled translation switch which regulates balance of cell death and survival. Full length DAP5/p97 drives expression from its own IRES in normally growing cells (left). Following and apoptotic trigger, a truncated variant of DAP5, p86, is generated by caspase cleavage, and subsequently drives IRES-mediated translation of a cohort of cellular mRNAs encoding proteins that regulate cell cycle and apoptosis. Green lines indicate positive, while red lines negative impact.

3.7.1 Regulation of apoptosis and cell cycle

DAP/p86 regulates cIAP1 IRES-mediated translation during UPR. Activation of cIAP1 in the context of the UPR is dependent on caspase activation and requires proteolytic processing of DAP5/p97 into DAP5/p86 [80, 124]. Under UPR-induced conditions of reduced cap-dependent translation, cIAP1 mRNA is selectively recruited to the polysomes *via* IRES, and this process is dependent on DAP5/p86, as reducing the levels of endogenous DAP5 by siRNA abrogated both the translation and IRES activity of cIAP1 during ER stress. The precise mechanism of how DAP5/p86 enhances cIAP1 IRES activity is not known but it is likely that it involves association with other accessory proteins. For example, the cIAP1 ITAF NF45 (see above) is also required for cIAP1 IRES activity in response to ER stress [46]. Whether NF45 and DAP5/p97 interact with each other, however, remains to be determined.

Several other mRNAs that encode proteins involved in the regulation of apoptosis require DAP5/p97 for their IRES-mediated translation. For example, expression of the pro-apoptotic Apaf-1 was shown to be enhanced by DAP5/p86 [127, 128]. In contrast, cells depleted of DAP5/p97 show reduced expression of Bcl-2, reduced Bcl-2 IRES activity and translation, as well as increased M-phase specific apoptosis [129]. The same study also identified an IRES in the 5'UTR of CDK1 and suggested that DAP5/p97 regulates CDK1 IRES-mediated regulation since DAP5/p97 knock-down decreased CDK1 translation and phosphorylation of CDK1 substrates in the M phase. Importantly, re-expression of Bcl-2 or CDK1 partially blocked caspase activation which is caused by DAP5/p97 knockdown [129]. Similarly, Bcl-xL protein levels were also reduced in DAP5/p97-depleted cells without concomitant changes in Bcl-xL mRNA levels, although it was not formally tested if the activity of Bcl-xL IRES was abrogated in these conditions [130]. By regulating translation of

Bcl-2 and Bcl-xL, DAP5/p97 may possibly also regulate cell cycle progression and cell division. Indeed, DAP5/p97 depleted cells appear smaller and less spread-out in culture, and most are arrested in the S-phase following thymidine block, compared to control cells that are arrested in G1/S. Furthermore, when released from thymidine block, DAP5/p97 depleted cells progress in the cell cycle one step ahead of control cells, thus entering mitosis faster [130]. Since Bcl-2 and Bcl-xL are known to be involved in apoptosis as well as cell cycle regulation, it is possible that by modulating their IRES-dependent translation, DAP5/p97 protein levels in the cell may control the fate of cells entering mitosis, either to progress through the cell cycle or to undergo apoptosis [130].

3.7.2 Regulation of p53-dependent cell survival

Another possible link of DAP5/p97 to cancer is its regulation of p53 IRES-mediated translation. The p53 mRNA contains two IRES elements that control the expression of the p53 full length protein and of the N-terminal truncated Δ40-p53 (or ΔN-p53) isoform, respectively [131, 132]. DAP5/p97 was recently shown to differentially regulate translation from these two IRESes, preferentially stimulating translation from the second IRES [133]. Therefore, DAP5/p97 depletion in H1299 or A549 lung carcinoma cells results in more pronounced downregulation of the Δ40-p53 isoform, compared to the full length p53 protein, and subsequent decrease in 14-3-3σ transcription, a known target of Δ40-p53, whereas transcription of p21 was not significantly altered [133]. Hence, by regulating the ratio between p53 and Δ40-p53 isoforms, DAP5/p97 may play an important role in deciding of the fate of the cell upon stress. It has been suggested that the two p53 isoforms are differentially expressed during the cell cycle, and that Δ40-p53 acts as a dominant-negative isoform that

antagonizes p53-mediated transcription and growth regulation [132, 134]. Importantly, H1229 cells overexpressing the Δ40-p53 isoform are more resistant to Doxorubicin-induced apoptosis [135], although whether it is the DAP5/p97 regulation of p53 IRES-mediated translation which contributes to this apoptotic resistance remains to be investigated.

These observations show that DAP5/p97 and its stress-induced DAP5/p86 variant play important roles in the regulation of apoptosis, cell cycle and mitosis (Figure 3.5). Given the critical role of DAP5-regulated mRNAs in cancer, the link between DAP5/p97 and carcinogenesis can be postulated; however, further work is needed to validate this link in tumour models *in vivo*.

3.8 Conclusion and perspectives

It is evident that IRES trans-acting factors play important roles in cancer development and progression. By modulating the expression status of a cohort of mRNAs involved in cell survival, proliferation, cell cycle or migration/invasion, single or multiple ITAFs can simultaneously impinge on the regulation of several hallmarks of cancer such as tumour growth, cell cycle progression, resistance to apoptosis , angiogenesis, and migration (Figure 3.6). Interestingly, expression and subcellular localization of most ITAFs is misregulated in tumour cells [136], further hinting at their involvement in carcinogenesis. Therefore, there is now enough evidence to consider ITAFs for potential targeted therapy in certain types of cancer, either alone or in combination with other therapies. One way of achieving this would be to restore ITAFs expression levels in cancer cells to normal. For example, for PDCD4 this

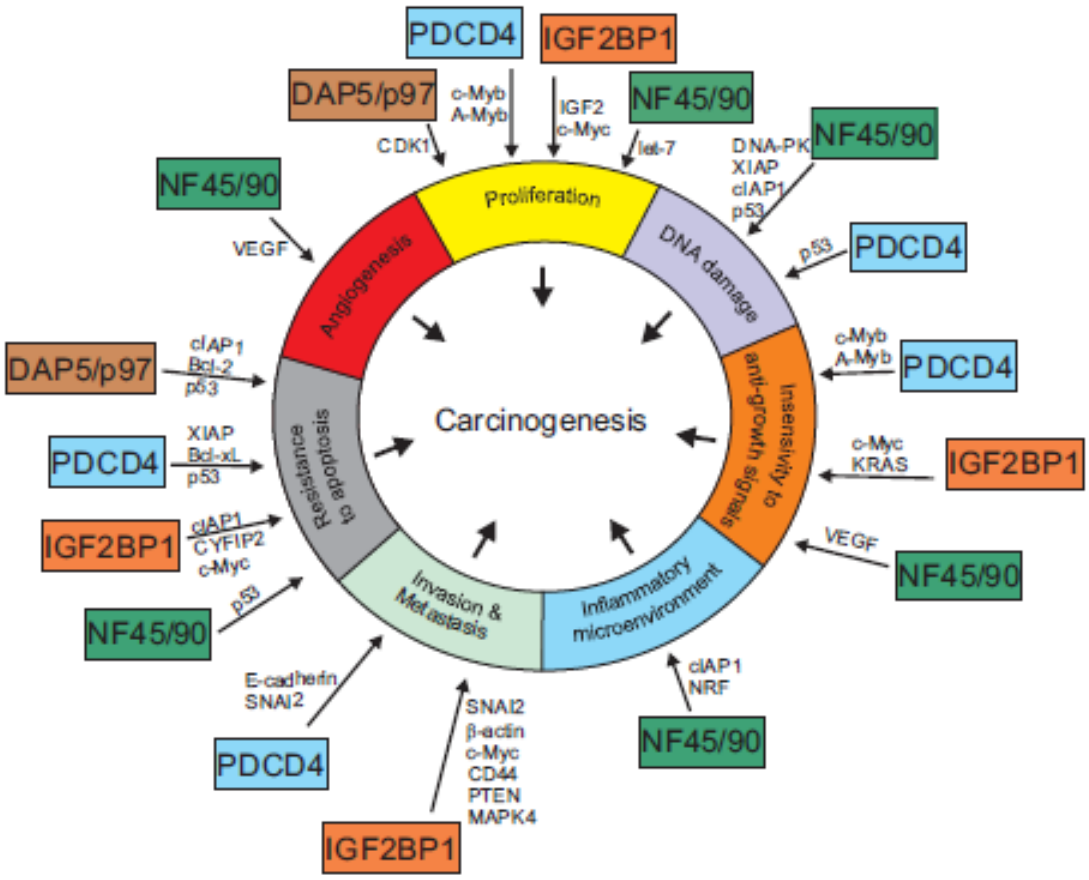
PDCD4
IGF2BP1
DAP5/p97
c-Myb
A-Myb
IGF2
c-Myc
NF45/90
CDK1
let-7
DNA-PK
XIAP
cIAP1
p53
NF45/90
NF45/90
VEGF
Proliferation
DNA damage
p53
PDCD4
Angiogenesis
c-Myb
A-Myb
PDCD4
DAP5/p97
cIAP1
Bcl-2
p53
Carcinogenesis
Insensitivity to anti-growth signals
c-Myc
KRAS
IGF2BP1
PDCD4
XIAP
Bcl-xL
p53
Resistance to apoptosis
VEGF
IGF2BP1
cIAP1
CYFIP2
c-Myc
NF45/90
p53
Invasion & Metastasis
Inflammatory microenvironment
cIAP1
NRF
NF45/90
E-cadherin
SNAI2
PDCD4
SNAI2
β-actin
c-Myc
CD44
PTEN
MAPK4
IGF2BP1

Figure 3.6: The impact of IRES trans-acting factors on the hallmarks of cancer. The hallmarks of cancer are shown in a colour-coded inner circle. The ITAFs, whose misregulation has been shown to affect the given hallmark, are shown on the outside, and their colour-coding coincides with that on previous Figures. The key factors whose mRNAs the ITAFs act upon are shown along the arrows. Only factors relevant to this review are shown.

could be achieved by targeting its regulation by miR-21, as strategies developed to reduce miR-21 overexpression in cancer cells should also impact PDCD4 expression and re-establish its function as a tumour suppressor in these cells [137]. Similarly, drugs designed to block S6 kinases activity should also block PDCD4 phosphorylation and its subsequent proteasomal degradation. However, in the case of ITAFs whose expression control is not well established, such as IGF2BP1, this therapeutic approach is not yet feasible. Instead, therapy strategies could focus on the downstream target mRNAs of these ITAFs, in particular those with known relevance to cancer. For example, in rhabdomyosarcomas which display elevated levels of IGF2BP1, its target cIAP1 was shown to be the main effector of increased apoptotic resistance. Therefore, Smac mimetics, drugs that inhibit cIAP1 were fully effective in triggering cancer cell death and significantly improving the survival of mice bearing RMS xenograft tumours [26]. Similarly, Bcl-xL, which is elevated
in glioblastoma multiforme (GBM) as a consequence of the loss of PDCD4, was shown to mediate enhanced chemoresistance of GBM, and direct inhibition of Bcl-xL by the small molecule antagonist ABT-737 was effective in sensitising glioblastoma cells to Doxorubicin [110]. A third avenue of ITAF targeted therapy could be to physically disrupt the interaction between ITAFs and their target mRNAs is cancer cells through the use of small molecule compounds. Recently, cardiac glycoside compounds were identified as inhibitors of c-Myc and VEGF IRES-dependent translation, possibly by blocking their interaction with regulatory ITAFs, although the exact mechanism of inhibition remains to be established [138]. As new research uncovers additional roles for ITAFs in cancer and sheds light on their mechanisms of action, this will further strengthen their importance as potential therapeutic targets.

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CHAPTER 4

Nucleotide Composition of Cellular IRES Defines Dependence on NF45 and Predicts a Post-transcriptional Mitotic Regulon

4.1 Preamble

‘‘Nucleotide composition of cellular IRES defines dependence on NF45 and predicts a post-transcriptional mitotic regulon’’ is a research article that is published in the journal Molecular and Cellular Biology (Volume 33, January 2013). This article describes an in silico approach to identifying cellular IRES mRNAs that are regulated by the NF45 ITAF solely based on the AU-richness of the IRES sequence. It shows that indeed, high AU content of IRES-containing 5’ UTRs serves as an excellent predictor of NF45 dependence. Moreover, it provides evidence that cells deficient in NF45 ITAF activity exhibit reduced IRES-mediated translation of XIAP and cIAP1 mRNAs. This in turn leads to dysregulated expression of their respective targets, Survivin and cyclin E, thus explaining in part the multinucleated phenotype of NF45-deficient Hela cells. Hence this article highlights the importance of NF45 regulated IRES translation in mitosis and cellular homeostasis.

Author list

Mame Daro Faye, Tyson E. Graber, Peng Liu, Nehal Thakor, Stephen D. Baird, Danielle Durie, and Martin Holcik

Author contributions

MDF, TEG and MH wrote the paper. MDF, TEG and MH conceived experiments. MDF, TEG, PL, DD performed experiments. SDB helped with compiling the IRES databases and writing perlscripts. MDF designed and performed experiments pertaining to all figures except for figures 4.1, 4.2A, 4.3, 4.4D, 4.5A and 4.5B and 4.6.

4.2 Abstract

The vast majority of cellular mRNAs initiate their translation through a well-defined mechanism of ribosome recruitment that occurs at the 5'terminal 7-methylguanosine cap with the help of several canonical protein factors. A subset of cellular and viral mRNAs contain regulatory motifs in their 5' untranslated regions (UTR) termed Internal Ribosome Entry Sites (IRES) that sidestep this canonical mode of initiation. On cellular mRNAs, this mechanism requires IRES trans-acting protein factors (ITAFs) that facilitate ribosome recruitment downstream of the cap. While several ITAFs and their target mRNAs have been empirically identified, *in silico* prediction of targets has proved difficult. Here, we report that high AU content (>60%) of IRES-containing 5'UTRs serves as an excellent predictor of dependence on the recently identified ITAF, NF45. Moreover, we provide evidence that cells deficient in NF45 ITAF activity exhibit reduced IRES-mediated translation of XIAP and cIAP1 mRNAs that in turn leads to dysregulated expression of their respective targets, Survivin and cyclin E. This specific defect in IRES translation in part explains the cytokinesis impairment and senescent-like phenotype observed in HeLa cells expressing NF45 RNAi. This study uncovers a novel role for NF45 in regulating ploidy and highlights the importance of IRES-mediated translation in cellular homeostasis.

4.3 Introduction

Translation is a process that is tightly regulated to ensure rapid and specific protein expression in response to different stimuli.[1] Translation regulation occurs primarily at the initiation step, through a canonical mechanism in which the ribosome is recruited at the 5' terminal 7-methylguanosine cap with the help of several protein factors.[2] However, a subset

of cellular and viral mRNAs contains regulatory motifs in their 5'UTR termed Internal Ribosome Entry Sites (IRES) that sidestep this canonical mode of translation initiation. First discovered in picornaviruses,[3] IRES are specific RNA elements that permit the recruitment of the ribosome to the translation initiation start, independently of the cap.[1, 3]. Unlike some viral IRES, which can directly recruit the ribosome, eukaryotic IRES appear to require IRES trans-acting protein factors (ITAFs) that facilitate ribosome recruitment by acting either as scaffold proteins or RNA chaperones (reviewed in [4] and [5]). While several ITAFs and their target mRNAs have been empirically identified, *in silico* prediction of targets has proved difficult due to lack of consensus in IRES binding motifs and overall poor understanding of the biology of cellular IRES.[6]

We have previously characterised NF45 as an ITAF that regulates the cellular Inhibitor of Apoptosis Protein 1 (cIAP1) IRES during the unfolded protein response.[7] Indeed, NF45 was shown to interact directly with the cIAP1 IRES and positively affect its activity, resulting in increased translation of its mRNA during thapsigargin-induced ER stress. This was the first report of NF45 having a *bona fide* function in IRES-mediated translation although there have been reports of NF45 and its binding partner NF90 involvement in transcription,[8, 9] viral replication[10-12] and microRNA processing.[13, 14]

More recently, NF45 has been implicated in mitotic control of HeLa cells. Indeed, NF45/NF90 complexes depletion by RNA interference leads to the generation of large multinucleated cells, a result of impaired cytokinesis and cell growth.[15, 16] Multinucleation can arise from defects in DNA replication, cell proliferation or mitosis, and from aberrant expression of proteins regulating these key cellular events. One such protein that is essential to mitosis and whose aberrant expression has been linked to multinucleation is Survivin.[17, 18]

Survivin (BIRC5), a member of the IAP family, has been shown to play a dual role in cell division, first by regulating microtubule dynamics,[19] but also as a member of the chromosomal passenger complex (reviewed in [20]). Cyclin E is another protein that is crucial for cell cycle progression through its interaction with Cdk2 which leads to Retinoblastoma protein (Rb) phosphorylation, release of E2F transcriptional activity and expression of genes that drive G1 to S transition.[21-23]

In this study, we developed a screen to identify new targets of NF45 regulation and show that high AU content of IRES-containing 5' UTRs serves as an excellent predictor of NF45 dependence. Moreover, we provide evidence that cells deficient in NF45 ITAF activity exhibit reduced IRES-mediated translation of XIAP and cIAP1 mRNAs. This in turn leads to dysregulated expression of their respective targets, Survivin and cyclin E, thus explaining in part the multinucleated phenotype of NF45-deficient cells. Hence, we report the identification of an NF45-regulated operon that impinges on the regulation of cell division and cell cycle progression.

4.4 Material and Methods

4.4.1 Cell culture, expression constructs and transfection.

HeLa cells expressing NF45 shRNA (*c* and *d5*) were previously characterized[16] and were maintained under selective pressure with 400 μg/ml of G418 (Invitrogen, Burlington, ON, Canada) in serum-, antibiotic- and sodium-pyruvate- supplemented Dulbecco's modified Eagle's medium (DMEM). HEK293 and HeLa cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and routinely cultured in serum-, antibiotic and L-Glutmaine supplemented DMEM media media. Transient DNA

transfections were performed using Lipofectamine Plus Reagent (Invitrogen, Burlington, ON, Canada) or JetPRIME (Polypus transfection, Illkirch, France) as per manufacturer's protocol. For western blot analysis, cells were transfected with 50 nM of NF45 or control (non-targeting) siRNAs using RNAiMax reagent (Invitrogen, Burlington, ON, Canada) for 72h or 96h. Unless otherwise stated, all assays were performed 24 hours following transfection, except for the rescue experiments, performed at 48h post-transfection. GFP-fusion expression plasmids were constructed by first ligating Turbo GFP (from pTurboGFP-dest1, Evrogen, Moscow, Russia) into a pcDNA3 backbone (Invitrogen, Burlington, ON, Canada), followed by ligation of the appropriate ORF (NF45 or XIAP). The GFP-NF45R construct was obtained by introducing a silent mutation that is resistant to the *d5* shRNA to the GFP-NF45 plasmid by site-directed mutagenesis. All plasmid constructs were verified by nucleotide sequencing.

4.4.2 Cloning of IRES and bicistronic assays.

A non-redundant human 5'UTR sequence database and a database containing known IRES sequences were used to calculate AU content of known human IRESes and 5'UTRs.[6] The AQP4, cIAP1, ELG, NRF, and XIAP IRES have been previously cloned into pβ-GAL/CAT bicistronic vector and characterized.[7, 24] In addition to these IRES, we cloned the previously characterized NRF,[25] SNM1,[26] MYT2,[27] UNR,[28] TEK,[29] and SCAMPER[30] IRES into the pβ-GAL/CAT bicistronic vector.[31] The SCAMPER and MYT2 IRES were synthesized with flanking NheI/XhoI sites and cloned into a pUC57 vector (Bio Basic, Inc., Toronto, ON, Canada) followed by sub-cloning into the pβ-GAL/CAT bicistronic vector. A portion of the SNM1 5'UTR containing IRES activity described in Zhang et al[26] (nucleotides -669 to -1,

forward primer: CTC TTC CT GCT AGC GGG ATT GTT CAT TGC TGC, reverse primer: CTC TTC CT CTC GAG GGC AAA ATG ATT TTA TCA), the UNR 5'UTR containing IRES activity described in Cornelis et al[28] (nucleotides -462 to -1, forward primer: CTC TTC CT GCT AGC TGC TGC TTA TGG CGG CGC, reverse primer: CTC TTC CT CTC GAG CGC AGT GAT ACT CAA ATA), and the TEK 5'UTR containing IRES activity described in Park et al [29] (nucleotides -354 to -1, forward primer: CTC TTC CT GCT AGC GCA GCA GCA AAA GCA GCA, reverse primer: CTC TTC CT CTC GAG GCT TCC CCA AAT CTC TCC), were amplified by PCR from mammalian gene collection clones (SNM1, Genbank Accession:BI770136; UNR, GB:BI546285; TEK; GB:BI546285, all purchased from Thermo Fisher Scientific, Inc., Ottawa, ON, Canada) using primers with NheI/XhoI sites. The NRF 5'UTR (nucleotides -653 to -1, forward primer: CTC GAG CAG AGT AAT GAC ATG GTT CC, reverse primer: CTC TTC CAA GCG TGG GCT GTA CC) was PCR-amplified from a liver cDNA library using primers with NheI/XhoI sites. The amplified UTRs were then sub-cloned into the pβ-GAL/CAT bicistronic vector. All constructs were verified by sequencing and basal IRES activity in *c* cells (relative to cells transfected with empty bicistronic vector) was confirmed. β-GAL and CAT assays were performed as previously described.[31]

4.4.3 Polysome profiling and quantitative RT-PCR analysis.

c and *d5* cells were grown to 70-80% confluency in 15 cm plates in sodium-pyruvate supplemented DMEM. For rescue experiments, cells were transfected with 10 μg of GFP or 20 μg of GFP-NF45R for 24h prior to harvesting. Cells were then incubated with 0.1 mg/ml cycloheximide for 3 min, washed with cold PBS-cycloheximide and lysed in cold polysome

lysis buffer (15mM Tris-HCl (pH 7.4), 15mM $MgCl_2$, 300mM NaCl, 1% (v/v) Triton X-100, 0.1 mg/ml cycloheximide, 100 U/ml RNasin). Equal OD254 units were loaded onto 10–50% linear sucrose gradients (10 ml) and centrifuged at 39,000 r.p.m. for 90 min at 4^0C. Gradients were fractionated from the top into 1 ml fractions using an ISCO gradient fractionation system (Teledyne ISCO Inc., Lincoln, NE, USA) and RNA/protein was monitored at 254 nm. 100 ng of *in vitro* synthesized CAT RNA was supplemented to each fraction as an internal control and total RNA was isolated from individual fractions by proteinase K digestion followed by phenol/chloroform extraction. Equal volumes of RNA from each fraction were used to generate cDNA from the qScript cDNA SuperMix reverse transcription kit (Quanta Biosciences, Gaithersburg, MD, USA). PCR primers specific for XIAP (forward AGGGCACATGTATGTCATGG; reverse TAGAGGGTGGCTCAGGAAAA), cIAP1 (forward TCTGGAGATGATCCATGGGTAGA; reverse TGGCCTTTCATTCGTATCAAGA), NRF (forward AAATCTGGTGAGGGCATACG; reverse TCAAATCTGTGTGGCTCTCG), SNM1 (forward TTCCTAGCCATTGCTGATGTT; reverse TTGCATCATTGGGAGAAGGT), ELG (forward AAACCCATCACCCCTAAATG; reverse GGGCGGGGAATATATAAAGG), APAF1 (forward CTTGAGCCCTGGAGTTTGAG; reverse TGCATGAACTGCCATGAAAT), and CAT (forward GCGTGTTACGGTGAAAACCT; reverse GGGCGAAGAAGTTGTCCATA) were used to quantify mRNAs by qPCR using PerfeCTA SYBR Green Supermix (Quanta Biosciences, Gaithersburg, MD, USA). mRNA distribution profiles were plotted for each fraction (normalised to CAT RNA) and ratios of heavy-polysomes (fractions 5 to 10) to light-polysomes (fraction 2 to 4) (HP/LP) were calculated for each transcript. For steady-

state mRNA levels, RNA was extracted from cells using the Absolutely RNA Miniprep kit (Agilent Technologies, Cedar Creek, TX, USA), cDNA and qPCR analysis performed as described above.

4.4.4 RNA immunoprecipitation (RIP).

Hela *c* cells were plated in 10-cm plates at 50% confluency and transfected the next day with 10 μg of pcDNA3-FLAG or pcDNA3-FLAG-NF45 plasmid using Jetprime transfection reagent (Polypus transfection). 24h later, 1% formaldehyde was added to the cells to cross-link RNA-protein complexes for 30 min at room temperature. 0.2M Glycine was added to quench the cross-linking and cells were lysed 30 min at 4 ^{0}C in RIP buffer (50 mM HEPES-KOH pH7.5, 140 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate) supplemented with 1 μg/ml each of aprotinin, leupeptin, pepstatin, PMSF and 40U/ml of RNAse inhibitor (Promega)). Cell lysates were sonicated and treated with DNAse I for 30 min. Immunprocepitation was carried out using 20 μl of anti-FLAG-M2 affinity gel (Sigma) per sample for 2h at 4 ^{0}C. Beads were washed 3 times in PBS, treated with 20 μg of proteinase K at 55 ^{0}C for 1h and the cross-linking reversed at 70 ^{0}C for 45 min. RNA was extracted by phenol-chloroform purification and cDNA was generated using the qScript cDNA supermix (Quanta Biosciences). RNA immunoprecipitates were detected using primers specific to XIAP, cIAP1 (QuantiTect Primer Assay, Qiagen), NRF, ELG and APAF1.

4.4.5 Western blot analysis.

Cells were lysed in RIPA buffer for 30 minutes at 4°C, followed by centrifugation at 10,000 x g to remove debris. Equal amounts of protein were resolved by 10% SDS-PAGE, transferred to PVDF membranes using a wet transfer protocol and probed with antibodies to NF45,[7] XIAP (anti-RIAP3[24]), Survivin (6EA, Cell Signaling, Danvers, MA, USA), cyclin E (Clone C-19, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) or GAPDH (BD Biosciences, Mississauga, ON, Canada). Membranes were incubated with Alexa 680-conjugated (Invitrogen, Burlington, ON, Canada) or IR800-conjugated (LI-COR Biotechnology, Lincoln, NE, USA) secondary antibodies followed by detection using the LI-COR Odyssey infrared scanner (LI-COR Biosciences, Lincoln, NE, USA). Densitometry analyses were performed using the LI-COR Odyssey software.

4.4.6 Metabolic labeling and immunoprecipitation.

Cells were plated in 10-cm dishes and transfected respectively with 50 nM of non-targeting siRNA or NF45 siRNA. After 96h of knock-down, cells were metabolically labeled with ^{35}S-methionine for 45 min at 37 ^{0}C and lysed as previously described.[7] Co-immunoprecipitation of cIAP1, XIAP and GAPDH proteins was performed at 4 ^{0}C overnight, using Protein G/ Protein A-Agarose beads (EMD Chemicals) coated with these respective antibodies: anti-cIAP1 (R&D Systems, MN, USA) at a 1:150 dilution, anti-GST-XIAP[32] (AEgera) at 1:150 and anti-GAPDH (Advanced ImmunoChemical; clone 6C5, CA, USA) at a 1:250 dilution. The immunoprecipitated beads were then washed with cold wash buffer (50mM Tris (pH 7.4), 300mM NaCl, 0.1% Triton X-100), resuspended in Laemmli buffer and boiled to elute bound proteins. The immunoprecipitated proteins were separated on a 10% SDS-PAGE along with 5% of the input proteins. The gel was stained with Coomassie Blue, incubated

with Amplify fluorogenic reagent (GE Biosciences, QC, Canada) for 30min and dried before exposure to film. Input proteins were also analysed by Western blot to verify the knock-down of NF45.

4.4.7 RNA-Streptomycin affinity chromatography.

S10 cytoplasmic lysate from HeLa cells was prepared as previously described.[7] Briefly, HeLa-S cells (2 ml packed cell volume; PCV) (Biovest International, Tampa, FL, USA) were resuspended in 2 ml of hypotonic buffer [10 mM Tris-HCl (pH 7.6), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT] containing EDTA-free protease inhibitor cocktail (Roche) and lysed using a dounce homogenizer (30 strokes, pestle B). A mixture of 4 ml of HeLa S10 cytoplasmic lysate and 12 ml binding buffer [20 mM Tris (pH 7.6), 10 mM $MgCl_2$, 120 mM KCl, 8% sucrose, 2 mM dithiothreitol] containing EDTA-free protease inhibitor cocktail (Roche) and ribonuclease inhibitor (Promega) was incubated at 37°C for 10 min. An *in vitro* transcribed, polyA tailed and strepto-tagged XIAP IRES or β-hemoglobin 5' UTR (HG) RNAs were added to the mixture and further incubated for 10 min at 37°C. RNA-dihydrostreptomycin affinity chromatography was performed as previously described[33] and the RNA associated proteins were analyzed using Western blot analysis.

4.4.8 Fluorescence microscopy.

$1.0x10^5$ *d5* and *c* cells were seeded in 12-well flat-bottom plates. For HeLa cells immunofluorescence, $1.0x10^5$ cells were seeded on coverslips in 6-well plates and transfected with 50 nM of non-targeting or NF45-targeting siRNA for 72h. 24h later, cells were fixed in 3.7% formaldehyde, permeabilised with 0.1% Triton X-100, and

simultaneously stained for nuclei (5 μg/ml Hoechst 3342) and F-actin filaments (0.2 U/ml Alexa-568 conjugated Phalloidin) for 30 minutes at room temperature. Staining was captured for *d5* and *c* cells using a Cellomics ArrayScan VTI automated fluorescence imaging system (Thermo Fisher Scientific, Inc., Ottawa, ON, Canada) and for HeLa cells using an Olympus Fluoview FV1000 confocal microscopy (Richmond Hill, Ontario, Canada).

4.4.9 Propidium Iodide staining and flow cytometry.

$3.0x10^5$ *d5* and *c* cells were seeded in 6-well flat-bottom plates. 24h post-transfection, cells were scraped from the plates, resuspended in 500 μL of PBS and fixed in 500 μL of chilled ethanol for 30 min. at 4˚C. Cells were then spun down at 300 x g for 5 min. at 4˚C and resuspended in 500 μL of PBS. DNase-free RNase (Qiagen, Toronto, ON, Canada) was added to the suspension at 9 μg/ml, the samples were briefly vortexed, and incubated for 1h at 37˚C. Propidium iodide (Sigma, Oakville, ON, Canada) was added to a final concentration of 13 μg/ml and the samples incubated for 30 min. at room temperature. Flow cytometry was performed using a BD FACSCanto flow cytometer (BD Biosciences, Mississauga, ON, Canada).

4.4.10 Statistical analysis.

All data are expressed as mean +/- standard error of mean (SEM). Unless otherwise stated, all results were obtained through a minimum of three independent experimental replications. *t*-test analysis was performed to determine data significance using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego).

4.5 Results

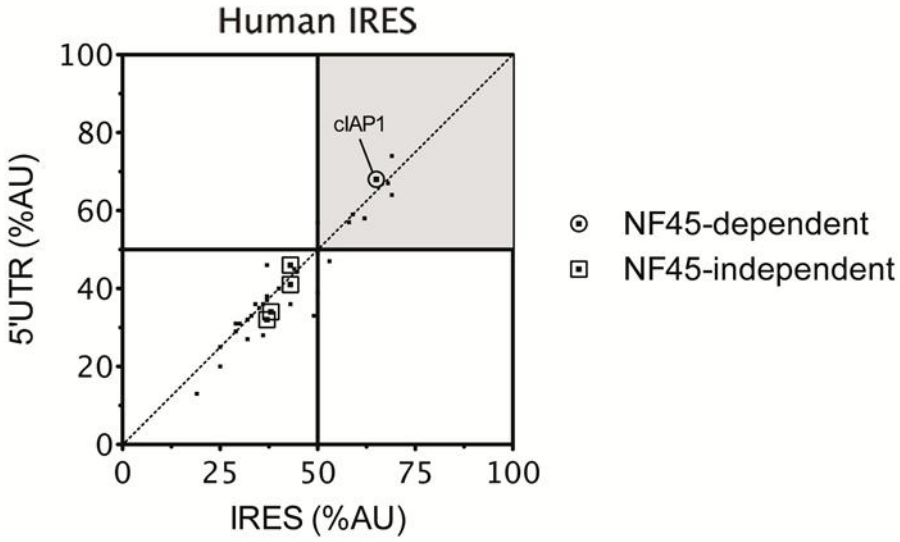
4.5.1 AU content of 5'UTRs correlates with NF45-dependent IRES activity.

We initially identified NF45 as an ITAF that positively regulates the cIAP1 IRES during the unfolded protein response.[7] In that study, NF45 was found to interact with an AU-rich stem-loop within the cIAP1 IRES. We noticed that the entire cIAP1 IRES region and indeed its entire 5'UTR were unusually AU-rich (68%). This was in stark contrast to several GC-rich IRES whose activities were found to be unaffected by changes in NF45 expression (Bcl-xL, DAP5, APAF1, VCIP; Figure 4.1A, square data points). It is important to note that the nucleotide composition of cellular IRES identified to date does not significantly deviate from that of their 5'UTRs.[6] Therefore, on its own, this constraint cannot be used to predict novel IRES. We hypothesized, however, that we could predict whether known IRES with a high AU-content would be dependent on NF45 for their activity.

To address this hypothesis, we surveyed the set of known IRES-containing RNAs[6] and calculated the AU content of their 5'UTR and, if known, minimal IRES regions (Figure 4.1A). We ranked a list of 58 known eukaryotic IRES and 38 viral IRES based on their AU content and further considered only human IRES with AU content greater than 50% (Figure 4.1A). This resulted in a list of 9 IRES (including cIAP1) that we would anticipate to be dependent on NF45 and have decided to test in the wet laboratory, along with the GC-rich IRES of Bcl-xL, DAP5, VCIP and APAF1 (Figure 4.1B).

Figure 1

A



B

% AU 5'UTR (IRES)	IRES name	IRESite ID	Unigene symbol	Unigene ID
74 (69)	XIAP	109	XIAP	Hs.356076
68 (65)	cIAP1	259	BIRC2	Hs.696238
67 (68)	NRF	243	NKRF	Hs.437084
64 (69)	ELG	492	C17ORF85	Hs.120963
59 (59)	SNM1	58	DCLRE1A	Hs.1560
58 (62)	MYT2	42	MYT2	Hs.123048
57 (58)	UNR	81	CSDE1	Hs.69855
57 (50)	AQP4	491	AQP4	Hs.315369
54 (54)	TEK	N/A	TEK	Hs.89640
46 (43)	Bcl-xL	N/A	BCL2L1	Hs.516966
41 (43)	DAP5	630	EIF4G2	Hs.183684
34 (38)	VCIP	N/A	PPAP2B	Hs.405156
32 (37)	APAF1	110	APAF1	Hs.728891

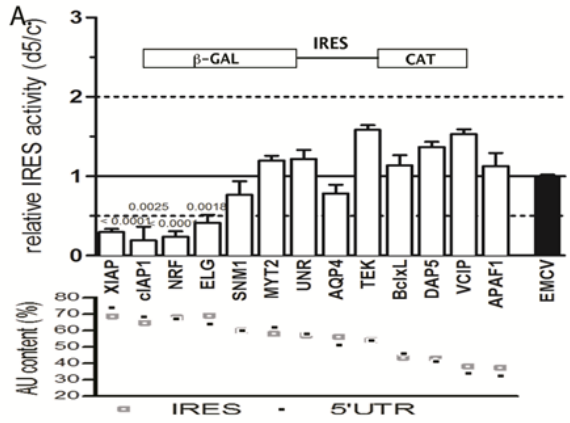
Figure 4.1: AU content of 5'UTRs correlates with NF45-dependent IRES activity.

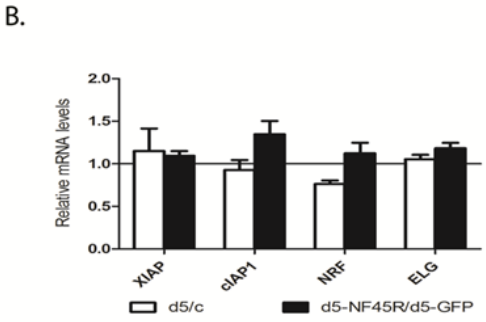
(**A**) Diagonal plot of AU content of human 5'UTRs and IRES. We have previously shown[7] that NF45 expression does not affect Bcl-xL, DAP5, APAF1, and VCIP IRES activities (squares=NF45-independent IRES) but does impact cIAP1 IRES (circle=NF45-dependent). (**B**) List of IRES-containing human mRNAs that have >50% AU content within their 5'UTRs. The percent AU content of the minimal IRES (if unknown, content is equivalent to that of 5'UTR) is indicated in brackets.

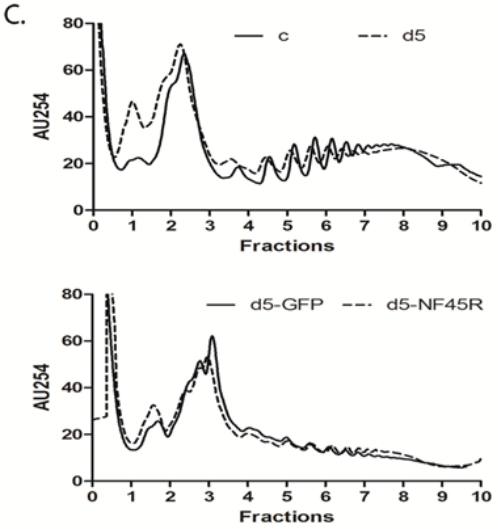
4.5.2 AU-rich 5'UTRs harbouring IRES are regulated by NF45.

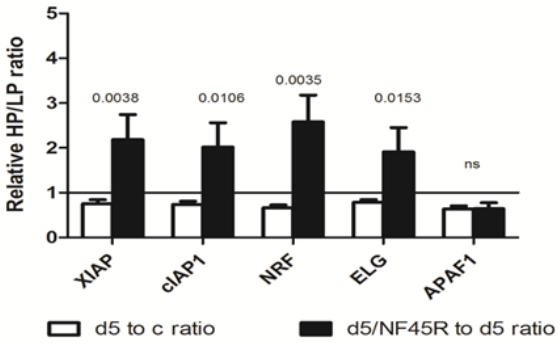
To determine whether NF45 acts as an ITAF for AU-rich IRES, we measured IRES activity using an experimental system consisting of HeLa cells that stably express shRNA targeting NF45 (*d5* cells) and cells expressing a non-targeting shRNA (*c* cells). These cells have been previously characterized and exhibit significantly reduced expression of NF45.[7, 16] We transiently transfected these cells lines with bicistronic pβ-GAL/CAT vectors harbouring selected cellular or viral IRES and determined IRES activity in cells deficient in NF45 relative to cells expressing normal levels of NF45. Using this system we have shown in our previously published assessment of six cellular IRES that decreased expression of NF45 considerably affects the activity of the AU-rich cIAP1 IRES (>2-fold decrease in activity) but spares the activity of GC-rich IRES such as APAF1, Bcl-xL, DAP5, and VCIP.[7] In the present study, we have extended the screen to include the AU-rich IRES that are predicted to be NF45-dependent (Figure 4.1B). We observed that the ratio of IRES activity in *d5* versus *c* cells was significantly less (<50% of that in *c* cells) for cIAP1, XIAP, NRF, and ELG[1] IRES (Figure 4.2A) and was indeed significantly affected compared to the EMCV IRES used as a control. The EMCV IRES (60% GC content) does not require most of the known cellular ITAFs for its optimal activity, except for PTB and La proteins,[34, 35] and therefore is not affected by the lack of NF45 in *d5* cells (Figure 4.2A, black bar). IRES with an AU content of less than 60% (MYT2, UNR, AQP4, TEK, Bcl-xL, DAP5, VCIP, and APAF1) were largely unaffected by decreased expression of NF45 in *d5* cells (Figure 4.2A). Importantly, the decrease in IRES activity observed for XIAP, NRF and ELG in *d5* cells was not due to an increase in β-Gal expression but to a decrease in CAT protein (data not shown). We also

[1] "ELG1" is another name for the gene *ATAD5* that is involved in DNA repair. "ELG" is the alias for the C17ORF85 ORF. In this study, we'll use "ELG" to designate the C17ORF85-related IRES.


A
relative IRES activity (d5/c)
IRES
β-GAL
CAT
0.0025
0.0018
0.0005
0.000
XIAP
cIAP1
NRF
ELG
SNM1
MYT2
UNR
AQP4
TEK
BclxL
DAP5
VCIP
APAF1
EMCV
AU content (%)
IRES - 5'UTR


B.
Relative mRNA levels
XIAP
cIAP1
NRF
ELG
d5/c
d5-NF45R/d5-GFP


C.
c
d5
AU254
Fractions
d5-GFP
d5-NF45R
AU254
Fractions


Relative HP/LP ratio
0.0038
0.0106
0.0035
0.0153
ns
XIAP
cIAP1
NRF
ELG
APAF1
d5 to c ratio
d5/NF45R to d5 ratio

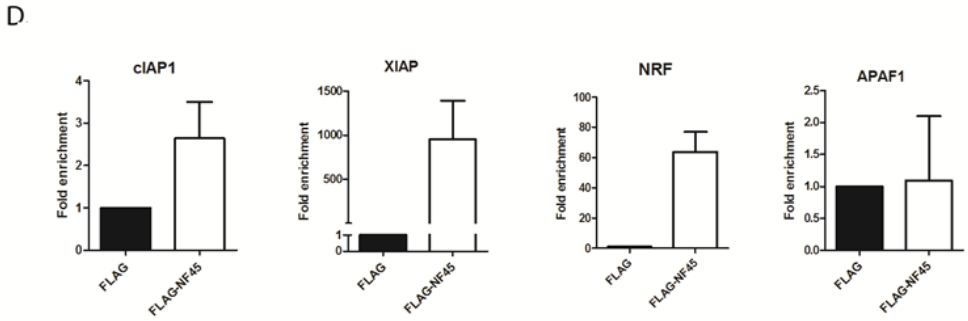

D
cIAP1
XIAP
NRF
APAF1
Fold enrichment
FLAG
FLAG-NF45

Figure 4.2: AU-rich 5‘UTRs harbouring IRES are regulated by NF45. (**A**) IRES activity was tested in *d5* (NF45 shRNA) stable cells relative to *c* (non-targeting shRNA) cells using a pβ-GAL/IRES/CAT-based bicistronic assay (schematic) with the IRES listed in Fig. 1B, as described in Materials and Methods. Note that a ratio of 1.0 is indicative of no change in IRES activity between the two cell lines. IRES are ranked in order of decreasing IRES activity and 5‘UTR AU content from left to right (bottom panel). p values are shown for the identified NF45-dependent IRES compared to the EMCV IRES used as a control (**B**) Steady-state mRNA levels of the indicated mRNAs were determined by qPCR in *c* and *d5* cells as well as *d5* cells transfected with GFP (*d5*-GFP) or a GFP-NF45R (*d5*-GFP-NF45R). Expression was normalized to that of β-actin and expressed as a ratio of *d5* to *c* or *d5*-NF45R to *d5*-GFP for each transcript. (**C**) Polyribosomes-associated mRNAs from indicated cell lines were determined as described in Materials and Methods. General polysome profiles as well as heavy to light polysomes ratios (HP/LP) are shown for *d5* relative to *c* (*d5* to *c* ratio) and for *d5*-GFP-NF45R relative to *d5*-GFP (*d5*-NF45R to *d5* ratio). (**D**) Detection and enrichment of cIAP1, XIAP and NRF mRNAs from FLAG-NF45 RNA immunoprecipitation compared to a FLAG control, showing that NF45 specifically associates with these transcripts *in vivo*.

controlled for possible splicing of the bicistronic pβ-Gal/CAT plasmids used and found that the ratios of β-Gal to CAT transcripts were equal in all cases (data not shown), showing that NF45 regulation of these AU-rich IRES is specific and not due to spurious events associated with the use of reporter constructs. Although we did see a measurable decrease in SNM1 activity (59% AU content, 24% decrease) it was not significant when compared to the EMCV IRES; therefore we did not consider it further. Altogether, these data strongly argue that NF45 is required for optimal activity of AU-rich IRES.

Next, we analysed the steady-state mRNA levels of the affected IRES-containing mRNAs by quantitative RT-PCR and found that there were not significantly decreased in *d5* relative to *c* cells, or in *d5* cells overexpressing a GFP-NF45 construct harbouring a silent mutation resistant to the *d5* shRNA (GFP-NF45R) (d5-NF45R, Figure 4.2B). These results further confirmed that the decrease in IRES activity observed for cIAP1, XIAP, NRF and ELG in *d5* cells is not due to changes at the mRNA level (transcription or mRNA stability) but likelythrough a specific effect on protein translation. To ensure that the NF45-dependent changes in IRES activity assay reflect the translational efficiency of endogenous mRNAs, polysome profiling of select IRES-containing mRNAs was performed. Cycloheximide treated *d5* and *c* cell extracts were fractionated on a 10-50% linear sucrose gradient by ultracentrifugation. *In vitro*-transcribed CAT RNA was introduced into each fraction to serve as an internal control and total RNA was extracted. The distribution of specific transcripts was analysed by quantitative RT-PCR and normalized to the CAT RNA. NF45 knock-down in *d5* cells resulted in a decrease in XIAP, cIAP1, NRF, and ELG polysomal loading as shown by a decrease in the ratio of heavy (HP) to light (LP) polysomes (Figure 4.2C right, white bars), reflective of a shift in their mRNA distribution towards less charged polysomes.

Importantly, this decrease in polysome loading could be rescued for all target mRNAs by overexpression of the GFP-NF45R (Figure 4.2C, right black bars). Unexpectedly, we found that the polysome loading of the GC-rich IRES, APAF1, was also decreased in *d5* cells. However, this reduction could not be rescued by NF45 overexpression and possibly reflects an indirect effect of NF45-NF90 transcriptional regulation of a wide array of genes. These results confirm that the translation of AU-rich IRES-containing mRNAs of XIAP, cIAP1, NRF and ELG is specifically enhanced by NF45. Moreover, NF45 knock-down or overexpression did not affect general translation rates as indicated by polysome profiles (Figure 4.2C left) or by ^{35}S-methionine incorporation (Figure 4.4C). These observations further demonstrate that NF45 is not a general modulator of translation but a regulator of IRES-mediated translation of specific transcripts.

To demonstrate that NF45 specifically interacts with the cIAP1, XIAP, ELG and NRF IRES in cells, we set out to identify an interaction between NF45 and these mRNAs by RNA-immunoprecipitation. Hela *c* cells were transfected with a pCDNA3-FLAG-NF45 plasmid or a control pcDNA3-FLAG plasmid and RNA immunoprecipitation was carried out using anti-FLAG-M2 affinity beads. Immunoprecipitates were analysed by qPCR for the presence of the target mRNAs using specific primers. Indeed, we observed an enrichment of the cIAP1, XIAP and NRF mRNAs in FLAG-NF45 compared to FLAG immunoprecipitates, indicating a specific interaction between NF45 and these transcripts in cells (Figure 4.2D). However, we could not detect the ELG mRNA in any of the precipitates (Figure 4.2D). ELG has two different transcripts of which the IRES-containing one, ELGa,[24] is not very abundant in c and *d5* Hela cells, and is therefore likely below the PCR detection limit in our assay. Importantly, the GC-rich IRES mRNA APAF1, whose polysome distribution was unaffected

by NF45 rescue (Figure 4.2C), was not present in FLAG-NF45 immunoprecipitates, further suggesting that NF45 specifically interacts with a select group of AU-rich IRES mRNAs.

Taken together, these data demonstrate that NF45 is an ITAF that specifically regulates the IRES activity and translation of the human AU-rich IRES-containing mRNAs of cIAP1, XIAP, ELG and NRF.

4.5.3 Prediction of NF45-dependent IRES.

We wished to use our results from the above IRES screen to establish specific constraints that could be used to predict NF45-dependent IRES *in silico*. From a plot of $\log_2$-transformed mean IRES activity versus the AU content of the IRES-containing 5‘UTRs, we can see that those IRES whose activity is reduced by more than 2-fold ($\log_2 < -1$) in NF45-deficient cells all lie within 5’UTRs with an AU content greater than or equal to 60% (Figure 4.3A). From this data, we propose that IRES-containing 5’UTRs with an AU content greater than 60% can be predicted to require NF45 for optimal IRES activity. To confirm this prediction on an unrelated AU-rich IRES that was not part of the original screen, we tested for activity of the *Canis familiaris* IRES, SCAMPER, in *d5* cells. The AU content of the SCAMPER 5’UTR and its respective IRES is 63% and therefore fits our constraint. As predicted, we found that SCAMPER IRES activity was decreased by 50% in *d5* relative to *c* cells, confirming that SCAMPER expression is regulated by human NF45 (Figure 4.3B). It is important to note that *C. familiaris* NF45 is 99.7% identical to that of the *H. sapiens*, suggesting functional NF45-IRES interactions in *C. familiaris*.

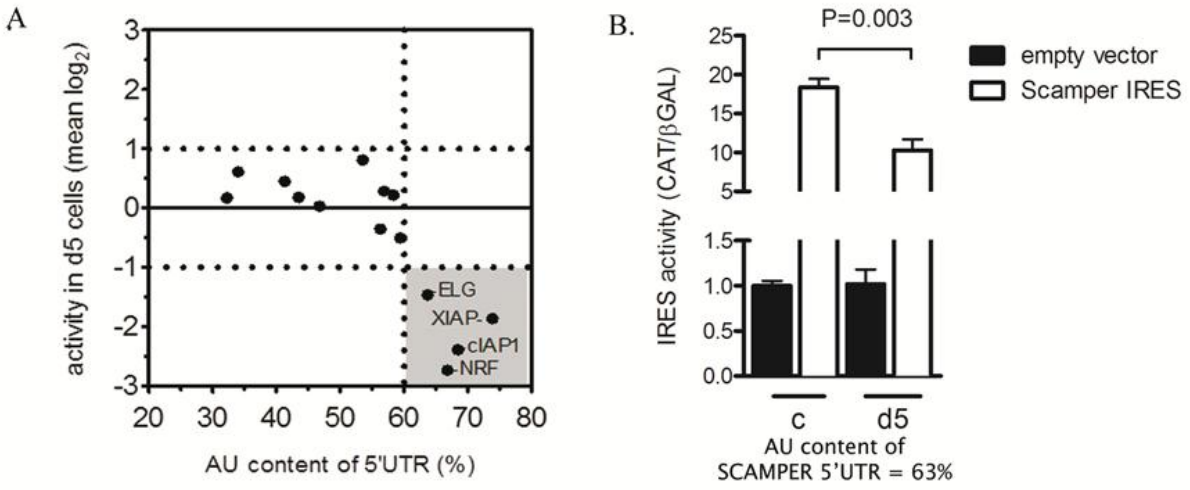

A
activity in d5 cells (mean log2)
AU content of 5'UTR (%)
ELG
XIAP
cIAP1
NRF
B.
P=0.003
empty vector
Scamper IRES
IRES activity (CAT/βGAL)
c
d5
AU content of
SCAMPER 5'UTR = 63%

Figure 4.3: Prediction of NF45-dependent IRES.

(**A**) Determination of threshold AU content for NF45-dependent IRES. IRES activity data from Figure 2A was transformed into log_2-space and plotted against the AU content of the 5'UTRs tested. A constraint of >60% AU content was chosen based on the clustered activities of IRES that changed by more than 2-fold up or down in d5 versus c cells (shaded quadrant). (**B**) Conservation of NF45-IRES interactions allows prediction of NF45-dependent IRES in Canis familiaris. The SCAMPER IRES (63% AU content) was cloned into a bicistronic vector as described in Materials and Methods and was tested for IRES activity as in Figure 2A (n=3, mean ±SD).

4.5.4 NF45 regulates XIAP protein levels through interaction with its IRES.

We have previously investigated the effect of NF45 on modulating cIAP1 IRES activity in the context of endoplasmic reticulum stress.[7] To further characterize NF45 regulation of predicted IRES-containing mRNAs, we next focused our attention on the other inhibitor of apoptosis protein from our screen, XIAP, as its IRES[31, 36, 37] and downstream cellular functions are well demarcated (reviewed in [38]). Our initial screen of XIAP IRES activity in *d5* versus *c* cells revealed a significant (75%) decrease in activity (Figure 4.2A). Next, we assessed the steady-state levels of XIAP protein in *c* and *d5* cells by Western blot to determine whether they correlated with impaired IRES activity and translation. As previously reported,[7] we observed a significant decrease in cIAP1 protein levels in d5 cells compared to the control c cells (Figure 4.4A). Similarly, there was a 50% decrease in XIAP protein levels in *d5* cells relative to the control cell line (Figure 4.4A). We next attempted to rescue XIAP protein expression in HeLa cells transiently knocked-down for NF45 expression, since the chronic state of *d5* cells makes them refractory to efficient steady-state protein rescue. Similarly to the stably transfected cells, the transient knock down of NF45 in HeLa cells caused a comparable decrease (41%) in XIAP protein when compared to control siRNA transfected cells (Figure 4.4B). In addition, the transient NF45 knock-down recapitulated the multinucleated phenotype observed in *d5* cells (Figure 4.5A, bottom right panel), showing that this system can be used to study pathways associated with the *d5* cells phenotype. Importantly, the reduction in XIAP protein expression caused by NF45 knock-down was significantly rescued by overexpression of the siRNA-resistant GFP-NF45 construct (GFP-NF45R, Figure 4.4B).

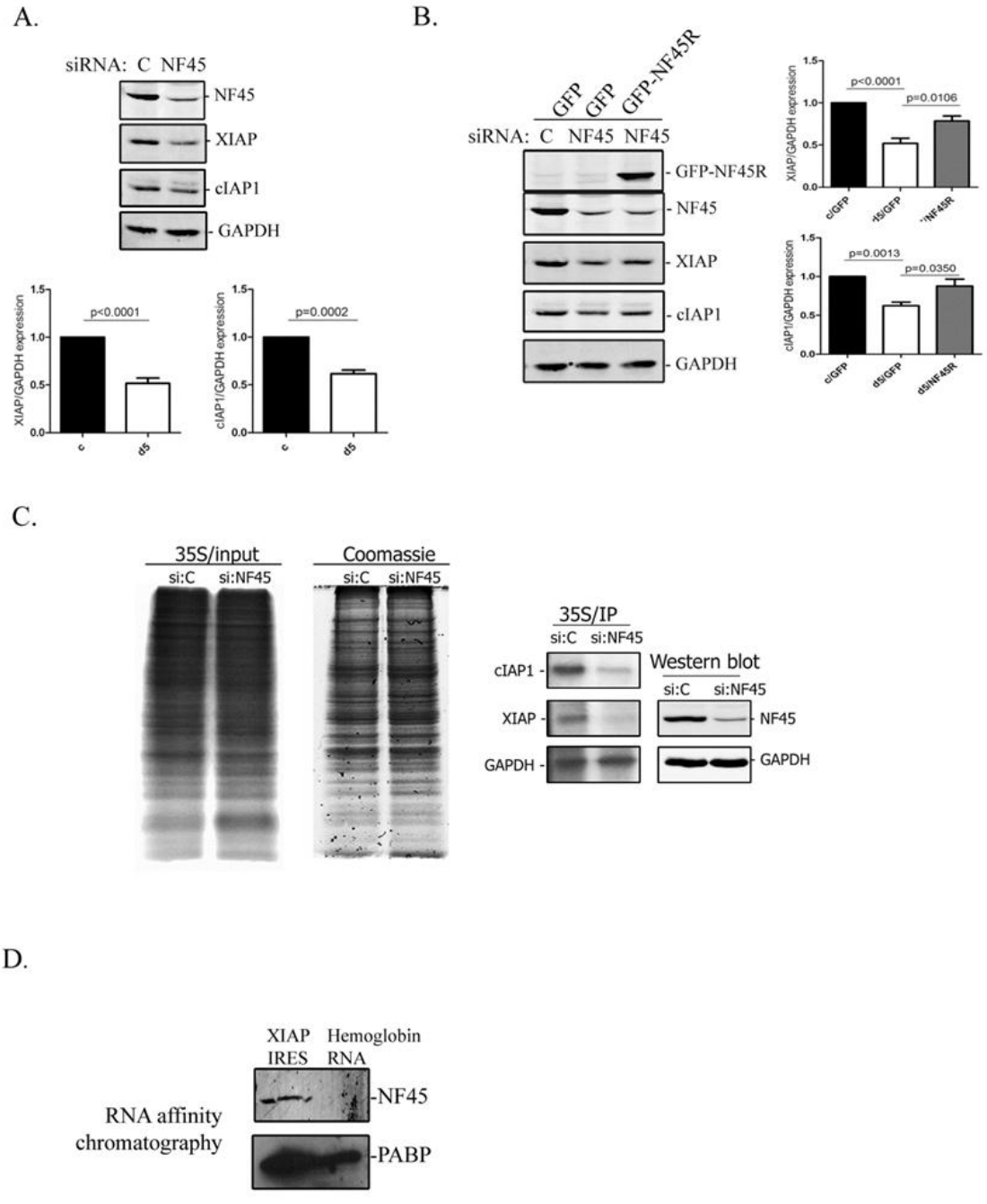

A.
siRNA: C NF45
NF45
XIAP
cIAP1
GAPDH
XIAP/GAPDH expression
p<0.0001
cIAP1/GAPDH expression
p=0.0002
B.
GFP
GFP
GFP-NF45R
siRNA: C NF45 NF45
GFP-NF45R
NF45
XIAP
cIAP1
GAPDH
XIAP/GAPDH expression
p<0.0001
p=0.0106
cIAP1/GAPDH expression
p=0.0013
p=0.0350
C.
35S/input
si:C si:NF45
Coomassie
si:C si:NF45
35S/IP
si:C si:NF45
cIAP1
XIAP
GAPDH
Western blot
si:C si:NF45
NF45
GAPDH
D.
XIAP IRES
Hemoglobin RNA
RNA affinity chromatography
NF45
PABP

Figure 4.4: NF45 regulates XIAP translation through interaction with its IRES.

(**A**) Western blot of endogenous XIAP and cIAP1 protein expression in *d5* cells relative to *c* cells. Blots and densitometry analysis are representative of at least three experiments. (**B**) NF45 re-expression in NF45-deficient HeLa cells rescues XIAP protein expression. HeLa cells were transfected with 50 nM of a control, non-targeting siRNA or NF45 siRNA (*d5* siRNA) for 48h, followed by GFP or GFP-NF45R overexpression for an additional 48h. Cell extracts were analysed by Western blot for NF45, XIAP, cIAP1 and GAPDH expression and protein expression was quantified. (**C**) *De novo* protein expression of cIAP1 and XIAP in NF45-deficient cells. *c* cells were transfected with 50 nM of control or NF45 siRNA and pulse-labeled with ^{35}S-Methionine. ^{35}S-labeled and Coomassie-stained total protein as well as specific cIAP1, XIAP and GAPDH immunoprecipitates are shown. (**D**) NF45 interacts specifically with the XIAP IRES. NF45 and PABP Western blots of an RNA affinity chromatography performed using the XIAP IRES or Hemoglobin RNA.

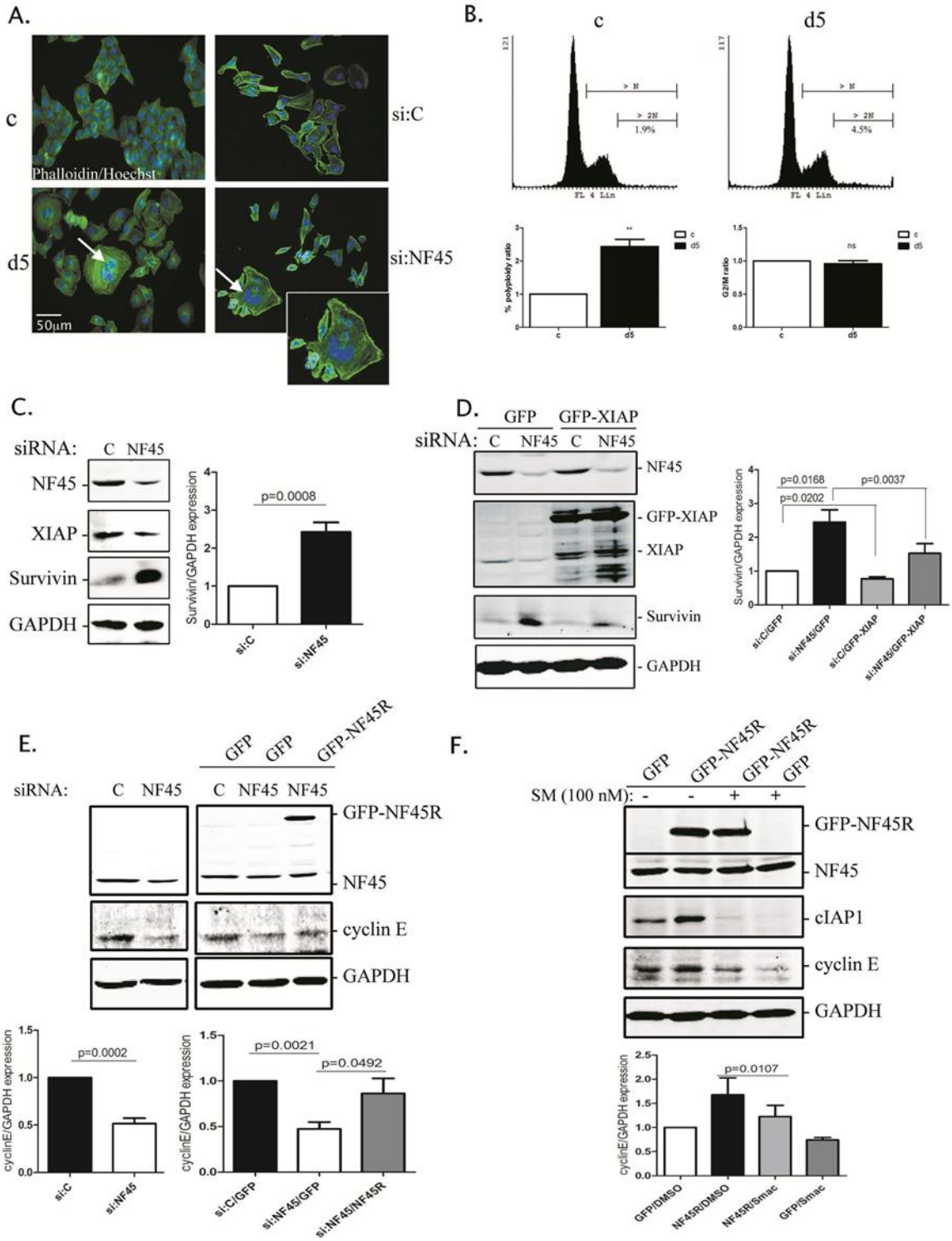

A.
c
d5
si:C
si:NF45
Phalloidin/Hoechst
50μm
B.
c
d5
> N
> 2N
1.9%
4.5%
FL 4 Lin
% polyploidy ratio
G2/M ratio
ns
C.
siRNA: C NF45
NF45
XIAP
Survivin
GAPDH
Survivin/GAPDH expression
p=0.0008
si:C
si:NF45
D.
GFP GFP-XIAP
siRNA: C NF45 C NF45
NF45
GFP-XIAP
XIAP
Survivin
GAPDH
p=0.0168
p=0.0037
p=0.0202
si:C/GFP
si:NF45/GFP
si:C/GFP-XIAP
si:NF45/GFP-XIAP
E.
GFP GFP GFP-NF45R
siRNA: C NF45 C NF45 NF45
GFP-NF45R
NF45
cyclin E
GAPDH
cyclinE/GAPDH expression
p=0.0002
p=0.0021
p=0.0492
si:C/GFP
si:NF45/GFP
si:NF45/NF45R
F.
GFP GFP-NF45R GFP-NF45R GFP
SM (100 nM): - - + +
GFP-NF45R
NF45
cIAP1
cyclin E
GAPDH
p=0.0107
GFP/DMSO
NF45R/DMSO
NF45R/Smac
GFP/Smac

Figure 4.5: NF45 regulates Survivin and cyclin E expression downstream of XIAP and cIAP1.

(**A**) Immunofluorescence images showing the multinucleation phenotype of the *d5* HeLa cell line (arrow, bottom left panel) compared to the *c* HeLa cell line. The same phenotype can be reproduced by transient siRNA knock-down of NF45 for 72h (arrow, bottom right panel and inset). Cells were stained with Phalloidin-Alexa568 for F-actin and Hoechst for nuclei. (**B**) *c* and *d5* cells phenotype was quantified by propidium iodide staining and flow cytometry analysis. The number of multinucleated cells expressed as a percentage of total number of viable cells was quantified and normalized to that of the control cell line (bottom). The number of cells in G2/M is also shown. (**C**) Western blot and densitometry analysis showing increased Survivin expression in HeLa cells treated with 50 nM of NF45 siRNA for 96h compared to control siRNA. (**D**) Survivin expression is blunted by XIAP overexpression in NF45 knocked-down HeLa cells. HeLa cells were transfected with 50 nM NF45 or control siRNA and 48h later with GFP-XIAP for an additional 48h. Protein extracts were analysed by Western blot and densitometry for Survivin, XIAP, NF45 and GAPDH. (**E**) NF45 regulates cyclin E expression. HeLa cells were treated with 50 nM of NF45 or control siRNA for 96h and expression of indicated proteins was analysed by Western blot and densitometry. (**F**) NF45 controls cyclin E protein levels through its regulation of cIAP1 IRES-mediated translation. HeLa cells were treated with 100 nM of Smac mimetic or DMSO for 24h and transfected with a pcDNA3-GFP or pcDNA3-GFP-NF45R plasmid for an additional 24h. Protein extracts were analysed by Western blot for NF45, cIAP1, cyclin E and GAPDH expression, and densitometry performed. Results are representative of three different experiments.

To further confirm NF45 regulation of XIAP protein translation, we measured the *de novo* protein synthesis. Control or NF45-targeting siRNA transfected cells were pulse-labeled with ^{35}S-methionine and the lysates immunoprecipitated with XIAP, cIAP1 or GAPDH antibodies. NF45 knock-down did not alter general protein synthesis as shown by equal incorporation of ^{35}S-methionine into newly synthesized proteins (Figure 4.4C, left and middle panel). In contrast, cIAP1 and XIAP *de novo* protein synthesis was significantly reduced by NF45 knock-down, whereas GAPDH translation remained unchanged (Figure 4.4C, right panel). This decrease in XIAP *de novo* protein synthesis in NF45 deficient cells, together with a decrease in IRES activity, polysome distribution and steady-state protein levels confirm that NF45 specifically regulates the IRES-mediated translation of the inhibitor of apoptosis XIAP.

Previously published data from our laboratory showed that NF45 directly interacts with an AU-rich stemloop structure present within the cIAP1 IRES.[7] Hence we wanted to determine if NF45 directly interacts with the XIAP IRES. To determine if NF45 interacts with the XIAP IRES, we used an RNA affinity chromatography approach to isolate XIAP IRES-binding proteins from a Hela cytoplasmic lysate using *in vitro* transcribed XIAP IRES as bait. A Western blot of this affinity preparation revealed that indeed, NF45 specifically interacts with the XIAP IRES but not with a control β-hemoglobin 5' UTR RNA (Figure 4.4D). The presence of poly-A binding protein (PABP) shows that both the XIAP IRES and the β-hemoglobin 5' UTR RNA were functional in this assay. We next attempted to map NF45 binding site(s) on the XIAP IRES by *in vitro* UV-crosslinking RNA binding assay. However, we were unable to detect any complex formation between the purified recombinant NF45 and the XIAP IRES (data not shown), indicating either that NF45 needs

to be post-translationally modified in cells before binding to the XIAP IRES or doesn't bind directly to the IRES in the absence of other proteins. Nevertheless, our results show that NF45 is a *bona fide* ITAF that interacts with the XIAP IRES and positively regulates its activity, thus driving XIAP protein expression.

4.5.5 NF45 regulates downstream proteins involved in cell cycle progression and cytokinesis.

We next set out to investigate the link between the impaired IRES-mediated translation observed in *d5* cells with their overt cellular phenotype. The *d5* HeLa stable cell line exhibits a striking senescent-like morphology[16] typified by an overall flat, non-elliptical shape, and increased F-actin expression together with an overall increase in cell size (Figure 4.5A, left panel). These cells also display multinucleation, suggesting either a cell fusion event or a defect in cytokinesis (Figure 4.5A, arrow). Quantitative analysis of propidium iodide-labeled *d5* cells by flow cytometry indicated a 2.4-fold increase in the multinucleated population compared to control cells, but no change in the number of cells arrested in G2/M (Figure 4.5B), as previously reported.[16] Importantly, this multinucleation phenotype could be reproduced by transient siRNA knock-down of NF45 for 72h in HeLa cells (Figure 4.5A, bottom right panel).

The slow growth phenotype observed in *d5* cells could be indicative of dysregulated NF-κB signaling, a key transcriptional program that affects cellular proliferation. Given that the IAPs have been well established as mediators of NF-κB signaling (reviewed in [39]), we decided to assess NF-κB activity in cells lacking NF45. However, we found no significant difference in activity between *d5* and *c* cells, measured using a luciferase-based NF-κB

reporter system (data not shown), although there was an increase in basal NF-κB activity in both *c* and *d5* cells when NF45 was overexpressed. Furthermore, the reduced cIAP1 expression in *d5* cells might have been expected to trigger the transcriptional activation of cIAP2 expression by NF-κB activity.[40] However, we didn't observe any changes in cIAP2 mRNA expression between the two cell lines, consistent with NF-κB signaling being unaffected (data not shown).

Another pathway through which slow growth and multinucleation could also arise is through dysregulation of Survivin function.[17, 41] Survivin is a member of the IAP family that plays important roles in apoptosis inhibition and in microtubule spindle checkpoint regulation.[17, 18] Survivin has been shown to play a dual role in cell division, first by regulating microtubule dynamics, through its association with polymerized tubulin,[19] but also as a member of the chromosomal passenger complex in which it associates with regulators of cytokinesis such as INCENP, Aurora B kinase and Borealin (reviewed in [20]). Interestingly, Survivin protein stability is regulated by XIAP.[42] Thus, we hypothesized that the loss of NF45 leading to the down-regulation of XIAP IRES-mediated translation will result in altered Survivin expression and deregulation of mitosis. We used HeLa cells in which we transiently knocked-down NF45 using the *d5* siRNA and set out to rescue Survivin expression through overexpression of GFP-XIAP. We observed that the NF45 knock-down caused a 2.5-fold increase in Survivin protein levels (Figure 4.5C). Moreover, GFP-XIAP overexpression in NF45 siRNA-treated cells significantly blunted Survivin expression compared to the GFP control (Figure 4.5D), while Survivin steady-state mRNA levels remained unchanged in NF45 knocked-down cells (data not shown).

Although cIAP1 is primarily known as a regulator of the NF-κB signaling pathways, a recent report showed that nuclear cIAP1 transcriptionally upregulates cyclin E expression through its interaction with the E2F1 transcription factor.[43] Given that NF45 is necessary for IRES-mediated cIAP1 expression,[7] we wanted to test whether knock-down of NF45 alters cyclin E levels in HeLa cells thus contributing to the distinct phenotype of NF45-deficient cells. Indeed, NF45 transient knock-down caused a 50% decrease in cyclin E protein levels (Figure 4.5E, left panel). Importantly, this decrease in cyclin E was almost entirely rescued by GFP-NF45R expression in NF45 depleted cells (Figure 4.5E, right panel). To further confirm that cIAP1 is required for cyclin E regulation downstream of NF45, we overexpressed GFP-NF45R in the presence of a Smac mimetic compound that causes rapid degradation of cIAP1 protein.[44] As expected, NF45 overexpression increased cyclin E protein relative to the GFP control in DMSO treated cells (Figure 4.5F). However, when cells were treated with Smac mimetic, which leads to depletion of cIAP1, the NF45-driven upregulation of cyclin E was blunted.

These results identified a new pathway in which NF45 regulates cIAP1 and XIAP IRES-mediated translation, which in turn regulates Survivin and cyclin E expression. The decrease in cyclin E expression coupled with the increased expression of Survivin protein likely contributes to the senescence-like and multinucleated phenotype observed in NF45-deficient cells.

4.6 Discussion

We have shown for the first time that NF45 regulates the translation of a cohort of IRES-containing transcripts that were initially predicted solely on the basis of the unusually

high AU content (>60%) of their respective 5‘UTRs. We then confirmed that NF45 specifically regulates the IRES-mediated translation of XIAP and cIAP1 mRNAs and that reduced expression of these proteins causes changes in the ploidy of HeLa cells through dysregulated expression of XIAP and cIAP1 respective downstream targets Survivin and cyclin E.

IRES trans-acting factors are cellular proteins that help in the recruitment of the ribosome by acting either as scaffold proteins or RNA chaperones.[4, 5] We have previously identified and characterised NF45 as an ITAF that positively regulates the AU-rich cIAP1 IRES during the unfolded protein response.[7] In this study, we show that high AU content (>60%) of 5’UTRs that contain IRES can serve as an excellent predictor of dependence on NF45. Indeed, we show that the IRES activity of the AU-rich IRES of cIAP1, XIAP, NRF, and ELG is significantly decreased in *d5* cells lacking NF45; similarly, the loading of these mRNAs onto translating polysomes is reduced in NF45-deficient cells (Figure 4.2). Importantly, this decrease in translation efficiency can be rescued by re-expression of NF45 (Figure 4.2C) and all of these AU-rich IRES bearing transcripts were detected in NF45 immunocomplexes, with the exception of ELG (Figure 4.2E). From these data, we propose that IRES-containing 5’UTRs with an AU content greater than 60% can be predicted to require NF45 for optimal IRES activity. Further validation experiments such as polysome profiling, *de novo* protein synthesis and RNA binding assays would be necessary to confirm that NF45 is a *bona fide* ITAF for a particular IRES, as we’ve previously done for the cIAP1 IRES [7] and as we have shown in this study for the XIAP IRES.

The non-AU-rich IRES APAF1 was also decreased in *d5* cells (Figure 4.2C). However, this reduction could not be rescued by NF45 overexpression, showing that it

wasn't due to a direct effect of NF45 on translation. NF45 was first identified as an NFAT-related transcription factor that regulates interleukin-2 transcription, together with its binding partner NF90.[8] Therefore, a general reduction in polysome loading of APAF1 could reflect an indirect transcriptional effect of NF45 and its binding partner, NF90, in a complex that is known to target a wide array of genes.[9, 45]. Moreover, we have previously shown that the attenuation of cIAP1 IRES-dependent translation induction in *d5* cells during ER stress can be rescued by NF45 but not NF90 re-expression [7], further arguing that the NF45-NF90 transcriptional effects are independent of NF45 function as an ITAF.

NF45 protein structure is well conserved across mammalian species, thus we predicted that its function also ought to be, and that IRES dependency for NF45 could be held across species. To test this prediction, the IRES activity of the *Canis familiaris* IRES, SCAMPER (63% AU content) was determined in *d5* cells. Indeed, SCAMPER IRES activity was decreased by 50% in *d5* relative to *c* cells (Figure 4.3), confirming that AU-content could be used to predict NF45 regulation of IRES-containing mRNAs in other species. We have also calculated the AU content of 5'UTR and minimal IRES regions from known rodents, drosophila and viruses IRES-containing mRNAs and identified several that could potentially be regulated by NF45 (Figure 4.6). For instance, outside of class Mammalia, the CG5641 protein in *D. melanogastor* is homologous to mammalian NF45 and may regulate IRES in this species. Of the few *D. melanogastor* IRES identified to date, grim, hid, ultrabithorax, reaper, hsp70, and hsp90 possess more than 60% AU content in their respective 5'UTRs, and would therefore be predicted to be regulated at the level of translation by CG5641. It would be interesting to test whether these AU-rich IRES transcripts are indeed regulated by NF45 in *D. melanogastor.* Interestingly, no NF45

orthologue exists in *S. cerevisiae*, perhaps reflecting the apparent distinct mechanism of IRES regulation in this species that requires short stretches of adenosine nucleotides to recruit ribosomes through interaction with the poly-A binding protein PAPB.[46] Collectively, these

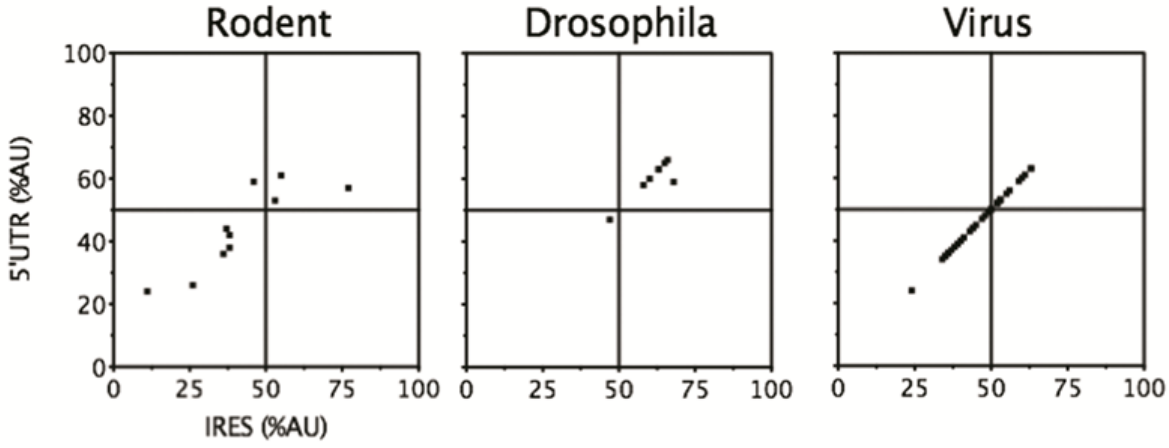

Rodent
Drosophila
Virus
5'UTR (%AU)
IRES (%AU)
0
20
40
60
80
100
0
25
50
75
100

Figure 4.6: AU content of 5'UTRs and IRES from other species

Diagonal plot of the AU content of 5'UTRs and IRES from rodent, *Drosophila* and virus showing that AU-rich IRES are present across species.

observations highlight the importance of NF45 ITAF activity in the regulation of AU-rich IRES across eukaryotic species.

Next, we wanted to further characterize NF45 regulation of predicted IRES-containing mRNAs and the functional consequences of the loss of NF45. ELG is one of the IRES whose activity and polysomal loading was significantly reduced in NF45-depleted cells, although we unable to verify its *in vivo* interaction with NF45 due to low transcript abundance (Figure 4.2). Unfortunately, the ill-defined function of the ELG/C17ORF85 gene product and lack of antibodies prevented us from further validating NF45 effects on ELG IRES-mediated translation. We were also unable to detect NRF protein in either the *d5* or *c* cells by Western blot (data not shown). We therefore focused our attention on the XIAP IRES and the previously characterized cIAP1 IRES and their potential role in the *d5* cells phenotype. The *d5* cell line exhibits a striking senescent-like and multinucleated phenotype compared to control *c* cells (Figure 4.5A and B). Guan et *al*[16] were the first to characterize the *d5* cells and showed that their slow growth was due to a block in cell cycle and defects in DNA synthesis, whereas the multinucleated cells were problably due to a defect in cytokinesis. Furthermore, recent studies using time-lapse microscopy of nuclear division were able to recapitulate the phenotype observed in *d5* cells and indicated that multinucleated cells arose from incomplete cytokinesis of daughter cells followed by fusion of several bi-nucleated cells.[15, 47] These observations pointed to a role for NF45 in regulating important cellular functions such as cell cycle and cell division. Looking downstream of XIAP and cIAP1 for regulators of cell cycle or mitosis, we identified the Survivin and cyclin E proteins as potential targets. Survivin is well characterized for its important roles in apoptosis inhibition and microtubule spindle checkpoint regulation.[17, 18] Furthermore,

Survivin anti-apoptotic effects are mediated by its interaction with XIAP and the two regulate each other's protein stability through the proteasomal degradation pathway.[42] In addition, the formation of a complex between XIAP and XIAP-associated factor 1 (XAF1) leads to a proteasomal degradation of Survivin.[48] We found that NF45 knock-down in HeLa cells leads to an increase in Survivin protein due to a decrease in XIAP expression (Figure 4.5C and D).

Reduction or loss of Survivin in mammalian cells gives rise to a variety of cell division defects, including cytokinesis failure and multinucleated cells.[41, 49, 50] This phenomenon is due to Survivin's role in regulating microtubule dynamics, through its association with polymerized tubulin,[19] but also as a member of the chromosomal passenger complex (reviewed in [20]). However, Survivin overexpression has also been linked to defects in cytokinesis and generation of multinucleated cells. This is due to a reduction in microtubule dynamics such as microtubule growth, number of growing microtubules and disorganized mitotic spindles.[51-53]

On the other hand, our results show that NF45 knock-down in HeLa cells also leads to cyclin E downregulation which can be rescued by NF45 re-expression (Figure 4.5E) and is dependent on cIAP1 (Figure 4.5F). Indeed, it was previously shown that a nuclear form of cIAP1, as found in HeLa cells,[54] can stimulate cyclin E expression at the transcriptional level through its direct interaction with E2F1.[43] Our results thus confirm cyclin E regulation by cIAP1 and show an additional layer of regulation by NF45 through the control of cIAP1 IRES-mediated translation. Cyclin E downregulation in NF45-depleted cells could explain their scenescence-like phenotype, and together with an increase in Survivin, would lead to a block in cell cycle, mitotic catastrophe and defects in cytokinesis. Moreover, the significant

decrease in XIAP and cIAP1 expression in these cells would also be expected to reduce their apoptotic threshold. Surprisingly, although aneuploidy is increased in *d5* cells, the apoptotic index is unaffected,[16, 47] probably due to the absence of the p53 protein in this HeLa cell line derivative.[55] Recently, Shamanna et *al*. suggested that the *d5* multinucleated phenotype is caused by a defect in DNA damage repair. They identified the NF90/NF45 complex as a regulator of Non-Homologous End Joining DNA damage repair mediated by DNA-PK and suggested that structured RNA may modulate this process.[15] These data in combination with our observations suggest that a defect in DNA repair mechanisms, cell cycle progression and cytokinesis are the basis of the multinucleated phenotype of NF45-deficient cells.

In conclusion, we show that NF45 regulates the translation of a set of IRES-containing transcripts that were initially predicted only on the basis of their high AU content. This regulation is important in maintaining normal levels of Survivin and cyclin E and to control normal ploidy during HeLa cell cycle and mitosis.

4.7 Conflict of interest

The authors have no competing financial interests in relation to the work described in this manuscript.

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CHAPTER 5

IGF2BP1 controls cell death and drug resistance in rhabdomyosarcomas by regulating translation of cIAP1.

5.1 Preamble

This chapter is a research article entitled ‘‘IGF2BP1 controls cell death and drug resistance in rhabdomyosarcomas by regulating translation of cIAP1’’ published in the journal Oncogene (advance online publication, 7 April 2014; doi:10.1038/onc.2014.90). This work identifies IGF2BP1, one of the protein factors found to interact with the cIAP1 IRES, as a *bona fide* ITAF that positively regulates cIAP1 IRES-mediated translation. In this article, we also report that IGF2BP1 and cIAP1 are highly overexpressed in Rhabdomyosarcoma cancer, a type of cancer characterized by undifferentiated myoblasts and the most common soft tissue tumour of childhood. Importantly, we found that reducing the levels of cIAP1 in RMS, either by IGF2BP1 knock-down or by IAP antagonists sensitizes these cells to TNFα- or TRAIL-mediated cell death. In vivo, IAP antagonists also delay Kym-1 RMS tumour growth and significantly improve survival in mice. Hence, this study identified IGF2BP1 as a critical translational regulator of cIAP1-mediated apoptotic resistance in RMS.

Author list

Mame Daro Faye, Shawn Beug, Tyson E. Graber, Nathalie Earl, Xiao Xiang, Benjamin Wild, Stephanie Langlois, Jean Michaud, Kyle N. Cowan, Robert G. Korneluk and Martin Holcik

Author contributions

MDF and MH designed the experiments and wrote the manuscript. MDF, SB, NE, XX and BW performed the experiments. MDF performed all the experiments except for the ones pertaining to Figure 5.1C and Figure 5.6. TEG did the preliminary work leading to this study. SL and KNC provided all the RMS tumour samples and participated in the design of related experiments. JM did the scoring of the immunohistochemistry slides. RGK

participated in the design of the experiments relating to smac mimetic compounds. All authors reviewed the paper.

5.2 Abstract

Rhabdomyosarcoma (RMS), a neoplasm characterized by undifferentiated myoblasts, is the most common soft tissue tumour of childhood. Although aggressive treatment of RMS could provide long term benefit, resistance to current therapies is an ongoing problem. We report here that Insulin-like Growth Factor 2 Binding protein 1 (IGF2BP1), an oncofetal protein, is expressed in RMS patient-derived cell lines and in primary tumours where it drives translation of the cIAP1, a key regulator of the NF-κB signaling pathway and of caspase-8-mediated cell death. We demonstrate that reducing the levels of cIAP1 in RMS, either by IGF2BP1 knock-down or by IAP antagonists sensitizes these cells to TNFα-mediated cell death. Finally, we show that targeting cIAP1 by IAP antagonists delay RMS tumour growth and improve survival in mice. Our results identify IGF2BP1 as a critical translational regulator of cIAP1-mediated apoptotic resistance in RMS and advocate for the combined use of IAP antagonists and TNFα as a therapeutic approach for this type of cancer.

5.3 Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue tumour in children and represents 3-4% of all childhood cancers.[1] RMS are malignant tumours of the muscle typified by myoblast-like cells that have lost the capacity to fully differentiate.[2] If treated aggressively, RMS patients have a high long-term survival rate, however chemotherapeutic resistance remains a large problem.[1-3] Understanding the molecular basis of this resistance therefore provides an opportunity for targeted drug therapies.

The failure of current chemotherapeutic approaches to eradicate cancer cells is frequently due to defects in the execution of cellular apoptotic program.[4] Apoptosis is the

mechanism by which multicellular organisms orchestrate death and removal of damaged cells, thus maintaining tissue homeostasis. It is tightly regulated by pro- and anti-apoptotic factors. Among these factors, cellular inhibitor of apoptosis (cIAP1, also known as HIAP2, MIHB), a member of the inhibitor of apoptosis (IAP) family, is a key regulator of apoptosis and promotes cancer cell survival by controlling the NF-κB signaling and extrinsic cell death pathways (reviewed in [5]).

Expression of cIAP1 is tightly regulated at the level of protein synthesis through an internal ribosome entry site (IRES).[6-8] IRESes are discreet RNA elements found in the 5'UTR of a number of viral and cellular mRNAs, that facilitate recruitment of the ribosome to the translation initiation start independently of the 5' cap.[9] The mechanisms by which cellular IRESes mediate ribosome recruitment are still not fully understood. However, it has been shown that in addition to RNA structure and some canonical translation initiation factors, other cellular proteins are required for proper IRES function.[10, 11] These proteins, termed IRES trans-acting factors (ITAFs), are thought to help in the recruitment of the ribosome by acting either as scaffold proteins or RNA chaperones.[12] We have previously identified four potential ITAFs interacting with cIAP1 IRES, among which was the insulin-like-growth-factor-2-mRNA-binding-protein-1 (IGF2BP1).[8]

IGF2BP1(also known as IMP1 or ZBP1) is a member of the VICKZ family of RNA binding proteins[13] which was first identified in the human rhabdomyosarcoma cell line RD, along with its parologs IGF2BP2 and IGF2BP3, as factors that bind to the human IGF-II leader-3 mRNA and regulate its translation.[14] IGF2BP1 is emerging as a key regulator of mRNA metabolism with diverse role in the control of mRNA localization,[15] stabilization[16, 17] and translation.[14, 18-20] Importantly, IGF2BP1 is an oncofetal protein that is normally only

expressed during embryogenesis[21] but is re-expressed in a variety of cancers (reviewed in [13, 22]). Although only one study has directly linked IGF2BP1 expression to tumourigenesis *in vivo*,[23] there is substantial evidence *in vitro* pointing to its oncogenic potential.[14, 17-19, 24-26]

Here, we report that IGF2BP1 is overexpressed in primary human RMS tumours and cell lines where it drives high expression of cIAP1 by enhancing IRES-mediated translation of cIAP1. Importantly, reducing the levels of cIAP1 in RMS cell lines, either by IGF2BP1 knock-down or by Smac mimetic compounds (SMC) treatment sensitizes RMS cells to TNFα-mediated cell death. Finally, targeting cIAP1 by SMC inhibits the establishment and growth of RMS xenograft tumours in mice. Our results identify IGF2BP1 as a critical regulator of cIAP1 expression and apoptotic resistance in RMS and advocate for the combined use of IAP antagonists and TNFα as a potential therapeutic approach for rhabdomyosarcomas.

5.4 Material and Methods

5.4.1 Cell culture, reagents, expression constructs and transfection.

Frozen and FFPE human paediatric RMS tumours and normal skeletal muscle samples were obtained from the Children's Hospital of Eastern Ontario Department of Pathology after institutional ethics board approval. Control human and fetal skeletal muscle whole lysates were purchased from Novus Biologicals (Oakville, ON). Human RMS cell lines (RH18, RH30, RH36, RD, and RH41) were a generous gift from Dr. P. Houghton (Department of Hematology-Oncology, St. Jude Children's Research Hospital, Memphis, TN) and were cultured in RMPI 1640 media. The human RMS cell line Kym-1 was purchased from the JCRB (Japan) and cultured in DMEM-F12 media. Primary human skeletal muscle myoblasts

(HSMM) were purchased from Lonza (Mississauga, ON) and HSMM total RNA was purchased from amsbio (Lake Forest, CA). HEK293 cells were purchased from the ATCC (Manassas, VA) and cultured in DMEM media. HEK293 cells stably expressing a non-targeting or IGF2BP1 shRNA (Supplementary Table 1) from a tetracycline-inducible promoter (pTRIPz plasmid), were maintained in TET-free DMEM supplemented with 2 μg/ml Puromycin. Transient siRNA transfections (Suppl. Table 1) were performed using Lipofectamine RNAiMax reagent (Invitrogen, Burlington, ON). Transient DNA transfections were performed using JetPRIME reagent (Polypus transfection, Illkirch, France). The GFP-IGF2BP1 plasmid was a gift from Dr. Stefan Hüttelmaier (Institute of Molecular Medicine, Martin Luther University of Halle, Halle, Germany); the pTurboGFP-dest1 (Evrogen, Moscow, Russia) was used as a transfection control.

5.4.2 Western blot analysis.

Western blots were performed as previously described.[41] Membranes were probed with antibodies against IGF2BP1 (IMP1 clone D9, Santa Cruz Biotechnologies), cIAP1 (RIAP1[8]), XIAP (RIAP3,[10, 49]), GAPDH (BD Biosciences), cleaved PARP (clone D64E10, Cell Signaling) and cleaved caspase 3 (clone 5A1E, Cell Signaling).

5.4.3 5'UTR and IRES reporter assays.

The monocistronic and bicistronic cIAP1 5'UTR reporter constructs were previously described and validated.[6, 8, 10] Relative translation activity of cIAP1 5'UTR was assayed using monocistronic plasmids as previously described.[8] Relative cIAP1 IRES activity was assayed using bicistronic reporter plasmids as previously described.[8]

Supplementary Table 1: siRNAs and shRNAs sequences and conditions

siRNA/shRNA	Sequences	Conditions
Scrambled shRNA	Cat# RHS4827, Thermo Scientific	1 µg/ml Doxycycline
IGF2BP1 shRNA	5'-TCTGCAACTCGTTCACCGT-3'	1 µg/ml Doxycycline
Scrambled siRNA	5'-UUCUCCGAACGUGUCACGU[dT][dT]-3'	10 or 50 nM
IGF2P1 siRNA	5'-UUCCAACCGGGAGCAGACCAGGCAA-3'	50 nM
TNFR1 siRNA	Cat# J-005197-05, Thermo Scientific	10 nM

Supplementary Table 2: Primers sequences

	mRNA	Primers	Sequence
qPCR primers	cIAP1	Forward	TCTGGAGATGATCCATGGGTAGA
		Reverse	TGGCCTTTCATTCGTATCAAGA
	CAT	Forward	GCGTGTTACGGTGAAAACCT
		Reverse	GGGCGAAGAAGTTGTCCATA
	GAPDH	Forward	ACAGTCAGCCGCATCTTCTT
		Reverse	ACGACCAAATCCGTTGACTC
	RPL13A2	QuantiTect Primers	Hs_RPL13A_2_SG
cIAP1 IRES probes (T7-PCR primers)	Probe 1	Forward	TAATACGACTCACTATAGGGCGAGTGATTTCTTTTTGTGGTA
		Reverse	TATACTCTTAATGTTTTGATAC
	Probe 2	Forward	TAATACGACTCACTATAGGGCGAGCAGTACTGTCACCTACTC
		Reverse	TATACTCTTAATGTTTTGATAC
	Probe 3	Forward	TAATACGACTCACTATAGGGCGAGTGATTTCTTTTTGTGGTA
		Reverse	GAGTAGGTGACAGTACTGTTTGATAG
	Probe 4	Forward	TAATACGACTCACTATAGGGCGAGTGATTTCTTTTTGTGGTA
		Reverse	TTTGATAGCTAAAACATTCACATG
	Probe 5	Forward	TAATACGACTCACTATAGGGCGAGATGCACAAAACTGCCTCC
		Reverse	TATACTCTTAATGTTTTGATAC
cIAP1 IRES-5 deletion probes	Probe 5	Foward strand	TAATACGACTCACTATAGGGCGAGATGCACAAAACTGCCTCCC AAAGACTTTTCCCAGGTCCCTCGTATCAAAACATTAAGAGTATA
		Reverse strand	TATACTCTTAATGTTTTGATACGAGGGACCTGGGAAAAGTCTTT GGGAGGCAGTTTTGTGCATCTCGCCCTATAGTGAGTCGTATTA
	Probe5-Δ2A	Foward strand	TAATACGACTCACTATAGGGCGAGATGCACAAAACTGCCTCCC AATTTCCCAGGTCCCTCGTATCAAAACATTAAGAGTATA
		Reverse strand	TATACTCTTAATGTTTTGATACGAGGGACCTGGGAAATTGGGAG GCAGTTTTGTGCATCTCGCCCTATAGTGAGTCGTATTA
	Probe5-Δ2B	Foward strand	TAATACGACTCACTATAGGGCGAGATGCACAAAACTGCCTCCC AAAGACTTTTCCCAGGTCCCTCGTATCAAAAGAGTATA
		Reverse strand	TATACTCTTTTGATACGAGGGACCTGGGAAAAGTCTTTGGGAG GCAGTTTTGTGCATCTCGCCCTATAGTGAGTCGTATTA
	Probe5-Δ2AB	Foward strand	TAATACGACTCACTATAGGGCGAGATGCACAAAACTGCCTCCC AATTTCCCAGGTCCCTCGTATCAAAAGAGTATA
		Reverse strand	TATACTCTTTTGATACGAGGGACCTGGGAAATTGGGAGGCAGT TTTGTGCATCTCGCCCTATAGTGAGTCGTATTA

5.4.4 Polysome profiling and quantitative RT-PCR analysis.

HEK293 cells were transfected with 50 nM of non-targeting or IGF2BP1-targeting siRNA for 72h and polysome profiling performed as previously described.[41] cIAP1 and RPL13A polysome-associated transcripts were quantified by RT-qPCR using specific primers (Suppl. Table 2). Steady-state mRNA levels were determined by RT-qPCR, as previously described.[41]

5.4.5 UV cross-linking RNA binding assay.

UV-crosslinking RNA binding assays were performed as previously described[8] using [α-^{32}P]-UTP-labeled, *in vitro*-transcribed cIAP1 RNA probes (Suppl. Table 2) and recombinant GST-IGF2BP1.

5.4.6 Cell viability, cytotoxicity and caspase activity assays.

1x10^4 cells were transfected with siRNA for 48h, treated with increasing concentrations of recombinant human TNFα or TRAIL (Enzo Life Sciences, Brockville, ON) and 48h later assayed for cell respiration by Alamar Blue (Invitrogen). For cytotoxicity assays, siRNA-treated cells or cells treated with 100 nM of AEG407030[36] or LCL161[47, 50] (kindly provided by Novartis Pharmaceuticals) or 0.1% DMSO, were incubated with 100 nM of YOYO-1 dye (Molecular probes, Burlington, ON) in the presence of TNFα and incorporation monitored over 48h using the INCUCYTE^{TM} ZOOM Live-Cell Imaging System (Essen Bioscience, Ann Arbor, MI). Caspase 3 and 7 activities were assayed using 1 uM of CellPlayer^{TM} Caspase-3/7 reagent (Essen Bioscience) and monitored using the INCUCYTE^{TM} ZOOM. For cIAP1 rescue experiments, RH36 cells were transfected siRNA for 48h, followed by

transduction with LacZ (Adv-LacZ) or LacZ-cIAP1 (Adv-HIAP2) adenovirus before monitoring of cytotoxicity or cell death for 48h. Fold cytotoxicity or cell death activity were calculated as the number of green fluorescence positive cells divided by the total number of cells (confluence) at endpoint, compared to time zero.

5.4.7 Xenograft mouse model and Smac mimetic treatment.

Subcutaneous tumours were established by injecting $3x10^6$ Kym-1 cells in Matrigel in the right flank of 6-week old female CD-1 nude mice. For established tumours (~300-400 mm^3), 5 mice were treated with vehicle (30% 0.1 M HCl, 70% 0.1 M NaOAc pH 4.63) or 50 mg/kg LCL161[47, 50] *per os*, twice a week for three weeks. In a parallel experiment, vehicle or LCL161 treatments commenced one day post-implantation for two weeks. Animals were euthanized when the tumour burden exceeded 2000 mm^3. Tumour volume was calculated using $(\pi)(W)^2(L)/4$ where W = tumour width and L = tumour length. All mice were maintained under barrier conditions and experiments carried according to protocols approved by the University of Ottawa Animal Care Facility.

5.4.8 Immunohistochemistry.

Human RMS primary tumours and normal skeletal muscle FFPE sections were deparaffinized in xylene and rehydrated in 100%-70% ethanol gradient. Heat-induced antigen retrieval was performed in 0.1M EDTA and sections incubated with antibodies against IGF2BP1 (IMP1 clone D9) or cIAPs (cIAP Pan, clone 315301, R&D Systems) for one hour at 3 C DAB detection was performed using the Envision anti-mouse-HRP kit (Dako, Burlington, ON). The sections were analysed and scored for staining intensity and

percentage of positive cells by a pathologist. For Kym-1 xenografts, established tumours treated with vehicle or 50 mg/kg LCL161 for 24h were stained for cleaved caspase 3 (C92-605 antibody, BD Pharmingen) as described before.[48]

5.5 Results

5.5.1 IGF2BP1 is overexpressed in rhabdomyosarcomas and drives cIAP1 expression.

We initially identified IGF2BP1 as one of the proteins that specifically binds to the cIAP1 IRES.[8] Since IGF2BP1 was first discovered in an RMS cell line and has been reported to be overexpressed in a variety of tumours,[13, 22] we decided to investigate a possible link between IGF2BP1 and cIAP1 expression in RMS tumours and tumour-derived cell lines. We observed a striking pattern of IGF2BP1 overexpression (10 to 60 fold) in established RMS cell lines of different origin (alveolar and embryonic) compared to normal human skeletal muscle myoblasts (HSMM) (Figure 5.1A, Suppl. Figure 5.1A,B). Notably, cIAP1 showed the same pattern of expression, being overexpressed 10 to 32 fold compared to HSMM (Figure 5.1A, Suppl. Figure 5.1A,B). Elevated IGF2BP1 and cIAP1 expression was also noted in three out of four primary human eRMS tumours when compared to human fetal and adult skeletal muscle (Figure 5.1B). In addition, formalin-fixed, paraffin-embedded (FFPE) primary human RMS tumours were analysed by immunohistochemistry and compared to normal human skeletal muscle sections (Figure 5.1C). IGF2BP1 was moderately to highly overexpressed (score 2 or 3) in 6 out of 8 RMS cases analysed compared to normal muscle sections (score 1; 7 out of 9 cases), and this correlated well with

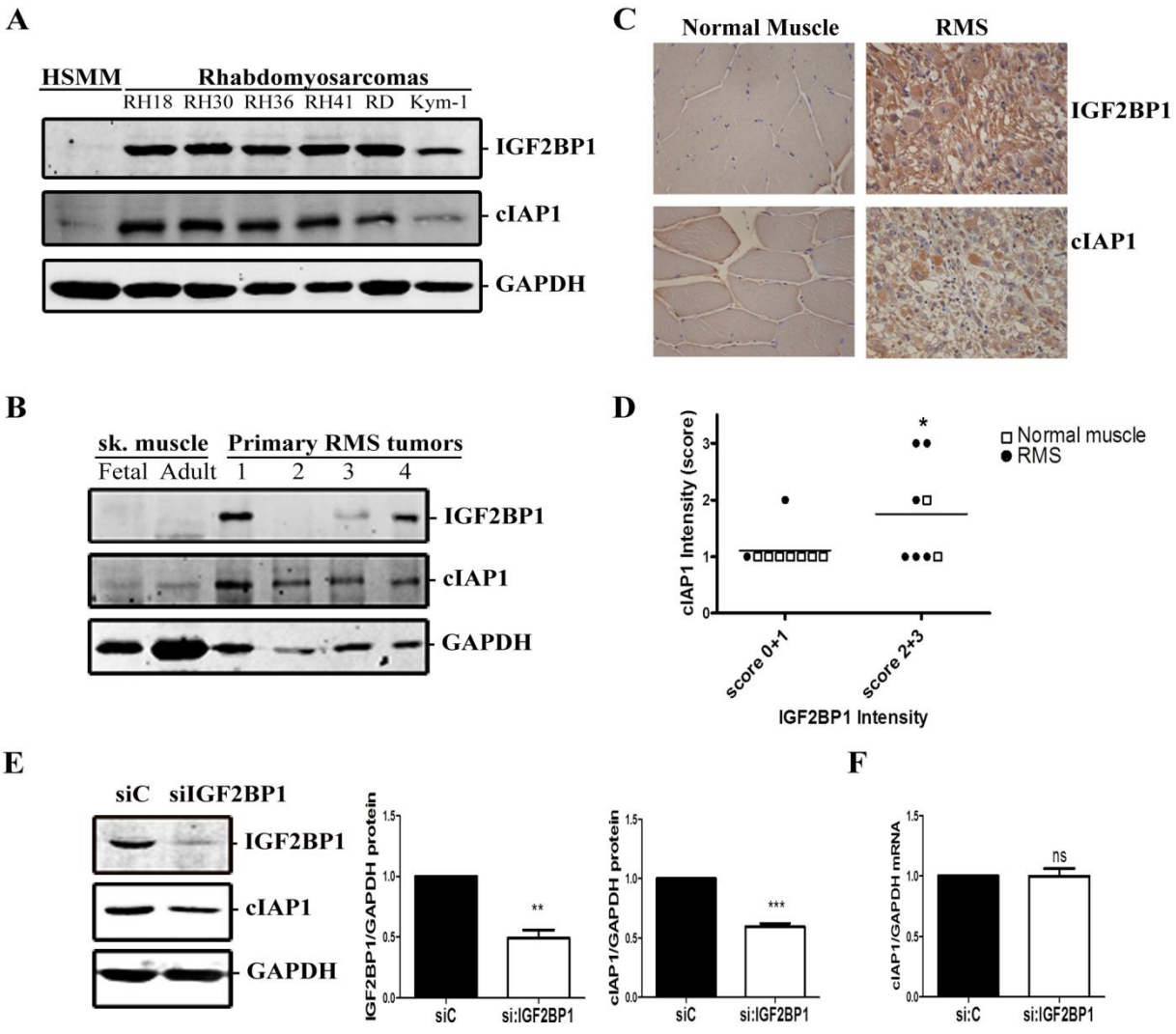
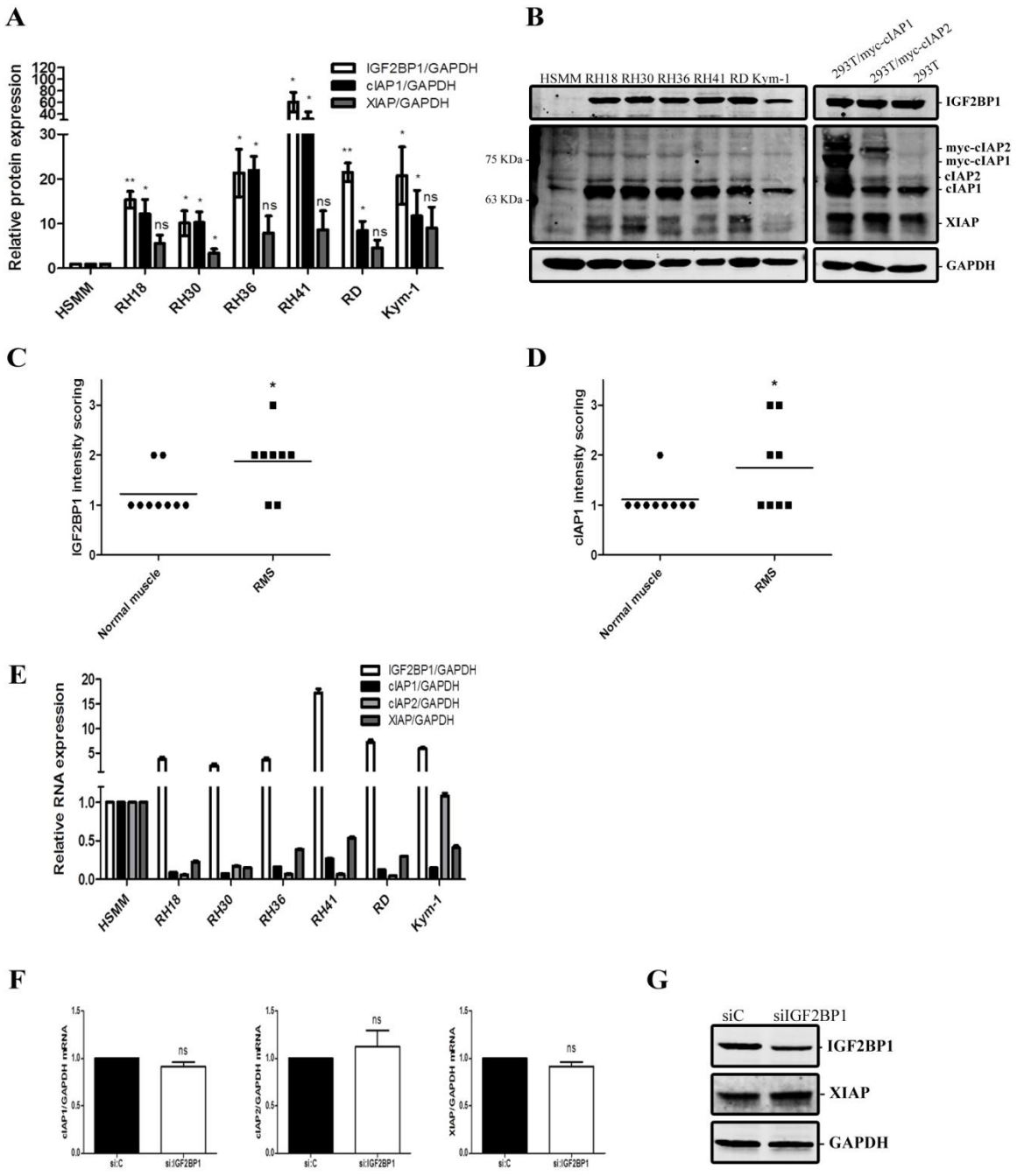

A
HSMM
Rhabdomyosarcomas
RH18 RH30 RH36 RH41 RD Kym-1
IGF2BP1
cIAP1
GAPDH
B
sk. muscle
Primary RMS tumors
Fetal Adult 1 2 3 4
IGF2BP1
cIAP1
GAPDH
C
Normal Muscle
RMS
IGF2BP1
cIAP1
D
cIAP1 Intensity (score)
Normal muscle
RMS
score 0+1
score 2+3
IGF2BP1 Intensity
E
siC siIGF2BP1
IGF2BP1
cIAP1
GAPDH
IGF2BP1/GAPDH protein
cIAP1/GAPDH protein
siC
si:IGF2BP1
F
cIAP1/GAPDH mRNA
ns
si:C
si:IGF2BP1

Figure 5.1: IGF2BP1 is overexpressed in Rhabdomyosarcoma and drives cIAP1 expression.

(A) RMS cell lines were analysed by western blot for IGF2BP1, cIAP1 and GAPDH protein expression compared to HSMM. **(B)** Western blot of four human primary eRMS tumours for IGF2BP1, cIAP1 and GAPDH protein expression, compared to fetal and adult human skeletal muscle. **(C)** Human RMS primary tumours and normal skeletal muscle FFPE sections were analysed by immunohistochemistry for IGF2BP1 and cIAP1 expression (brown staining) and representative images shown. **(D)** Intensity scoring of the sections analysed in (C), 1 = mild, 2 = moderate, 3 = strong expression. Spearman correlation p value of 0.033 between IGF2BP1 and cIAP1 staining intensity (n=8 RMS, 9 normal muscle). **(E)** RH36 cells were transfected with non-targeting siRNA (siC) or IGF2BP1 siRNA (siIGF2BP1) for 72h and IGF2BP1, cIAP1 and GAPDH protein expression analysed by western blot. Bar graphs show densitometry of IGF2BP1 and cIAP1 protein levels normalised to GAPDH. (**F)** RH36 cells were transfected with siC or siIGF2BP1 for 72h and cIAP1 mRNA levels analysed by RT-qPCR, compared to GAPDH. Results are representative of at least three biological replicates. Mean ±SEM. Student t-test: ns, non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.


A
Relative protein expression
IGF2BP1/GAPDH
cIAP1/GAPDH
XIAP/GAPDH
HSMM
RH18
RH30
RH36
RH41
RD
Kym-1
B
HSMM RH18 RH30 RH36 RH41 RD Kym-1
293T/myc-cIAP1
293T/myc-cIAP2
293T
IGF2BP1
75 KDa
63 KDa
myc-cIAP2
myc-cIAP1
cIAP2
cIAP1
XIAP
GAPDH
C
IGF2BP1 intensity scoring
Normal muscle
RMS
D
cIAP1 intensity scoring
Normal muscle
RMS
E
Relative RNA expression
IGF2BP1/GAPDH
cIAP1/GAPDH
cIAP2/GAPDH
XIAP/GAPDH
HSMM
RH18
RH30
RH36
RH41
RD
Kym-1
F
cIAP1/GAPDH mRNA
cIAP2/GAPDH mRNA
XIAP/GAPDH mRNA
siC
siIGF2BP1
G
siC
siIGF2BP1
IGF2BP1
XIAP
GAPDH

Supplementary Figure 5.1: IGF2BP1 and cIAP1 expression in RMS cell lines and primary tumours

(A) Densitometry of IGF2BP1, cIAP1 and XIAP protein levels normalised to GAPDH protein in the panel of RMS cell lines compared to HSMM. Mean ± SEM (n=3-5). Student t-test: ns, non-significant, * $P < 0.05$, ** $P < 0.01$. **(B)** A panel of RMS tumor-derived cell lines were analysed by western blot for IGF2BP1, cIAP1/2 (overexposed), XIAP and GAPDH protein expression compared to control human skeletal muscle myoblasts (HSMM). HEK293 were transfected with myc-cIAP1 and myc-cIAP2 overexpressing plasmids for 24h and used as positive controls for the cIAP1/2 antibody specificity. **(C, D)** Intensity scoring of human RMS primary tumours and normal skeletal muscle FFPE sections analysed by immunohistochemistry for IGF2BP1 and cIAP1 expression (1 = mild, 2 = moderate, 3 = strong expression). Student t-test: $p = 0.0171$ for IGF2BP1, $p = 0.0410$ for cIAP1 (n=8 RMS, 9 normal muscle). **(E)** IGF2BP1, cIAP1, cIAP2 and XIAP steady-state mRNA levels were analysed by qPCR and nornalised to GAPDH RNA in the panel of RMS cell lines compared to HSMM. **(F)** HEK293 cells were transfected with 50 nM of non-targeting (siC) or IGF2BP1 (siIGF2BP1) siRNA for 72h, RNA extracted and analysed for cIAP1, cIAP2 and XIAP steady-state mRNA levels by qPCR compared to GAPDH mRNA. **(G)** HEK293 cells were transfected with non-targeting or IGF2BP1 for 72h, total protein extracted and analysed for IGF2BP1, XIAP and GAPDH protein levels by western blot.

increased cIAP1 expression (Figure 5.1D, Spearman correlation p value of 0.033; Suppl.Figure 5.1C,D). We also examined the expression of two other members of the IAP family, cIAP2 and XIAP, in RMS cell lines and found that while cIAP2 protein was barely detectable, XIAP protein was not significantly increased, except for RH30 cells (Suppl. Figure 5.1A,B).

To demonstrate that high expression of IGF2BP1 drives cIAP1 expression in RMS, we knocked-down IGF2BP1 in the RH36 cell line and observed a concomitant decrease in cIAP1 protein levels compared to cells transfected with a non-targeting control siRNA (Figure 5.1E). Interestingly, the reduction in cIAP1 protein levels was not associated with changes in cIAP1 steady-state mRNA levels (Figure 5.1F). Furthermore, while IGF2BP1 steady-state mRNA levels were elevated in all six RMS cell lines, cIAP1 mRNA levels were significantly decreased (Suppl. Figure 5.1E), arguing for a post-transcriptional control of cIAP1 expression. Collectively, these data and the fact that we have previously identified IGF2BP1 as a cIAP1 IRES binding protein prompted us to hypothesize that IGF2BP1 is an IRES trans-acting factor that regulates cIAP1 IRES-mediated translation.

5.5.2 IGF2BP1 regulates cIAP1 translation.

We next set out to characterise the mechanism by which IGF2BP1 regulates cIAP1 expression. For these experiments we used HEK293 cells which recapitulate the IGF2BP1-mediated changes in cIAP1 expression seen in RMS cells (Figure 5.2A,B). Importantly, re-expression of IGF2BP1 in cells previously transfected with IGF2BP1 siRNA was able to rescue cIAP1 protein to levels comparable to that of control siRNA treated cells (Figure 5.2C). In addition, since IGF2BP1 knock-down didn't affect cIAP2 and XIAP expression at

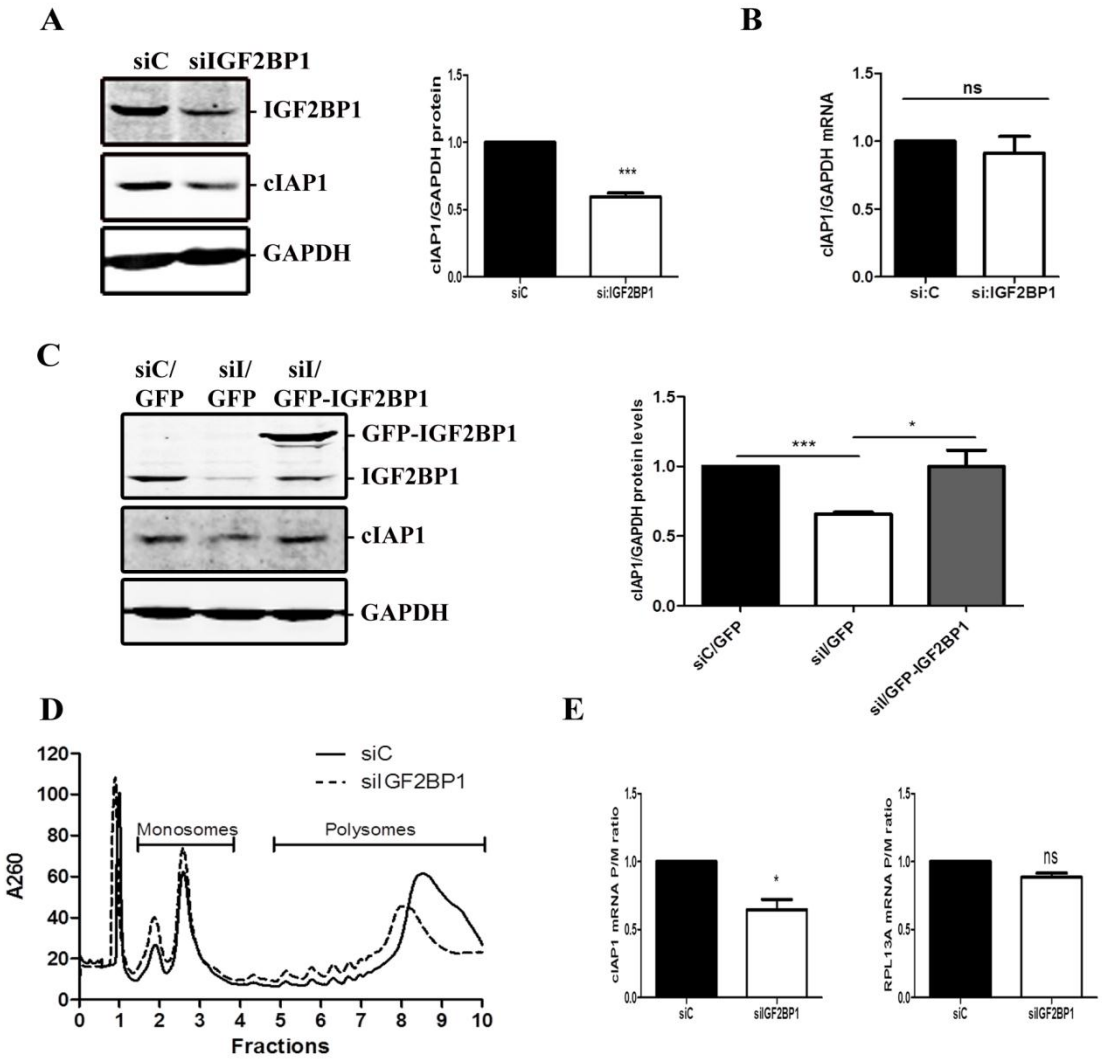

A
siC
siIGF2BP1
IGF2BP1
cIAP1
GAPDH
cIAP1/GAPDH protein
1.5
1.0
0.5
0.0
***
siC
si:IGF2BP1
B
cIAP1/GAPDH mRNA
ns
si:C
si:IGF2BP1
C
siC/ GFP
siI/ GFP
siI/ GFP-IGF2BP1
GFP-IGF2BP1
IGF2BP1
cIAP1
GAPDH
cIAP1/GAPDH protein levels
***
*
siC/GFP
siI/GFP
siI/GFP-IGF2BP1
D
siC
siIGF2BP1
Monosomes
Polysomes
A260
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
Fractions
E
cIAP1 mRNA P/M ratio
*
siC
siIGF2BP1
RPL13A mRNA P/M ratio
ns
siC
siIGF2BP1

Figure 5.2: IGF2BP1 regulates cIAP1 translation.

(A) HEK293 cells were transfected with non-targeting siRNA (siC) or IGF2BP1 siRNA (siIGF2BP1) for 72h and protein expression assessed by western blot. (**B)** HEK293 cells transfected with siC or siIGF2BP1 for 72h were analyzed by RT-qPCR for cIAP1 mRNA levels compared to GAPDH. (**C**) HEK293 cells were transfected with siC or siIGF2BP1 for 48h, followed by GFP or GFP-IGF2BP1 overexpression for 24h and analysis of IGF2BP1, cIAP1 and GAPDH protein levels by western blot. (**D)** Representative polysome profiles of HEK293 cells transfected with siC or siIGF2BP1 for 72h. (**E)** cIAP1 and RPL13A mRNA abundance in polyribosome fractions was analysed by RT-qPCR and shown as a ratio of mRNA content in polysome fractions compared to monosome fractions (P/M ratio). Mean ± SEM. Student t-test: ns, non-significant, * $P < 0.05$.

the mRNA or protein levels (Suppl. Figure 5.1F,G) these results demonstrate that IGF2BP1 specifically regulates cIAP1 protein levels.

To demonstrate that IGF2BP1 regulates cIAP1 translation, we performed polysome profiling to examine the association of endogenous cIAP1 mRNA with translating ribosomes upon IGF2BP1 knock-down (Figure 5.2D). RT-qPCR amplification of cIAP1 mRNA from individual ribosome fractions showed a decreased association with polysomes in cells with reduced IGF2BP1 levels, as shown by a decrease in cIAP1 mRNA polysome to monosome ratio (Figure 5.2E; Suppl. Figure 5.2A). In contrast, association of RPL13A, a representative house-keeping mRNA, with polysomes remained unchanged (Figure 5.2E; Suppl. Figure 5.2B). Importantly, IGF2BP1 knock-down did not affect the overall polysomes profiles except for a slight decrease in total polysome quantities (Figure 5.2D and Suppl. Figure 5.2C), which could be explained by reported association of IGF2BP1 with more than 1000 transcripts and possibly within polysomes.[27, 28] These results are consistent with the notion that IGF2BP1 directly regulates translation of the cIAP1 mRNA.

5.5.3 IGF2BP1 directly binds cIAP1 mRNA and mediates its translation via the 5'UTR IRES.

Since our data showed that IGF2BP1 controls translation of cIAP1 mRNA, we questioned whether this control is exerted through the 5'UTR of cIAP1, and specifically through its IRES. We first tested this possibility using a monocistronic reporter in which the cIAP1 5'UTR controls translation of the downstream CAT reporter gene We observed that knocking-down IGF2BP1 resulted in a significant decrease in CAT expression from the cIAP1-5'UTR-controlled reporter (cIAP1-5'UTR-pMC) when compared to non-targeting

siRNA transfected cells, whereas CAT expression from the empty reporter plasmid (pMC) remained unaffected (Figure 5.3A). Importantly, this decrease in CAT expression was not due to changes in CAT mRNA levels (Figure 5.3A, bottom) indicating that IGF2BP1 regulates cIAP1 translation through the 5'UTR To further determine if IGF2BP1 regulates cIAP1 the IRES-mediated translation, we performed a bicistronic reporter assay in which β-galactosidase (β−Gal) expression reports cap-dependent translation, while CAT expression is driven by the cIAP1 IRES (pBC-cIAP1-5'UTR; [8]). We found that cIAP1 IRES activity was specifically decreased in cells expressing IGF2BP1 shRNA but not in cells expressing the scrambled shRNA, or when a bicistronic reporter containing the inverted cIAP1-5'UTR was used (Figure 5.3B). Importantly, neither IGF2BP1 knock-down nor the directionality of the cIAP1 IRES affected β−Gal expression (Figure 5.3B, bottom), indicating that regulation of CAT expression through the IRES is separate from that of β−Gal, directed by the cap.

We next wished to examine whether IGF2BP1 binds directly to the cIAP1 IRES. UV cross-linking of incubated GST-IGF2BP1 and ^{32}P-labeled cIAP1 IRES RNA probes showed that GST-IGF2BP1 directly binds to the cIAP1 full-length IRES (probe 1) and to probes corresponding to the 3' end (probes 2 and 5) but not the 5' end of the IRES (probes 3 and 4) (Figure 5.3C). To further delineate IGF2BP1 binding region(s), we searched the cIAP1 IRES sequence for IGF2BP1 putative binding sites (GGACU/ACACC[29] or CAUH where H = A, U or C[28]) and identified two sites that fit these criteria (sites A and B, Figure 3D). While deletion of siteA (probe5-ΔA) did not abrogate binding of GST-IGF2BP1, deletion of siteB (probe5-ΔB) or both sites (probe5-ΔAB) resulted in a loss of binding (Figure 3E, left panel) However, when tested in the context of the full length IRES (probe 1), site B only accounted for part of GST-IGF2BP1 binding (Figure 5.3E, right panel).

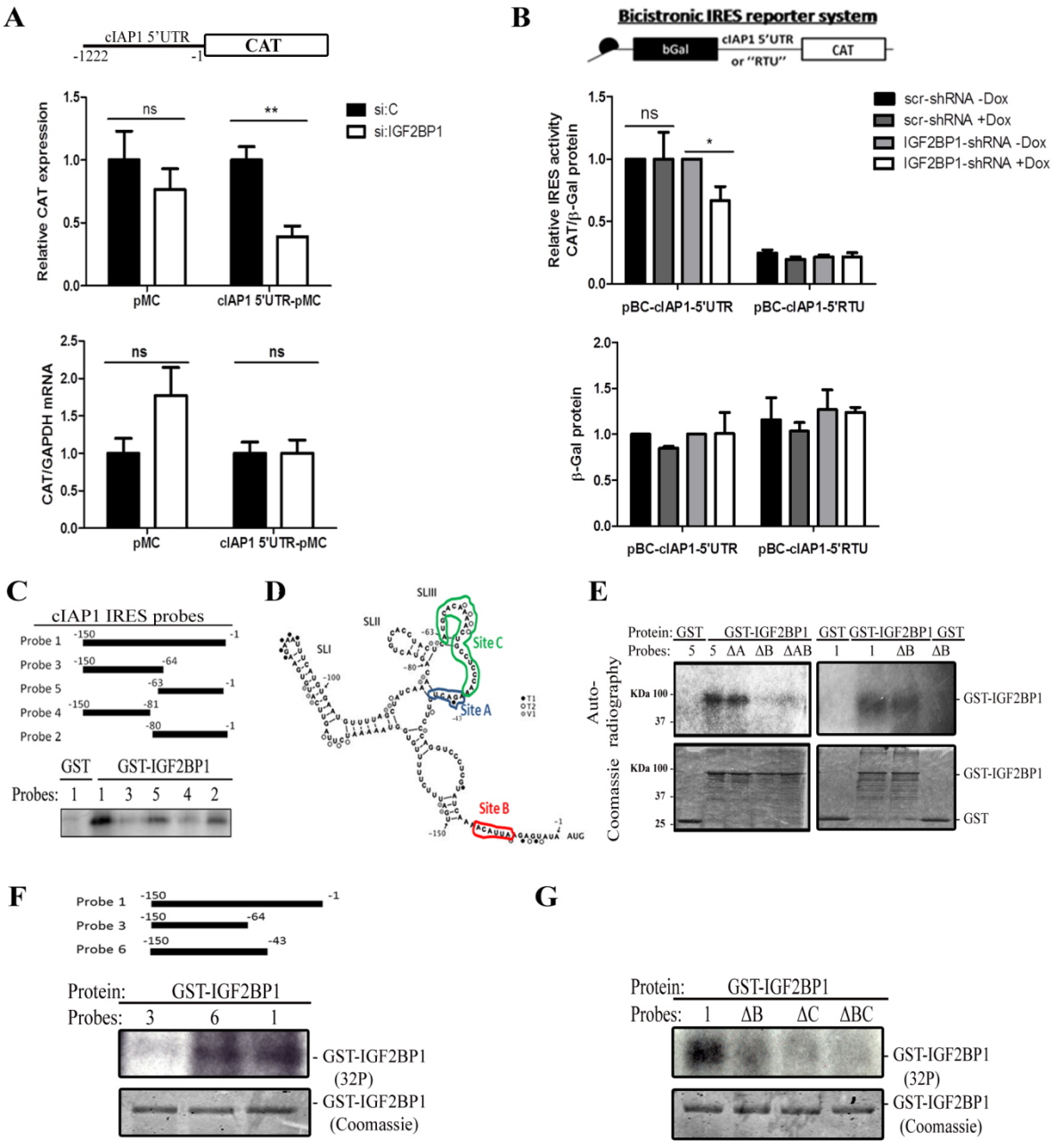

A
cIAP1 5'UTR
CAT
-1222
-1
Relative CAT expression
ns
**
si:C
si:IGF2BP1
pMC
cIAP1 5'UTR-pMC
CAT/GAPDH mRNA
ns
ns
pMC
cIAP1 5'UTR-pMC
B
Bicistronic IRES reporter system
bGal
cIAP1 5'UTR
or "RTU"
CAT
Relative IRES activity
CAT/β-Gal protein
ns
*
scr-shRNA -Dox
scr-shRNA +Dox
IGF2BP1-shRNA -Dox
IGF2BP1-shRNA +Dox
pBC-cIAP1-5'UTR
pBC-cIAP1-5'RTU
β-Gal protein
pBC-cIAP1-5'UTR
pBC-cIAP1-5'RTU
C
cIAP1 IRES probes
Probe 1
Probe 3
Probe 5
Probe 4
Probe 2
GST
GST-IGF2BP1
Probes: 1 1 3 5 4 2
D
SLIII
SLII
SLI
Site C
Site A
Site B
AUG
E
Protein: GST GST-IGF2BP1 GST GST-IGF2BP1 GST
Probes: 5 5 ΔA ΔB ΔAB 1 1 ΔB ΔB
Auto-radiography
KDa 100
37
GST-IGF2BP1
Coomassie
KDa 100
37
25
GST-IGF2BP1
GST
F
Probe 1
Probe 3
Probe 6
Protein: GST-IGF2BP1
Probes: 3 6 1
- GST-IGF2BP1 (32P)
- GST-IGF2BP1 (Coomassie)
G
Protein: GST-IGF2BP1
Probes: 1 ΔB ΔC ΔBC
GST-IGF2BP1 (32P)
GST-IGF2BP1 (Coomassie)

Figure 5.3: IGF2BP1 directly binds the cIAP1 mRNA and mediates its translation via the 5'UTR IRES.

(A) Relative cIAP1 5'UTR activity from a CAT reporter (cIAP1-5'UTR-pMC, schematic) and from a control plasmid (pMC) was assayed in cells transfected with control (siC) or IGF2BP1 (siIGF2BP1) siRNA and expressed as CAT protein/mRNA ratio. CAT mRNA levels were assayed by RT-qPCR (bottom panel). (**B)** Relative cIAP1 IRES activity from a bicistronic reporter containing the cIAP1 5'UTR in the forward (pBC-cIAP1-5'UTR) or inverted (pBC-cIAP1-5'RTU) orientation (schematic) was assayed in HEK293 inducibly expressing (+/- Dox) a scrambled (scr-shRNA) or IGF2BP1 (IGF2BP1-shRNA) shRNA and is shown as CAT/β–Gal protein ratio; basal β–Gal expression is shown (bottom panel). Mean ± SEM. Student t-test: ns, non-significant, * $P < 0.05$, ** $P < 0.01$. (**C**) GST-IGF2BP1 was incubated with different [α-^{32}P]-UTP-labeled cIAP1 IRES probes (schematic), the complexes UV cross-linked and analysed by SDS-PAGE and autoradiography. (**D)** Secondary structure of the cIAP1 IRES as previously determined by enzymatic probing [8]. Sites A and B represent putative IGF2BP1 binding sites. (**E)** cIAP1 IRES probes deleted of the putative IGF2BP1 binding site A and B were assayed as in **(C)**. **(F)** cIAP1 IRES probe 6 (-150 to -43) was assayed for GST-IGF2BP1 binding as described in **(C)**. **(G)** A cIAP1 IRES probe deleted of the SLIII (ΔC) was assayed for GST-IGF2BP1 binding compared to probe1 and probe1-ΔB

Further mapping revealed that GST-IGF2BP1 binds to probe 6 (-150 to -43 of cIAP1 IRES) but not to probe 3 (-150 to -64) (Figure 5.3F), suggesting that GST-IGF2BP1 binds to the region containing stem loops II and III (Figure 5.3D). To test this possibility, we generated sequential deletions of this region and found that deletion of site C-containing SLIII (Figure 5.3D, probe1-ΔC) resulted in a significant loss of binding whereas double deletion of sites B and C (probe1-ΔBC) resulted in a complete loss of binding (Figure 5.3G). These results show that GST-IGF2BP1 binds to SLIII of the cIAP1 IRES and to the 'ACAUUA' site (site B) proximal to the AUG start codon. To correlate GST-IGF2BP1 binding with cIAP1 IRES activity, we constructed pBGal/CAT bicistronic plasmids in which sites B and C of the cIAP1 minimal IRES are deleted individually or in combination. As expected, deletion of site B did not result in changes of cIAP1 IRES activity; however, the contribution of site C to cIAP1 IRES activity could not be confirmed as the construct harbouring this deletion was not functional (data not shown). Altogether, these results suggest that IGF2BP1 binding to the stemloop III is critical in mediating cIAP1 IRES activity and function in cells.

5.5.4 IGF2BP1 knock-down sensitizes rhabdomyosarcoma cells to TNFα mediated cell death.

IGF2BP1 has been emerging as an important oncogenic factor, in particular with respect to cellular proliferation and metastasis, through the regulation of proto-oncogenes such as MYC and KRAS.[17, 24] In contrast, the role of IGF2BP1 in controlling apotosis in cancer cells has not been investigated. Since cIAP1 is a key regulator of caspase-8-mediated cell death[5] and, as we have now shown, a post-transcriptional target of IGF2BP1, we asked whether elevated levels of IGF2BP1 play a role in RMS cell survival. Consequently, we

examined the effect of IGF2BP1 knock-down on RH36 cells treated with TNFα, since recent reports suggested that this RMS-derived cell line (amongst others) is resistant to TNFα-related apoptosis-inducing-ligand (TRAIL).[30-32]

Treatment of RH36 cells with increasing concentrations of TNFα did not affect their viability (Figure 5.4A). However, IGF2BP1 knock-down (Suppl. Figure 5.2E) significantly reduced RH36 cell viability when compared to non-targeting siRNA-transfected cells (Figure 5.4A). Similarly, IGF2BP1 knock-down significantly increased caspase-3/7 activity upon TNFα treatment, compared to untreated cells (Figure 5.4C,D). Importantly, IGF2BP1 knock-down alone didn't affect RH36 cell viability or caspase activity (Figure 5.4B,C,D) indicating that reducing IGF2BP1 levels does not cause general toxicity but instead sensitizes RH36 cells to TNFα-mediated death. Importantly, restoring cIAP1 expression by Adv-HIAP2 reduced caspase-3/7 activity in IGF2BP1 siRNA-transfected cells to levels comparable to that of non-targeting siRNA transfected ones, whereas a control Lac-Z adenovirus did not have the same effects (Figure 5.4E,F). These results show that IGF2BP1 controls TNFα-mediated cell death through the regulation of cIAP1 translation.

5.5.5 cIAP1 depletion by SMCs sensitizes rhabdomyosarcoma cells to TNFα mediated cell death.

We have shown that cIAP1 is the key factor downstream of IGF2BP1 which mediates RMS sensitivity to TNFα To test the therapeutic utility of our observations, we set out to directly target cIAP1 by the use of smac mimetic compounds (SMCs). SMC treatment triggers the auto-ubiquitination and proteasomal degradation of cIAPs, leading to activation of the non-canonical NF-κB pathway, activation of caspase-8, and cell death. SMCs can also

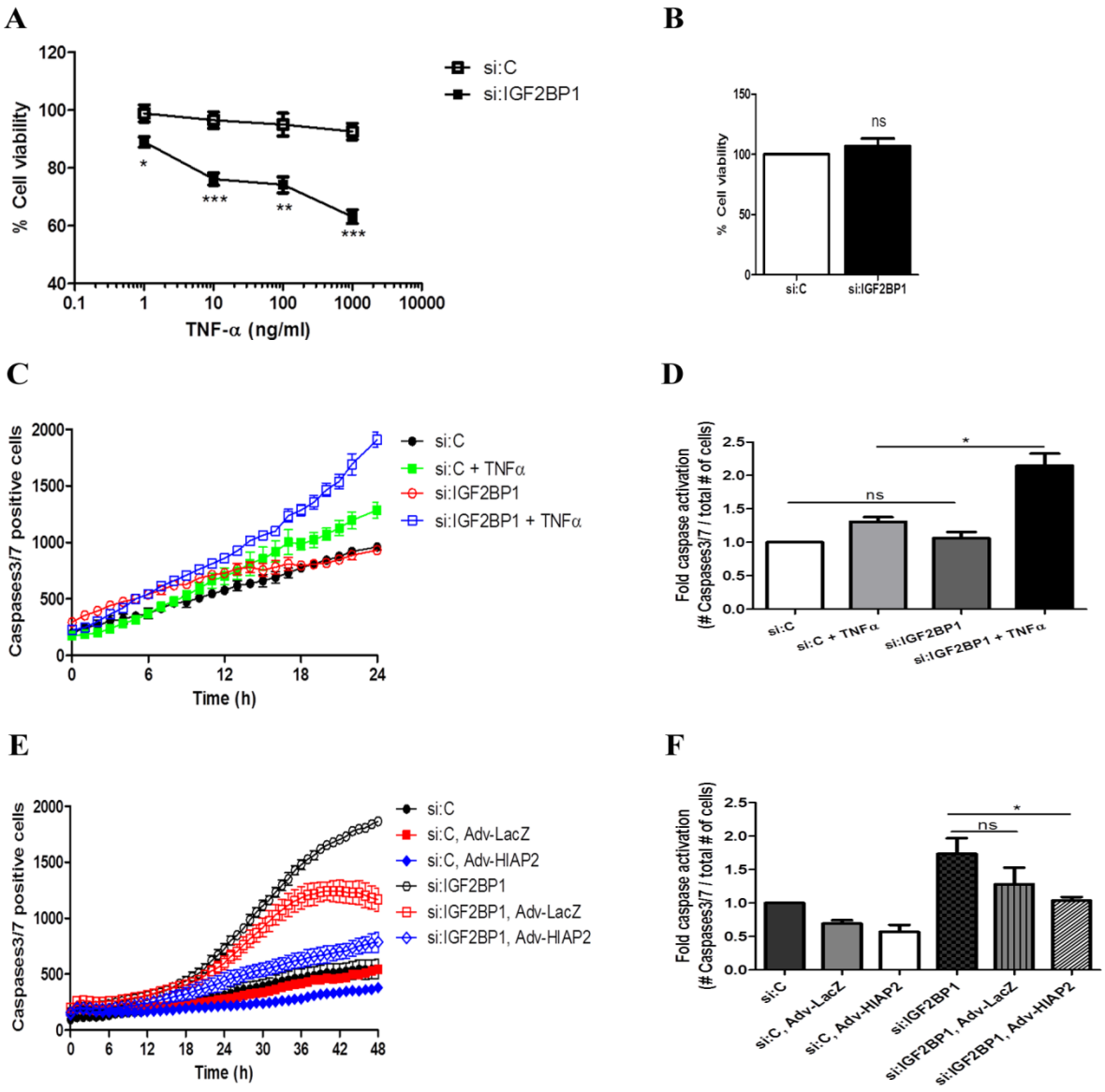

A
120
100
80
60
40
% Cell viability
si:C
si:IGF2BP1
*
***
**
***
0.1
1
10
100
1000
10000
TNF-α (ng/ml)
B
150
100
50
0
% Cell viability
ns
si:C
si:IGF2BP1
C
2000
1500
1000
500
0
Caspases3/7 positive cells
si:C
si:C + TNFα
si:IGF2BP1
si:IGF2BP1 + TNFα
0
6
12
18
24
Time (h)
D
2.5
2.0
1.5
1.0
0.5
0.0
Fold caspase activation
(# Caspases3/7 / total # of cells)
*
ns
si:C
si:C + TNFα
si:IGF2BP1
si:IGF2BP1 + TNFα
E
2000
1500
1000
500
0
Caspases3/7 positive cells
si:C
si:C, Adv-LacZ
si:C, Adv-HIAP2
si:IGF2BP1
si:IGF2BP1, Adv-LacZ
si:IGF2BP1, Adv-HIAP2
0
6
12
18
24
30
36
42
48
Time (h)
F
2.5
2.0
1.5
1.0
0.5
0.0
Fold caspase activation
(# Caspases3/7 / total # of cells)
*
ns
si:C
si:C, Adv-LacZ
si:C, Adv-HIAP2
si:IGF2BP1
si:IGF2BP1, Adv-LacZ
si:IGF2BP1, Adv-HIAP2

Figure 5.4: IGF2BP1 knock-down sensitizes RH36 rhabdomyosarcoma cells to TNFα mediated cell death.

RH36 cells were transfected with non-targeting (siC) or IGF2BP1 siRNA (siIGF2BP1) for 72h in the presence (**A**) or absence (**B**) of increasing TNFα concentrations and cell viability determined by Alamar Blue assay. **C**/ Caspase-3/7 activity in RH36 cells after IGF2BP1 knock-down and TNFα treatment (10 ng/ml) (**D)** Fold caspase activities at 24h from (**C)** were calculated as described in Material and Methods. (**E)** RH36 cells were transfected with siC or siIGF2BP1 for 48h followed by adenovirus expression of cIAP1 (Adv-HIAP2) or Lac-Z (Adv-LacZ) for an additional 48h, in the presence of 10 ng/ml TNFα The number of caspase3/7 positive cells over 48h is shown. (**F)** Fold caspase activities at 48h for (**E)** are shown. Mean ± SEM. Student t-test: ns, non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

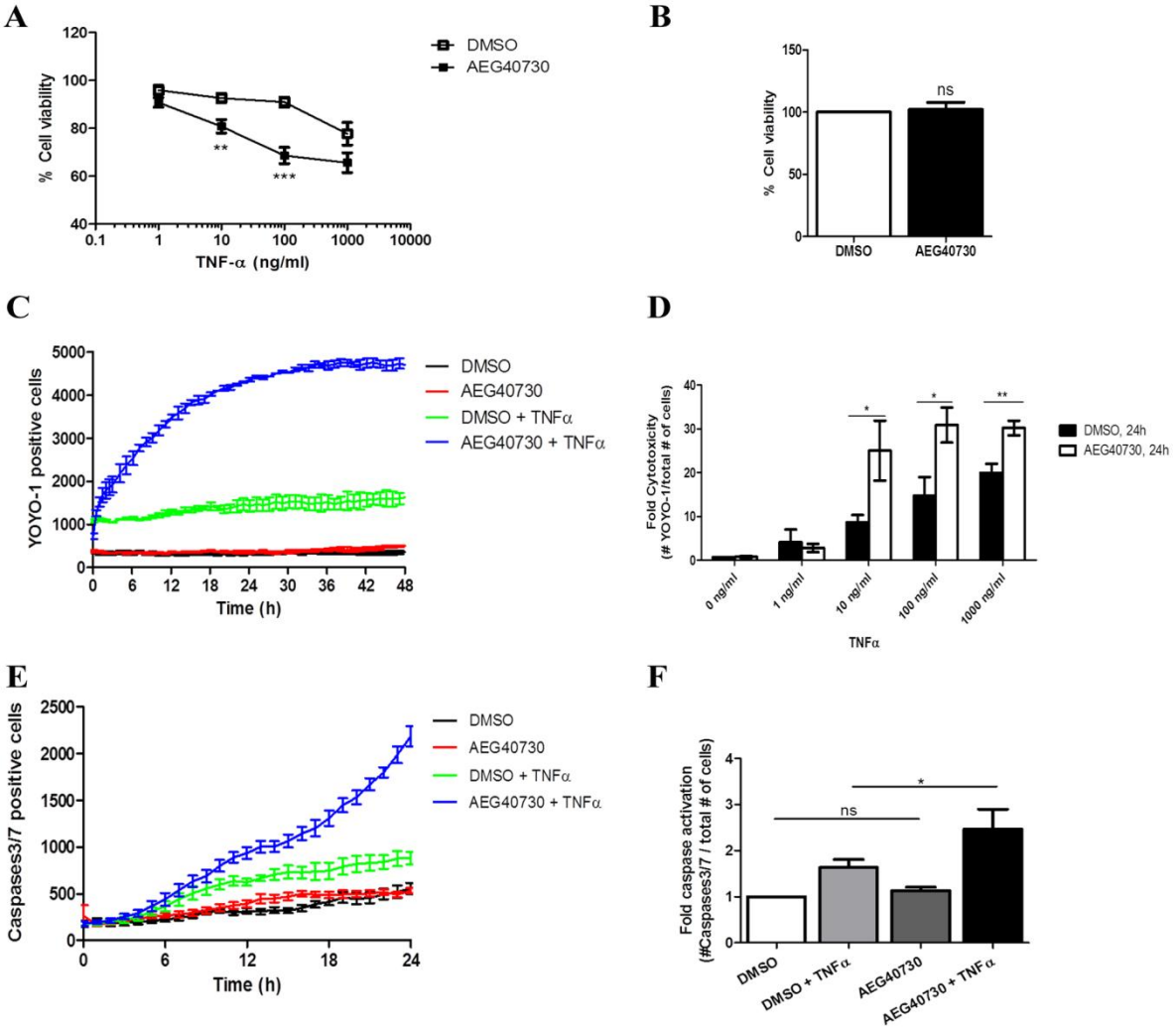
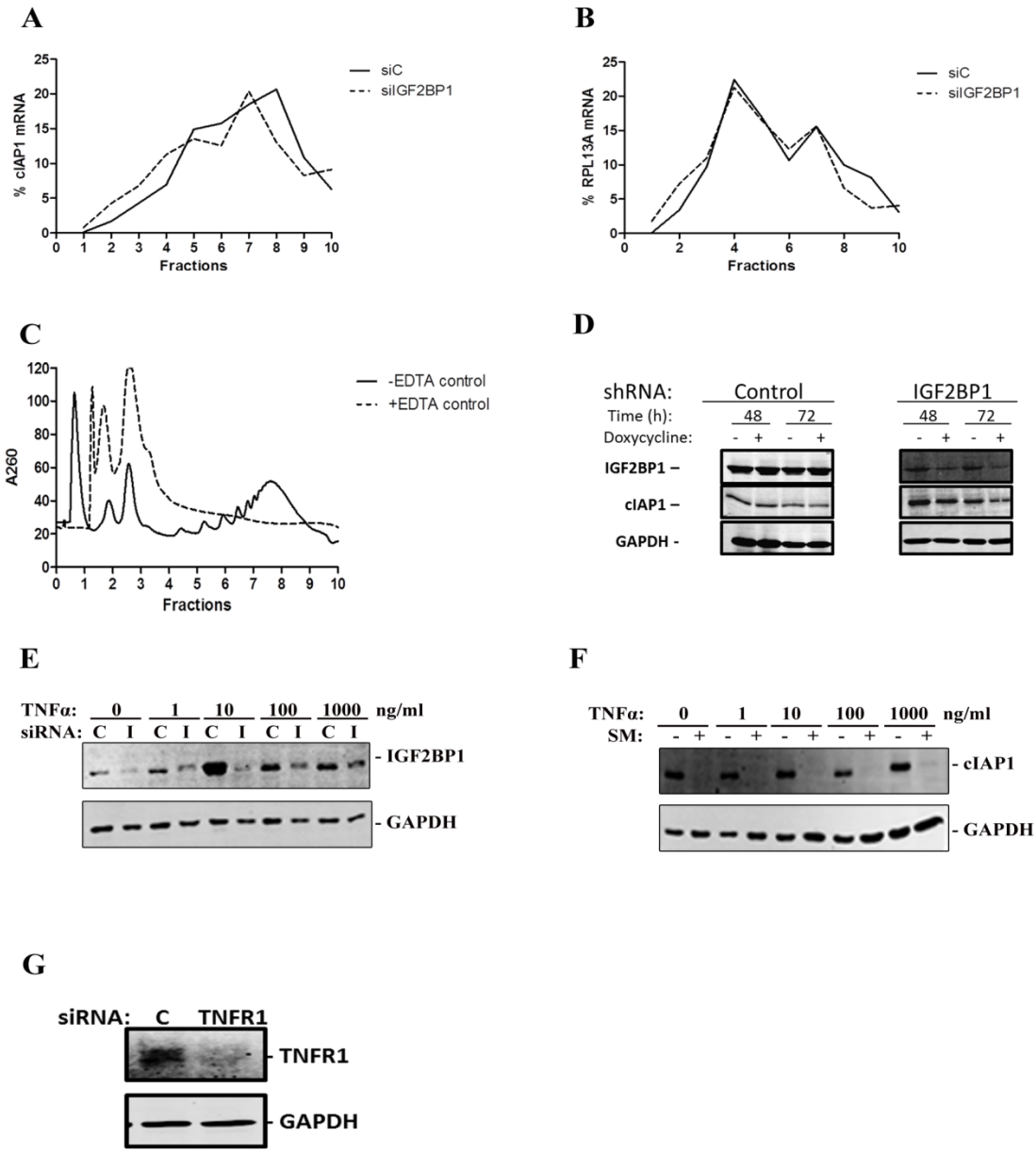

A
% Cell viability
TNF-α (ng/ml)
DMSO
AEG40730
**
***
B
% Cell viability
ns
DMSO
AEG40730
C
YOYO-1 positive cells
Time (h)
DMSO
AEG40730
DMSO + TNFα
AEG40730 + TNFα
D
Fold Cytotoxicity
(# YOYO-1/total # of cells)
TNFα
0 ng/ml
1 ng/ml
10 ng/ml
100 ng/ml
1000 ng/ml
DMSO, 24h
AEG40730, 24h
E
Caspases3/7 positive cells
Time (h)
DMSO
AEG40730
DMSO + TNFα
AEG40730 + TNFα
F
Fold caspase activation
(#Caspases3/7 / total # of cells)
ns
DMSO
DMSO + TNFα
AEG40730
AEG40730 + TNFα

Figure 5.5: cIAP1 depletion by Smac mimetic compounds sensitizes rhabdomyosarcoma cells to TNFα mediated cell death.

RH36 cells were treated with 0.1% DMSO or 100 nM of AEG40730 in the presence (**A**) or absence (**B**) of increasing TNFα concentrations and cell viability determined by Alamar Blue assay. (**C)** RH36 cells were treated as in (**A)** in the presence of 10 ng/ml TNFα and assayed for cytotoxicity by YOYO-1 dye incorporation for 48h. (**D)** Fold cytotoxicity at 24h from (**C)** and at other TNFα concentrations are shown (**E)** RH36 cells were treated with 0.1% DMSO or 100 nM AEG40730 in the presence or absence of 10 ng/ml TNFα and the number of caspase-3/7 positive cells monitored over 24h. **F/** Fold caspase activities at 24h for (**E)** were calculated. Mean ± SEM. Student t-test: ns, non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

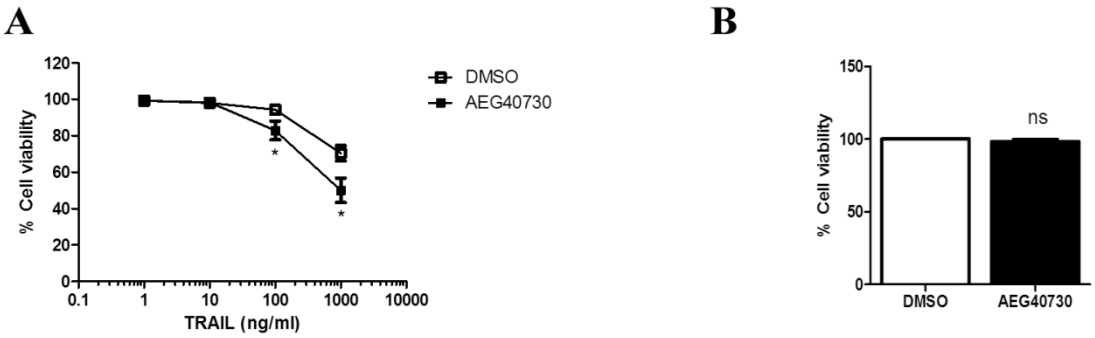

A
% cIAP1 mRNA
Fractions
siC
siIGF2BP1
B
% RPL13A mRNA
Fractions
siC
siIGF2BP1
C
A260
Fractions
-EDTA control
+EDTA control
D
shRNA:
Control
IGF2BP1
Time (h):
48
72
48
72
Doxycycline:
- + - + - + - +
IGF2BP1 –
cIAP1 –
GAPDH -
E
TNFα: 0 1 10 100 1000 ng/ml
siRNA: C I C I C I C I C I
- IGF2BP1
- GAPDH
F
TNFα: 0 1 10 100 1000 ng/ml
SM: - + - + - + - + - +
- cIAP1
- GAPDH
G
siRNA: C TNFR1
- TNFR1
- GAPDH

Supplementary Figure 5.2: Polysome profiles and western blots from cell death assays. Representative cIAP1 **(A)** and RPL13A **(B)** polysomal mRNA distribution. Ribosomes-bound mRNAs from HEK293 cells transfected with non-targeting (siC) or IGF2BP1-targeting (siIGF2BP1) siRNA were separated on a 10-50% sucrose gradient, 10 fractions collected. cIAP1 and RPL13A abundance from these fractions was analysed by RT-qPCR. **(C)** Representative polysome profiles of HEK293 cells untreated (-EDTA control) or treated with 20 mM EDTA (+EDTA control). The +EDTA control shows a complete collapse of polysome peaks, indicating that they represented (in –EDTA control) true polyribosomes bound mRNAs fractions. **(D)** HEK293 inducibly expressing a scrambled (control) or IGF2BP1 shRNA were treated (+) or (-) with 1 μg/ml of Doxycycline for 48h and 72h to assess the extent of IGF2BP1 knock-down. IGF2BP1, cIAP1 and GAPDH protein expression was analysed by western blot. **(E)** Protein lysates from RH36 cells treated with non-targeting (C) or IGF2BP1 (I) siRNA for 72h were harvested after a cell viability assay and analysed by western blot for IGF2BP1 and GAPDH expression. (**F)** Protein lysates from RH36 cells treated with 0.1% DMSO (-SM) or the SMC AEG407030 treated (+SM) for 48h were harvested after a cell viability assay and analysed for cIAP1 and GAPDH expression. (**G)** Western blot demonstrating TNFR1 knock-down in Kym-1 cells that were transfected with non-targeting (C) or TNFR1-targeting siRNA (TNFR1) for 96h.

antagonize XIAP inhibition of caspases through the binding to the BIR2 domain.[33-35] We observed that cIAP1 depletion from RH36 cells by the dimeric SMC AEG40730 ([36], Suppl. Figure 5.2F) greatly reduced RH36 viability in the presence of TNFα (Figure 5 5A,C,D) and increased caspases-3/7 activity by 3-fold compared to vehicle treated cells (Figure 5.5E, F). Importantly, SMC treatment alone did not affect the viability of RH36 cells (Figure 5.5B-F). We also found that AEG40730 sensitizes RH36 cells to TRAIL-mediated cell death (Suppl. Figure 5.3), as was previously reported.[3, 37, 38] Importantly, AEG40730 sensitisation to TNFα and TRAIL mediated cell death was also observed in another RMS cell line, RH41 (Suppl. Figure 5.4).

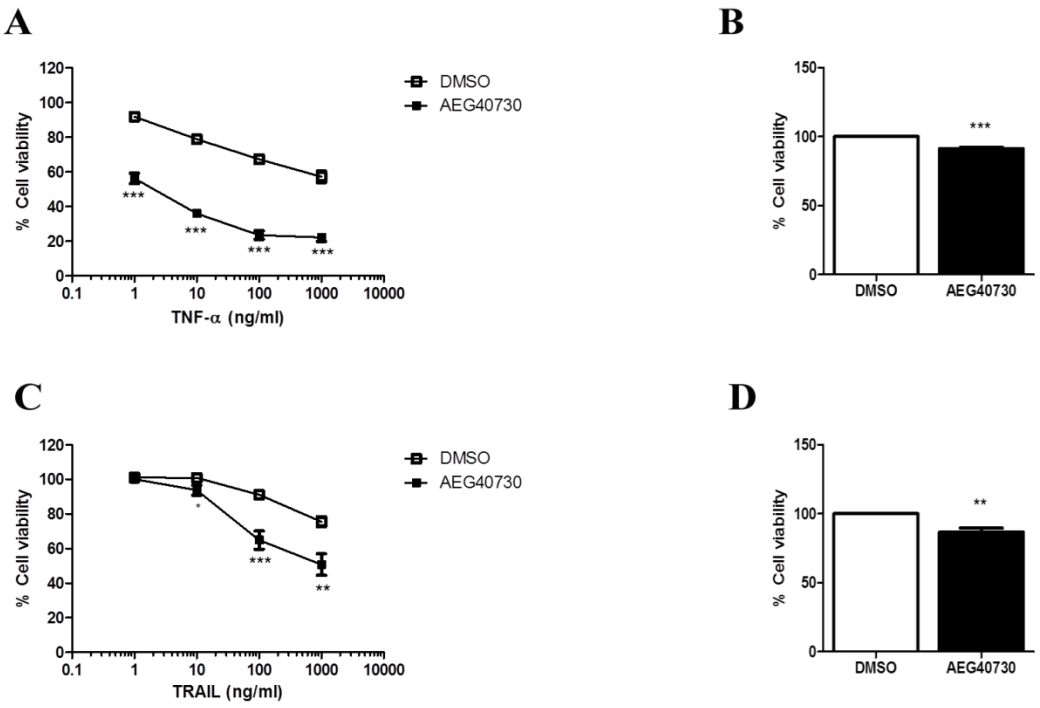
Because SMC can also target cIAP2 for degradation and inhibit XIAP activity, we examined their contribution to the sensitisation of RH36 cells to TNFα cIAP2 is not expressed in RH36 cells (Suppl. Figure 5.1A) and XIAP knock-down did not significantly sensitise RH36 to TNFα-mediated cytotoxicity compared to untreated cells, in stark contrast to cIAP1 knock-down (Suppl. Figure 5.5A,B). Furthermore, we did not observe any synergistic effect of AEG40730 with doxorubicin or etoposide on the viability of RH36 and RH41 cells (Suppl. Figure 5.5C-F), suggesting that SMC sensitization of RMS cells is primarily executed *via* cIAP1 and the extrinsic cell death pathway. Taken together, these results suggest that SMCs could be used in combination with TNFα as a therapeutic approach to trigger the death of RMS cancer cells.

As a proof of principle, we next decided to test this approach in the human RMS cell line Kym-1 that has autocrine TNFα production[39] and therefore should be sensitive to SMC treatment alone. Indeed, AEG40730 treatment of Kym-1 cells induced significant cytotoxicity (10 fold) and caspase activation (200 fold) when compared to a vehicle control


A
120
100
80
60
40
20
0
% Cell viability
0.1
1
10
100
1000
10000
TRAIL (ng/ml)
DMSO
AEG40730
*
*
B
150
100
50
0
% Cell viability
ns
DMSO
AEG40730

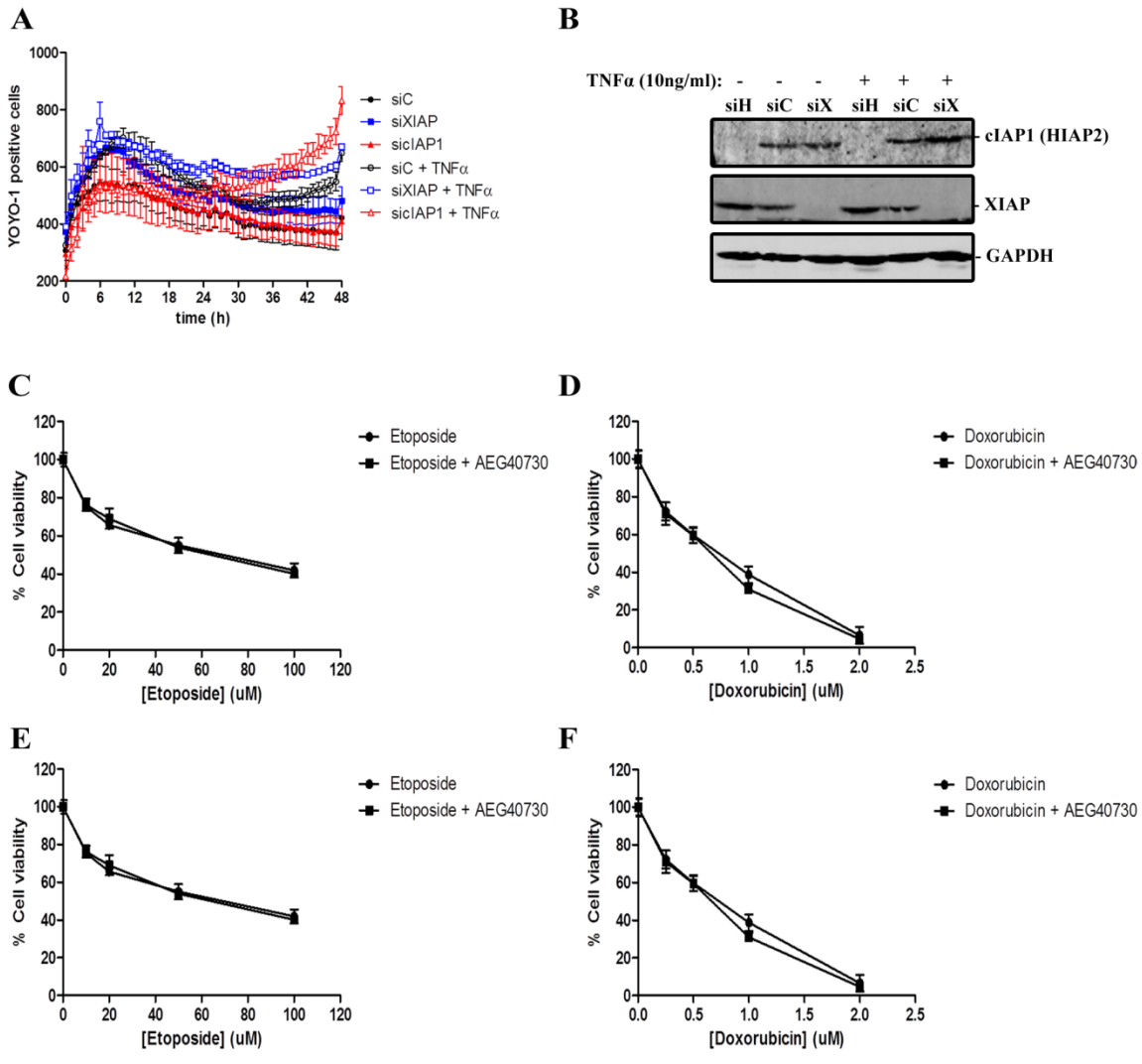
Supplementary Figure 5.3: RH36 cell viability assay in the presence of Smac mimetic AEG40730 and TRAIL

RH36 cells were treated with 0.1% DMSO or 100 nM of the smac mimetic compound AEG40730 in the presence (**A**) or absence (**B**) of increasing concentrations of TRAIL and cell viability determined by Alamar Blue assay


A
B
C
D
DMSO
AEG40730
% Cell viability
TNF-α (ng/ml)
TRAIL (ng/ml)
0.1
1
10
100
1000
10000
0
20
40
60
80
100
120
50
150
***
**
*
DMSO
AEG40730

Supplementary Figure 5.4: RH41 cell viability assays in the presence of Smac mimetic AEG40730 and TNFα or TRAIL

RH41 cells were treated with 0.1% DMSO or 100 nM of the smac mimetic compound AEG40730 in the presence **(A)** or absence **(B)** of increasing concentrations of recombinant human TNFα and cell viability determined by Alamar Blue assay The same experiment was performed in the presence **(C)** or absence **(D)** of increasing concentrations of TRAIL.


A
YOYO-1 positive cells
time (h)
siC
siXIAP
sicIAP1
siC + TNFα
siXIAP + TNFα
sicIAP1 + TNFα
B
TNFα (10ng/ml): - - - + + +
siH siC siX siH siC siX
cIAP1 (HIAP2)
XIAP
GAPDH
C
% Cell viability
[Etoposide] (uM)
Etoposide
Etoposide + AEG40730
D
% Cell viability
[Doxorubicin] (uM)
Doxorubicin
Doxorubicin + AEG40730
E
% Cell viability
[Etoposide] (uM)
Etoposide
Etoposide + AEG40730
F
% Cell viability
[Doxorubicin] (uM)
Doxorubicin
Doxorubicin + AEG40730

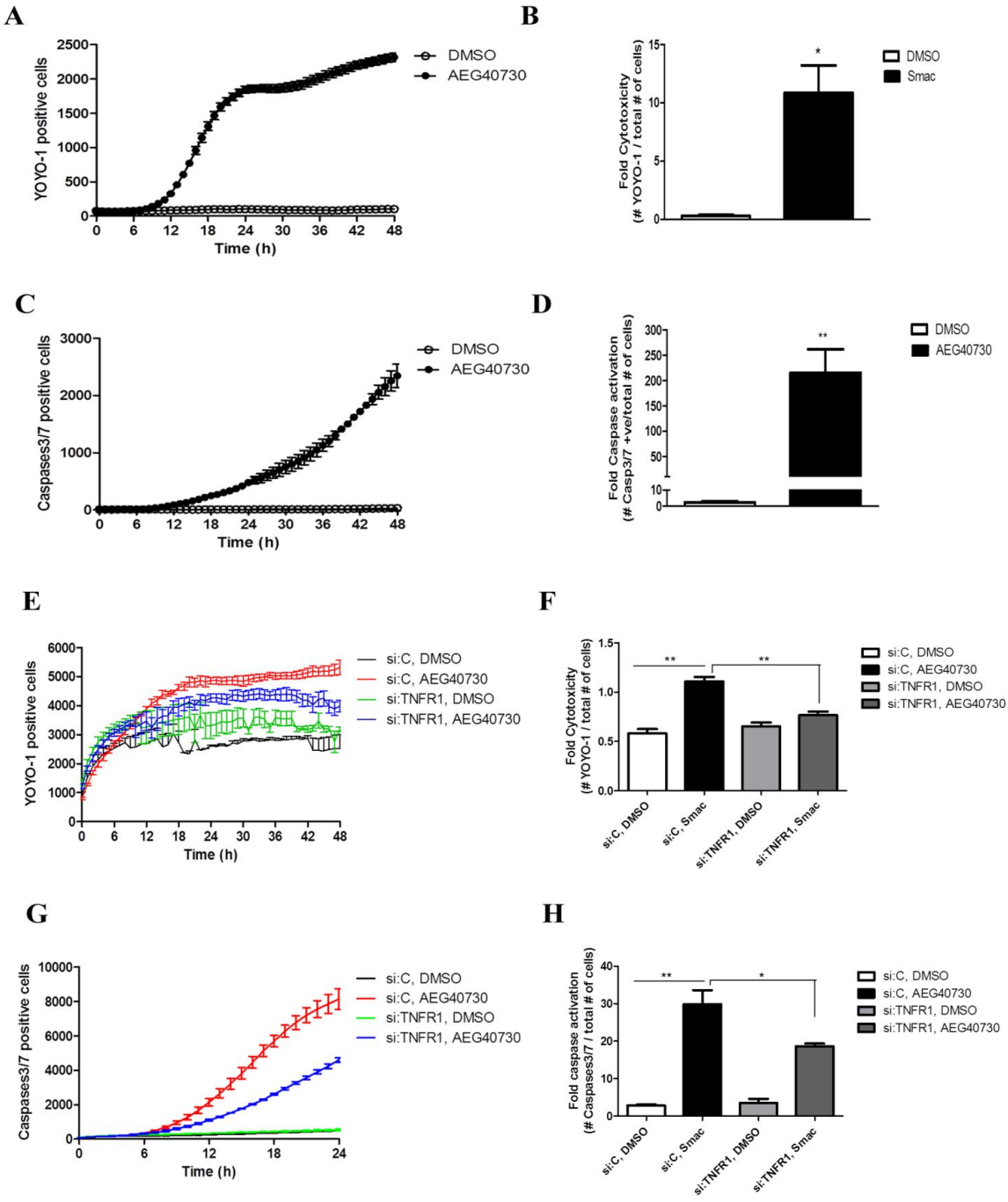
Supplementary Figure 5.5: XIAP depletion by siRNA or SM AEG40730 does not sensitize RMS cells to TNFα, Etoposide or Doxorubicin mediated cell death

(A) RH36 cells were transfected with 30 nM of non-targeting (siC), XIAP (siXIAP) or cIAP1 (sicIAP1) siRNA for 48h and treated or not with 10 ng/ml of TNFα for an additional 48h. Cell cytotoxicity was monitored over those 48h by YOYO-1 dye incorporation. **(B)** Western blot showing the extent of cIAP1 and XIAP knock-down in RH36 cells transfected with 30 nM of non-targeting (siC), XIAP (siX) or cIAP1 (siH) for a total of 96h (**A**) compared to control siRNA treated cells. **(C)** RH36 cells were treated with 0.1% DMSO or 100 nM of the SMC AEG40730 for 48h in the presence of increasing concentrations of etoposide and cell viability determined by Alamar Blue assay. **(D)** RH36 cells were treated with 0.1% DMSO or 100 nM of the SMC AEG40730 for 48h in the presence of increasing concentrations of Doxorubicin and cell viability determined by Alamar Blue assay. **(E)** RH41 cells were treated as in **(C)** and cell viability determined. **(F)** RH41 cells were treated as in **(D)** and cell viability determined.

(Suppl. Figure 5.6A-D). Furthermore, TNFR-1 knock-down (Suppl. Figure 5.2G) reduced cell death and caspase activation of Kym-1 cells in the presence of AEG40730, as compared to non-targeting siRNA-transfected cells (Suppl. Figure 5.6E-H). Similar data was obtained using the monomer SMC LCL161 that has better bioavailability *in vivo* (Suppl. Figure 5.7). These results demonstrate that cIAP1 depletion by SMCs sensitizes Kym-1 cells to cell death in a TNFR-1 dependent manner.

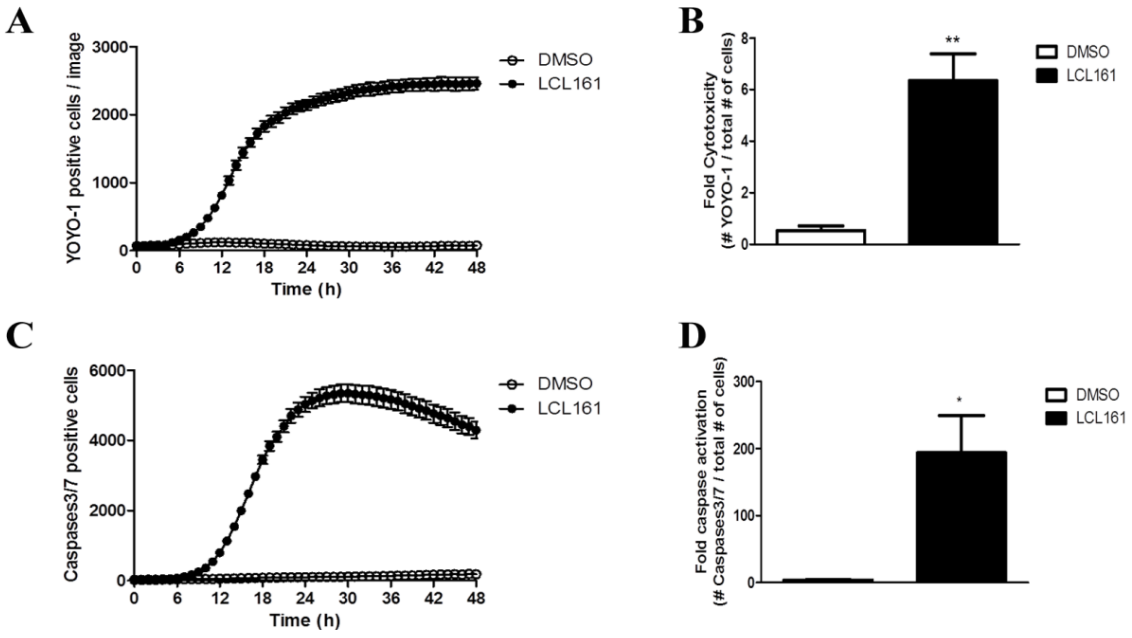
5.5.6 SMC treatment inhibits the growth of Kym-1 rhabdomyosarcoma xenograft tumours.

To test the efficacy of combined TNFα and SMC in a tumour model *in vivo*, we treated CD-1 female nude mice bearing established Kym-1 xenograft tumours with LCL161, or a vehicle control, twice a week for two weeks. LCL161 treatment significantly reduced the growth of Kym-1 tumours compared to vehicle-treated animals (Figure 5.6A). Concomitantly, the mean survival of LCL161-treated Kym-1 xenograft mice was significantly extended by 32 days compared to vehicle-treated mice (Figure 5.6B, $p = 0.002$). In a parallel experiment we implanted Kym-1 cells and initiated SMC treatment the following day. We observed an even more striking effect of LCL161 on tumour growth and survival; LCL161-treated mice did not develop detectable tumours by 120 days post-implantation whereas vehicle-treated animals developed sizable tumours within 35 days (Figure 5.6C). Consequently, this treatment strategy lead to the durable cure of LCL161-treated Kym-1 xenograft mice (Figure 5.6D, $p = 0.002$). Western blot analysis of excised tumours from Kym-1 xenograft treated with LCL161 for 24h confirmed cIAP1 depletion and an increase in cleaved PARP compared to vehicle-treated mice (Suppl. Figure 5.8),


A
YOYO-1 positive cells
Time (h)
DMSO
AEG40730
B
Fold Cytotoxicity
(# YOYO-1 / total # of cells)
DMSO
Smac
C
Caspases3/7 positive cells
Time (h)
DMSO
AEG40730
D
Fold Caspase activation
(# Casp3/7 +ve/total # of cells)
DMSO
AEG40730
E
YOYO-1 positive cells
Time (h)
si:C, DMSO
si:C, AEG40730
si:TNFR1, DMSO
si:TNFR1, AEG40730
F
Fold Cytotoxicity
(# YOYO-1 / total # of cells)
si:C, DMSO
si:C, Smac
si:TNFR1, DMSO
si:TNFR1, Smac
si:C, DMSO
si:C, AEG40730
si:TNFR1, DMSO
si:TNFR1, AEG40730
G
Caspases3/7 positive cells
Time (h)
si:C, DMSO
si:C, AEG40730
si:TNFR1, DMSO
si:TNFR1, AEG40730
H
Fold caspase activation
(# Caspases3/7 / total # of cells)
si:C, DMSO
si:C, Smac
si:TNFR1, DMSO
si:TNFR1, Smac
si:C, DMSO
si:C, AEG40730
si:TNFR1, DMSO
si:TNFR1, AEG40730

Supplementary Figure 5.6: AEG40730 sensitizes Kym-1 rhabdomyosarcoma cells to apoptosis in a TNFR1-dependent manner.

(A) 5,000 Kym-1 cells were treated with 0.1% DMSO or 100 nM of the SMC AEG40730 and assayed for cell cytotoxicity by YOYO-1 dye incorporation, using the INCUCYTE^{TM} Live-Cell Imaging System. The number of YOYO-1 positive cells over a period of 48h is shown. **(B)** Fold cytotoxicity from **(A)** is expressed as the number of YOYO-1 positive cells divided by the total number of cells at 48h, compared to time = 2h. (**C)** Kym-1 cells were treated as in (**A)** and the number of caspase-3/7 positive cells was monitored over 48h. (**D)** Fold caspase-3/7 activity for **(C)** at 48h is expressed as the number of caspase-3/7 positive cells divided by the total number of cells at 48h, compared to time = 2h. **(E)** 5,000 Kym-1 cells were transfected with 10 nM of non-targeting (siC) or TNFR1-targeting siRNA (siTNFR1) for 48h. Cells were then treated with 0.1% DMSO or 100 nM of the SMC AEG40730 and YOYO-1 dye incorporation was monitored over 48h. **(F)** Fold cytotoxicity from **(E)** at 48h is calculated as described in **(B)**. (**G)** Kym-1 cells were treated as in **(E)** and the number of caspase-3/7 positive cells monitored over 24h. (**H)** Fold caspase-3/7 activity for **(C)** at 24h is calculated as described in **(D)**. Mean ±SEM. Student's t-test: ns, non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.


A
YOYO-1 positive cells / image
3000
2000
1000
0
0 6 12 18 24 30 36 42 48
Time (h)
DMSO
LCL161
B
Fold Cytotoxicity
(# YOYO-1 / total # of cells)
8
6
4
2
0
**
DMSO
LCL161
C
Caspases3/7 positive cells
6000
4000
2000
0
0 6 12 18 24 30 36 42 48
Time (h)
DMSO
LCL161
D
Fold caspase activation
(# Caspases3/7 / total # of cells)
300
200
100
0
*
DMSO
LCL161

Supplementary Figure 5.7: LCL161 sensitizes Kym-1 rhabdomyosarcoma cells to cell death.

(A) 5,000 Kym-1 cells were treated with 0.1% DMSO or 100 nM of the SMC LCL161 and assayed for cell cytotoxicity by YOYO-1 dye incorporation, using the INCUCYTE^{TM} Live-Cell Imaging System. The number of YOYO-1 positive cells over a period of 48h is shown (**B)** Fold cytotoxicity from **(A)** is expressed as the number of YOYO-1 positive cells divided by the total number of cells at 48h, compared to time = 2h. (**C)** Kym-1 cells were treated as in **(A)** and caspase-3/7 positive cells monitored over 48h. (**D)** Fold caspase-3/7 activity for (**C)** is expressed as the number of caspase-3/7 positive cells divided by the total number of cells at 48h, compared to time = 2h. Mean ±SEM. Student's t-test: ns, non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

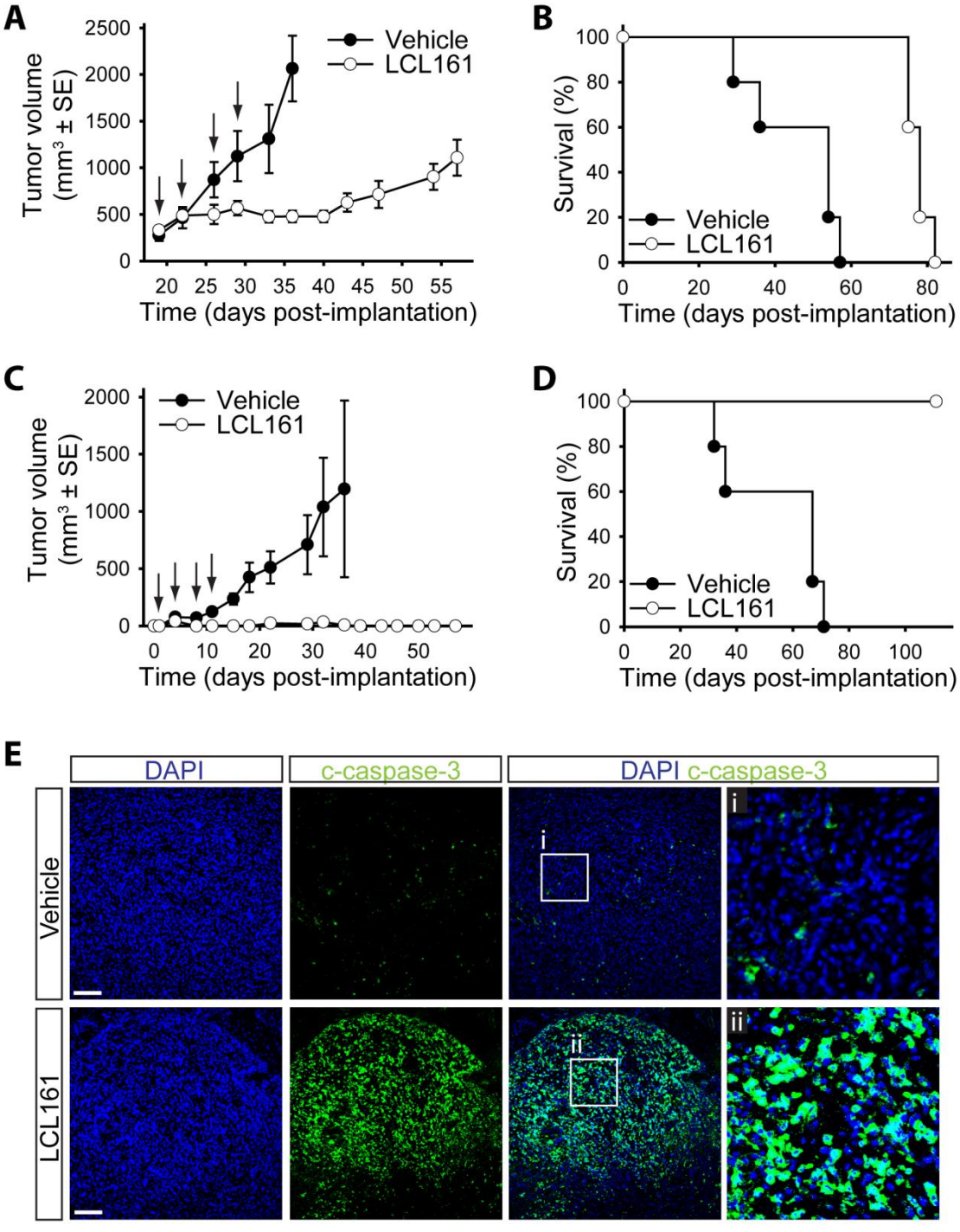
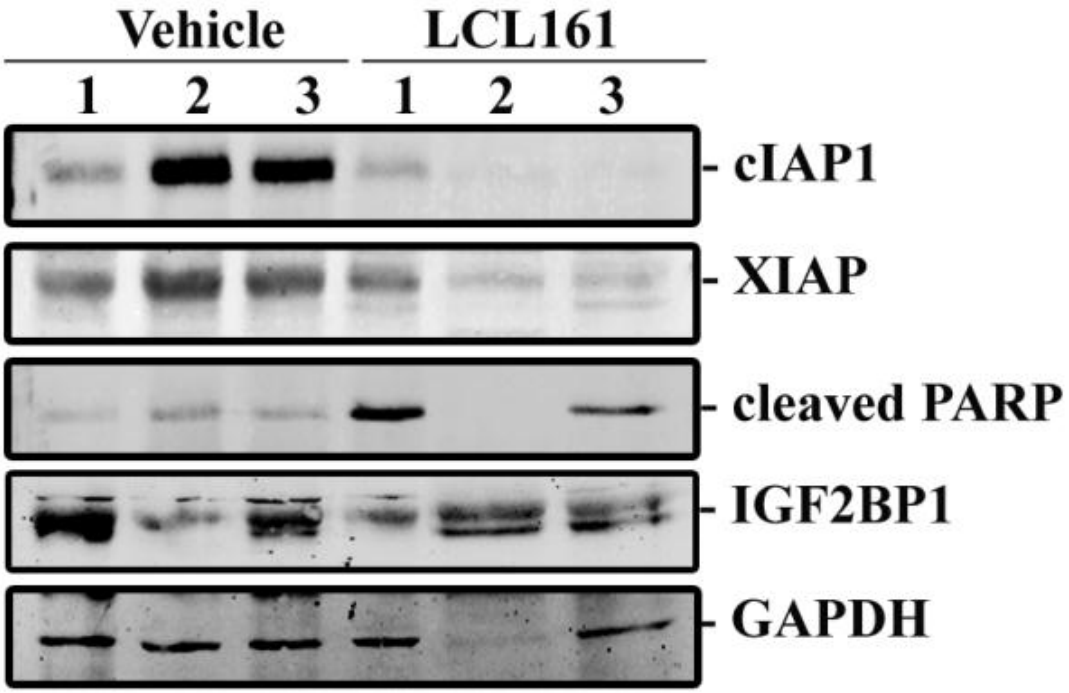

A
Tumor volume (mm³ ± SE)
2500
2000
1500
1000
500
0
20 25 30 35 40 45 50 55
Time (days post-implantation)
Vehicle
LCL161
B
Survival (%)
100
80
60
40
20
0
0 20 40 60 80
Time (days post-implantation)
Vehicle
LCL161
C
Tumor volume (mm³ ± SE)
2000
1500
1000
500
0
0 10 20 30 40 50
Time (days post-implantation)
Vehicle
LCL161
D
Survival (%)
100
80
60
40
20
0
0 20 40 60 80 100
Time (days post-implantation)
Vehicle
LCL161
E
DAPI
c-caspase-3
DAPI c-caspase-3
Vehicle
LCL161
i
ii

Figure 5.6: Smac mimetic compound treatment inhibits the growth of Kym-1 rhabdomyosarcoma xenograft tumours.

(A) Female CD-1 nude mice with established Kym-1 xenograft tumours were treated with vehicle or 50 mg/kg LCL161 and tumour volumes monitored. Arrows denote treatments. **(B)** Survival of the mice was monitored over time. Mean survival days were of 46 +/- 5.648 (SE) for vehicle-treated mice and 77.69+/- 1.288 (SE) for LCL161-treated ones. $p = 0.002$. **(C)** In a parallel experiment, vehicle or LCL161 treatments started one day post-implantation and tumour volumes monitored. **(D)** Survival of mice described in **(C)** was monitored over time. Mean survival days were of 54.6 +/- 8.465 (SE) for vehicle-treated mice and were still ongoing for LCL161-treated ones. Results are representative of 5 mice for each condition. (**E)** Cleaved-caspase-3 immunohistochemistry of Kym-1 xenografts treated with vehicle (inset i) or 50 mg/kg LCL161 (inset ii) for 24h.


Vehicle
LCL161
1
2
3
1
2
3
cIAP1
XIAP
cleaved PARP
IGF2BP1
GAPDH

Supplementary Figure 5.8: cIAP1 depletion by LCL161 in Kym-1 xenograft tumors after 24h of treatment

Western blot analysis of Kym-1 xenograft tumor extracts after 24h of vehicle or LCL161 treatment showing efficient depletion of cIAP1 in LCL161-treated tumors, increased PARP cleavage and no change in IGF2BP1 protein levels.

indicating that these tumour cells have undergone cell death. Immunohistochemistry analysis of these tumours also revealed a significant increase in cleaved-caspase-3 in LCL161-treated mice compared to vehicle (Figure 5.6E). IGF2BP1 protein levels were not affected by LCL161 treatment (Suppl. Figure 5.8).

Taken together, these results show that cIAP1 de5.pletion by LCL161 inhibits the growth of Kym-1 tumours by sensitizing them to TNFα-induced cell death, thus significantly increasing the survival of Kym-1 tumour-bearing mice. These results attest to the potential of using SMCs for the treatment of rhabdomyosarcoma tumours.

5.6 Discussion

cIAP1 is a key regulator of apoptosis and cancer cell survival by controlling the NF-κB signaling and extrinsic cell death pathways.[5] In an attempt to better understand the mechanism of translational regulation of cIAP1 expression, we previously conducted RNA chromatography on the cIAP1 IRES and identified four potential ITAFs binding specifically to it, including IGF2BP1.

Here, we found that IGF2BP1 is overexpressed in a panel of human RMS cell lines and in human primary RMS tumours when compared to skeletal muscle or myoblasts (Figure 1A-D, Suppl. Figure 1A-D). These findings are consistent with classification of IGF2BP1 as an oncofetal protein that is re-expressed in a variety of cancers (reviewed in[13, 22, 40]). Interestingly, IGF2BP1 mRNA levels are elevated in all RMS cell lines analysed compared to HSMM (Suppl. Figure 5.1E) and may explain in part the observed increase in protein. Currently, IGF2BP1 expression regulation is not well understood,[22] hence it would be interesting to investigate its expression pattern during human embryogenesis and carcinogenesis. cIAP1 showed the same pattern of expression as IGF2BP1 (Figure 5.1A and

B), although cIAP1 mRNA levels were significantly decreased in RMS cell lines compared to HSMM (Suppl. Figure 5.1E). This observation, along with the fact that IGF2BP1 was identified as a cIAP1 IRES binding protein led us to hypothesize that IGF2BP1 regulates cIAP1 IRES-mediated translation. Indeed, we confirmed that IGF2BP1 is a *bona fide* ITAF that drives cIAP1 protein expression by enhancing cIAP1 translation specifically through binding and modulation of its IRES activity (Figure 5.1-5.3). The mechanisms by which ITAFs modulate IRES-mediated translation are still not fully understood; it is possible that IGF2BP1 interacts with other known cIAP1 ITAFs, such as NF45[8, 41] and p97.[6, 42] Interestingly, IGF2BP1 was shown to enhance HCV IRES-mediated translation via the 3'UTR[17] and recent reports point to a role of IGF2BP1/2 in mediating IGF2 IRES-translation upon mTOR activation.[19, 43] IGF2BP1 is thus emerging as a critical ITAF that regulates the synthesis of viral and cellular proteins.

IGF2BP1 has also been emerging as an important oncogenic factor;[22] however, its role in facilitating the apoptotic resistance of cancer cells has not been previously determined. We found that reducing IGF2BP1 levels sensitizes RMS cells to TNFα-mediated cell death in a cIAP1-dependent way (Figure 5.4). Given the importance of cIAP1 in sensitizing RMS cells to TNFα-induced cell death, we used a class of pharmacologics that target the IAPs, smac mimetics.[44] SMCs have been shown to be effective in the treatment of several cancers and are currently in phase I and II clinical trials (reviewed in [5, 45]). As expected, SMCs sensitized RH36 and RH41 RMS cells to cell death, in the presence of TNFα or TRAIL, similar to IGF2BP1 downregulation (Figure 5.5, Suppl. Figures 5.3,5.4). We also observed the same sensitisation of Kym-1 cells to cell death in the presence of the SMCs AEG40730 and LCL161 without the addition of exogenous TNFα, in a TNFR1-

dependent manner (Suppl. Figures 5.6 and 5.7). Indeed, Kym-1 cells were previously shown to have autocrine TNFα production and to mediate cell death *via* the TNFR1 receptor.[39, 46] Importantly, our findings are corroborated by a recent paper showing that SMCs synergize with Lexatumumab (a TRAIL receptor2 agonist antibody) to induce cell death in a variety of RMS cells in a RIP1-dependent manner.[37]

Finally, we tested the synergistic effects of SMC and TNFα on the growth of rhabdomyosarcoma tumours *in vivo*. LCL161 significantly decreased the growth of established Kym-1 xenograft tumours and consequently extended the mean survival of mice by 32 days compared to vehicle-treated mice (Figure 5.6B). Importantly, LCL161 treatment was able to prevent the onset of tumourigenesis when SMC treatment commenced one day post-implantation tumour (Figure 5.6C). Taken together, these results attest to the efficacy of SMC in reducing Kym-1 tumour burden and to the potential of using this therapeutic approach for the treatment of rabdomyosarcoma, especially if the tumours are detected early. Our observations are of relevance since a recent report on the initial testing of LCL161 by the Pediatric Preclinical Testing Program did not find any significant delay in the growth of several rhabdomyosarcoma xenograft tumours when used as a single agent.[47] Although therapies involving TNFα or TRAIL [3] may not be a viable option because of these agents toxicity, our results suggest that therapeutic strategies that target IAPs in combination with other TNFR signaling pathway-inducing agents may be effective in the treatment of rhabdomyosarcomas, as we have recently shown with oncolytic viruses.[48]

In summary, we identified IGF2BP1 as a critical regulator of cIAP1 expression and cell death resistance in RMS. Our results not only strongly argue for combined use of SMCs and TNFR stimulating agents as a potential therapeutic approach for rhabdomyosarcomas,

they also hint at the potential use of IGF2BP1 as a biomarker to identify which tumours would be responsive to such a combinatorial therapy.

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

5.8 Acknowledgements

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CHAPTER 6

Modulators of innate immunity synergize with Smac mimetic compounds to induce rhabdomyosarcoma cancer cell death

6.1 Preamble

This chapter contains unpublished data that provides a rationale for combining cIAP1 protein depletion through the use of SMCs and induction of TNFα expression by modulators of innate immunity for the treatment of rhabdomyosarcoma cancer. Indeed, in the previous chapter, I have identified IGF2BP1 and cIAP1 as important regulators of TNFα- or TRAIL-mediated RMS cell death and shown that SMCs (or IAP antagonists) greatly sensitized RMS cancer cells to these apoptotic agents. However, given the cytotoxicity associated with recombinant cytokine therapy, combined use of TNFα and SMCs may not be a viable treatment option for RMS cancers. Hence this chapter explores the possibility of using modulators of innate immunity such as non-pathogenic viruses, poly-IC, CpG or interferons (already used in the clinic), as a source of TNFα or TRAIL production in the tumour milieu, and how they can synergize with SMCs to induce RMS cancer cell death.

Author list

Mame Daro Faye and Martin Holcik

Author contributions

MDF and MH designed the experiments. MDF performed the experiments and wrote the manuscript. MH contributed ideas and editorial support as well.

6.2 Abstract

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma arising in children. Despite important advances in the treatment of RMS cancers, patients with progressive or recurrent disease have poor prognosis, hence the need for better therapeutics. We have previously reported that the cellular inhibitor of apoptosis 1 (cIAP1) is overexpressed in human primary RMS tumours as well as patient-derived RMS cancer cell lines, and is responsible for the apoptotic resistance of these cells. Importantly, depletion of cIAP1 by the use of Smac mimetic compounds greatly sensitizes RMS cancer cells to TNFα-mediated apoptosis. In this study, we report that modulators of innate immunity such as interferon γ and the tumour necrosis factor-like weak inducer of apoptosis, synergize with the Smac mimetic compound LCL161 to induce apoptosis of Kym-1 RMS cancer cells and that this synergy is dependent on induction of TNFα production by these modulators of innate immunity

6.3 Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma arising in children. RMS cancers are classified in two goups, alveolar (aRMS) and embryonal (eRMS), based on histologic features, primary tumour sites and genetic alterations.[1] RMS cancers are generally thought to be of a myogenic origin as they typically arise in muscle tissues and display characteristics of undifferentiated myoblasts.[2] However, RMS can also arise in non-muscle tissues, such as the biliary tract, and some evidence point to a mesenchymal origin

and induce RMS cancer cell death. We report here that modulators of innate immunity such as VSVΔ51, Interferon γ (IFNγ) and the tumour necrosis factor-like weak inducer of apoptosis (TWEAK) synergize with the SMC LCL161 to induce apoptosis of Kym-1 RMS cancer cells and that this synergy is dependent on induction of TNFα production by these modulators of innate immunity.

6.4 Material and Methods

6.4.1 Cell culture, reagents, and transfections.

Human RMS cell lines (RH18, RH30, RH36, RH41 and RD) were a generous gift from Dr. P. Houghton (Department of Hematology-Oncology, St. Jude Children's Research Hospital, Memphis, TN) and were cultured in RPMI 1640 complete media (i.e supplemented with 10% fetal calf serum FCS, penicillin/streptomycin and L-glutamine). The human RMS cell line Kym-1 was purchased from the JCRB (Japan) and cultured in DMEM-F12 complete media. The Smac mimetic reagent LCL161 was provided by Novartis[24-26]. Recombinant TNFα and TRAIL were purchased from Enzo Life Sciences (Brockville, ON). Human recombinant IFN-α, IFN-β and IFN-γ, as well as mouse universal type I interferons were purchased from PBL Interferonsource (Oiscataway, NJ). Mouse recombinant IFN-γ was purchased from R&D Systems (Minneapolis, MN). Human recombinant soluble TWEAK (sTWEAK), fusion TWEAK (fTWEAK) and mouse fusion TWEAK (mfTWEAK) were a gift from Dr Linda Burkly from Biogen idec (Cambridge, MA). Poly(I:C) was purchased from Life Technologies (Burlington, ON) and LPS was from Sigma-Aldrich (Oakville, ON). CpG-ODN 2216 (5'-gggGGACGATCGTCgggggg-3', referred to as CpG) was synthesized

patients[14, 15]; therefore strategies aiming at increasing TNFα production in the tumour microenvironement are of great clinical relevance.

In recent years, the concept of cancer immunotherapy, which involves stimulation of the host immune responses to eliminate cancer cells recognised as 'non-self' entities, has been gaining a lot of ground and shown promising therapeutic potential.[16] However, some cancer cells can escape immune surveillance, develop more genetic lesions and become resistant to immunotherapy.[17] It has been proposed that combining immunotherapy with targeted therapy, for instance through the use of SMCs, may be even more effective in eliminating cancer cells and improving clinical outcomes.[18] Indeed, other than their well-known functions in inhibiting apoptosis, IAPs and in particular cIAP1 and 2, play key roles in modulating the immune system through their regulation of the NF-κB and MAPK signaling pathways (reviewed in [19, 20]). Inhibition of IAPs through the use of SMCs was shown to greatly enhance T cell activity upon T cell receptor engagement, and combination of SMCs with tumour cell vaccines resulted in greater inhibition of melanoma tumour growth compared to either monotherapies.[21] Furthermore, it was recently reported that stimulation of the innate immune system through the use of oncolytic viruses, poly(I:C) or CpG leads to TNFα or TRAIL production through activation of type-I interferons, and synergizes with SMCs to efficiently induce cell death and tumour regression in syngeneic glioblastoma and mammary cancer models.[22] Activation of the innate immune system, the body's first line of defense against foreign pathogens, leads to the production of pro-inflammatory cytokines such as TNFα which in turn regulate the inflammatory response.[23] Hence, in this study we looked at means of inducing TNFα production through the use of different modulators of innate immunity, in order to potentiate synergy with SMCs treatment

and induce RMS cancer cell death. We report here that modulators of innate immunity such as VSVΔ51, Interferon γ (IFNγ) and the tumour necrosis factor-like weak inducer of apoptosis (TWEAK) synergize with the SMC LCL161 to induce apoptosis of Kym-1 RMS cancer cells and that this synergy is dependent on induction of TNFα production by these modulators of innate immunity.

6.4 Material and Methods

6.4.1 Cell culture, reagents, and transfections.

Human RMS cell lines (RH18, RH30, RH36, RH41 and RD) were a generous gift from Dr. P. Houghton (Department of Hematology-Oncology, St. Jude Children's Research Hospital, Memphis, TN) and were cultured in RPMI 1640 complete media (i.e supplemented with 10% fetal calf serum FCS, penicillin/streptomycin and L-glutamine). The human RMS cell line Kym-1 was purchased from the JCRB (Japan) and cultured in DMEM-F12 complete media. The Smac mimetic reagent LCL161 was provided by Novartis[24-26]. Recombinant TNFα and TRAIL were purchased from Enzo Life Sciences (Brockville, ON). Human recombinant IFN-α, IFN-β and IFN-γ, as well as mouse universal type I interferons were purchased from PBL Interferonsource (Oiscataway, NJ). Mouse recombinant IFN-γ was purchased from R&D Systems (Minneapolis, MN). Human recombinant soluble TWEAK (sTWEAK), fusion TWEAK (fTWEAK) and mouse fusion TWEAK (mfTWEAK) were a gift from Dr Linda Burkly from Biogen idec (Cambridge, MA). Poly(I:C) was purchased from Life Technologies (Burlington, ON) and LPS was from Sigma-Aldrich (Oakville, ON). CpG-ODN 2216 (5'-gggGGACGATCGTCgggggg-3', referred to as CpG) was synthesized

by IDT (Kanata, ON). TNF receptor 1 (TNFR1) siRNA (Cat# J-005197-05) and control scrambled siRNA (Cat# RHS4827) were purchased from Thermo Scientific (Waltham, MA) and transient siRNA transfections were performed using Lipofectamine RNAiMax reagent (Life Technologies, Burlington, ON) as previously described.[5]

6.4.2 Western blot analysis.

Western blots were performed as previously described.[27] Membranes were probed with antibodies against cIAP1 (RIAP1 [28]), TNFR1 (3736), TNFR2 (3727), DR5 (3696), PARP (9541), caspase-3 (9661), caspase-8 (9746), caspase-9 (9508) from Cell Signaling Technologies (Danvers, MA); DR4 (06-744) from Upstate Biotechnology Inc. (Lake Placid, NY); IFNR1 (EP899) from Abcam (Toronto, ON); TLR4; IFNγRα (sc-700); FN14 (sc-56250) from Santa Cruz (Dallas, Texas); c-FLIP (ADI-AAP-440) from Enzo LIFE Sciences (Farmingdale, NY) and GAPDH (6C5) from Advanced Immunochemical Inc. (Cedarlane, Burlington, ON).

6.4.3 Quantitative RT-PCR analysis.

qRT-PCR was performed as previously described[27] using commercial primers from Realtimeprimers.com or Qiagen (See Table 1). Briefly, RNA was extracted from treated cells using RNAzol (Sigma-Aldrich, Oakville, ON) and 1 ug of total RNA used to make cDNA, using qScript cDNA Supermix (Quanta Biosciences, Gaithersburd, MD). 0.5 or 1 µl of cDNA was then used to perform quantitative real time PCR using SSoAdvanced SYBR Green Supermix (Bio-rad, Mississauga, ON) for all mRNAs except for FN14 that was

quantified using the PerfeCTa SYBR Green Supermix (Quanta Biosciences, Gaithersburd, MD).

Table 1: List of qRT-PCR primers used in this study

mRNA target	**Catalog Number**	**Company**
TNFα	VHPS-9415	Realtimeprimers.com
IRF1	VHPS-4626	Realtimeprimers.com
GAPDH	VHPS-3541	Realtimeprimers.com
FN14 (TNFRSF12A)	QT00221179	Qiagen (QuantiTect primers)

6.4.4 Cytotoxicity and caspase activity assays.

5,000 cells were seeded in 96-well plates and the next day treated with LCL161, IFNα, IFNβ, IFNγ, poly(I:C), CpG, LPS, sTWEAK, fTWEAK or BSA (control) at the indicated concentrations, in the presence of 100 nM of YOYO-1 dye (Molecular probes, Burlington, ON). Cytotoxicity was monitored over 48h by YOYO-1 dye incorporation into the cells and green fluorescence imaging using the INCUCYTE^{TM} ZOOM Live-Cell Imaging System (Essen Bioscience, Ann Arbor, MI). Caspase 3 and 7 activities were assayed using 1 uM of CellPlayer^{TM} Caspase-3/7 reagent (Essen Bioscience) and monitored using the INCUCYTE^{TM} ZOOM. For TNFR1 knock-down experiments, 5,000 cells were seeded in 96-well plates and the next day transfected with 20 nM of TNFR1 or control siRNA for 48h using RNAiMax as described by the manufacturer. 48h later, cells were treated with IFNγ (250 U/ml) or sTWEAK (100 ng/ml) in the presence of LCL161 (10nM) or DMSO control, and cytotoxicity or Caspase-3/7 activity were measured as described above. Fold cytotoxicity or cell death activity were calculated as the number of green fluorescence positive cells divided by the total number of cells at endpoint, compared to control treatment.

6.4.5 VSVΔ51 cell viability and cytotoxicity assays

The Indiana serotype of VSVΔ51 was previously described and in these assays, we used a recombinant derivative of it expressing red fluorescent protein (RFP-VSVΔ51) that was previously cloned and characterised.[22] 20 000 cells were seeded in 96-well plates and the next day treated with serial dilutions (10^{2} to 10^{-6} MOI) of RFP-VSVΔ51 in the presence of LCL161 or DMSO control for 24h and cell viability assayed by Alamar Blue. Cytotoxicity

was also measured in parallel treatments by YOYO-1 dye incorporation using the INCUCYTE™ ZOOM Live-Cell Imaging System.

6.4.6 Mouse splenocytes conditioned media experiments.

Splenocytes were extracted as previously described by passing the spleen of Balb/c mice through a 70 μm nylon mesh and lysing red blood cells with ACK buffer.[22] 900,000 splenocytes were then seeded in 3 mL of RPMI-1640 complete media supplemented with 10 μM of β-mercaptoethanol and treated with IFNγ (1000 U/ml) or BSA (control, 80 pg/ml) or were left untreated. 24h later, the supernatants were collected, centrifuged at 500 xg for 3 min to get rid of splenocytes. Conditioned media was applied to pre-plated RMS cells (5,000 cells/well in 96-well plates) in 2-fold dilutions series in the presence or LCL161 or DMSO control and in the presence of YOYO-1 dye. Conditioned media induced cytotoxicity was then monitored by YOYO-1 dye incorporation in the cells using the INCUCYTE™ ZOOM Live-Cell Imaging System.

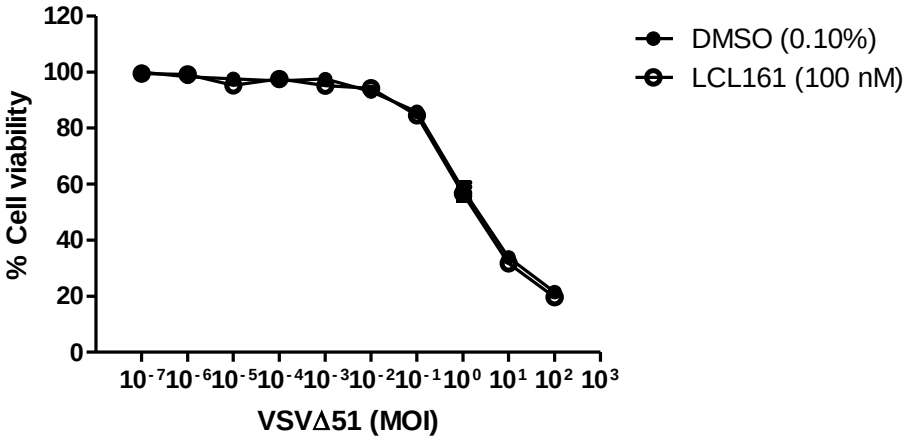
6.5 Results

6.5.1 RMS cells are sensitive to VSV-Δ51, but synergic cytotoxicity with LC161 is cell line specific.

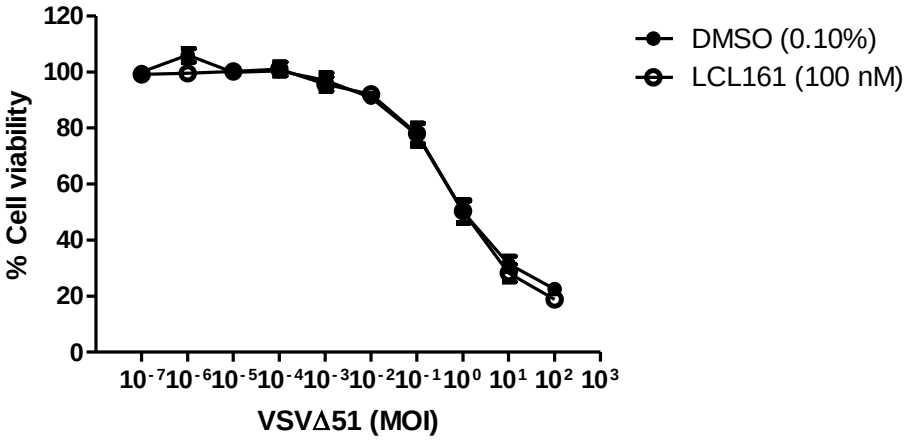
We have previously reported that human RMS cell lines RH36 (embryonic) and RH41 (alveolar) are highly resistant to TNFα or TRAIL induced cell death, and that depletion of cIAP1 by SMCs renders them highly sensitive to these death ligands.[5] In contrast, the embryonic human RMS cell line Kym-1, which has autocrine TNFα production,

is sensitive to SMC treatment alone.[5] It has been reported that VSVΔ51, an oncolytic rhabdovirus and a modulator of innate immunity can synergize with SMC to induce tumour cell death through induction of interferons and TNFα expression.[22] We therefore wished to test if RMS cells are also sensitive to VSVΔ51 and treated these three RMS cell lines with serial dilutions of RFP-VSVΔ51 in the presence or absence of LCL161 and assessed cell viability by Alamar blue assay. Interestingly, while all three cell lines were sensitive to RFP-VSVΔ51 alone, the synergy of RFP-VSVΔ51 with LCL161 was only observed in Kym-1 cells (Figure 6.1A-C). It was previously suggested that the synergy between VSVΔ51 and SMCs was occurs through a bystander effect; that is, the infection of tumour cells with the virus leads to production of TNFα, which in turn synergizes with SMC to induce cancer cells apoptosis.[22] Therefore, our results suggest that RFP-VSVΔ51 kills RH36 and RH41 cells by a lytic action, whereas in Kym cells, the cell death is likely occurring through both lytic action and a bystander effect.

A


RH36
% Cell viability
120
100
80
60
40
20
0
10-710-610-510-410-310-210-1100 101 102 103
VSVΔ51 (MOI)
DMSO (0.10%)
LCL161 (100 nM)

B


RH41
% Cell viability
120
100
80
60
40
20
0
10-710-610-510-410-310-210-1100 101 102 103
VSVΔ51 (MOI)
DMSO (0.10%)
LCL161 (100 nM)

C

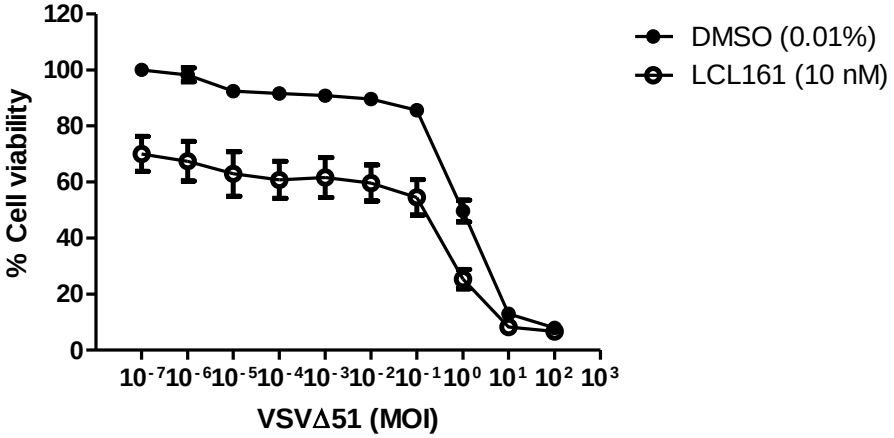

Kym1
% Cell viability
120
100
80
60
40
20
0
10-710-610-510-410-310-210-1100 101 102 103
VSVΔ51 (MOI)
DMSO (0.01%)
LCL161 (10 nM)

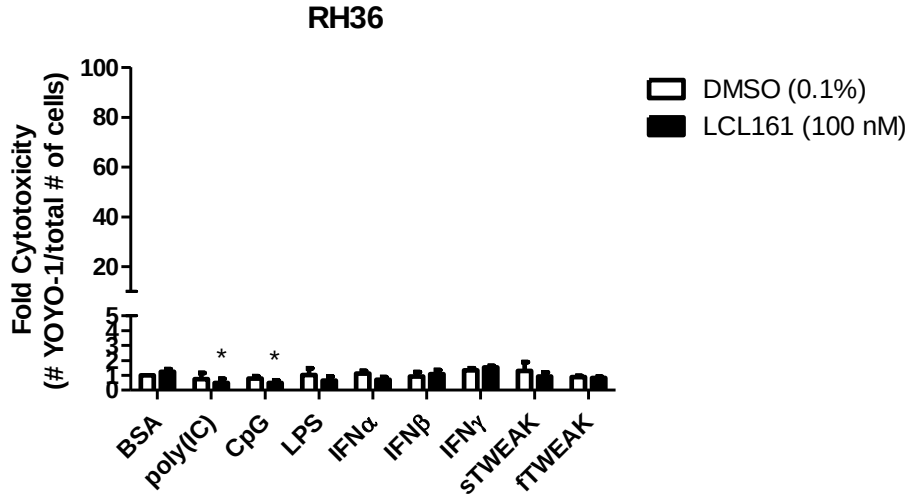
Figure 6.1: RMS cells are sensitive to VSV-Δ51 but synergic cytotoxicity with LC161 occurs only in Kym-1 cells

5,000 of RH36 **(A),** RH41 **(B)** and Kym-1 **(C)** cells were treated with 10-fold dilutions of RFP-VSVΔ51, in the presence of indicated concentrations of LCL161 or DMSO control. Cell viability was determined after 24h by Alamar Blue assay and normalised to PBS/DMSO control set at 100% viability.

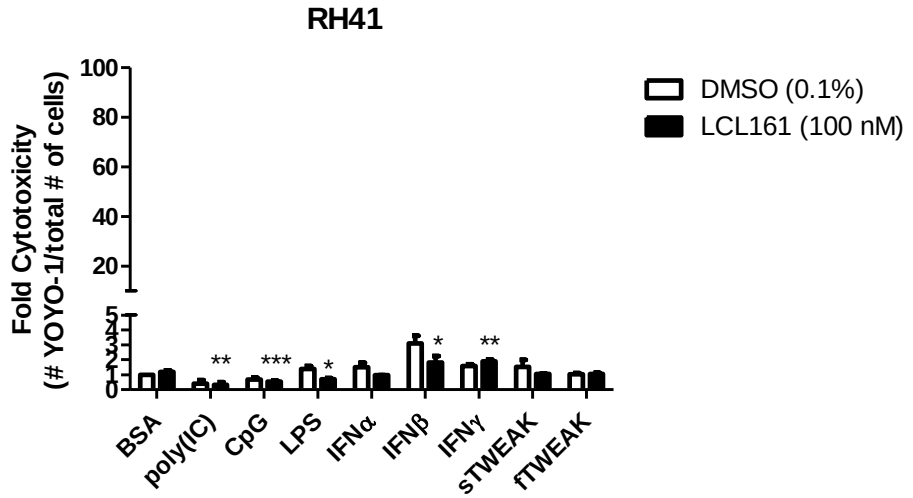
6.5.2 Kym-1, but not RH36 and RH41, are sensitive to combination of LCL161 and modulators of innate immunity

Next, we asked whether these three RMS cell lines would be sensitive to other modulators of innate immunity that could potentially lead to TNFα production, and whether SMC treatment would synergize with these modulators to induce RMS cell death. To this end, we treated RH36, RH41 and Kym-1 cells with the SMC LCL161 with the following modulators of innate immunity: microbial RNA mimetic poly(I:C), microbial DNA mimetic CpG, bacterial lipopolysaccharides (LPS), interferons (IFNα, β and γ), TWEAK (both soluble form (sTWEAK) and fused form (fTWEAK)), or a bovine serum albumin (BSA) control. Cytotoxicity was monitored over 48h by YOYO-1 staining. Treatment of RH36 or RH41 cells with 100 nM of LCL161 alone did not induce cytotoxicity when compared to a DMSO control (Figure 6.2A, B) as we previously reported.[5] Surprisingly, neither RH36 nor RH41 cells were overtly sensitive to the immune modulators tested either in the presence or absence of LCL161 (Figure 6.2A, B). Although statistically discernable differences were observed with IFNβ, IFNγ, polyI:C, CpG, and LPS treatments in these cells in the presence of LCL161, these changes were negligible (< 2 fold, Figure 6.2A, B). Because Kym-1 cells produce autocrine TNFα and are already sensitive to SMC treatment at 100 nM[5], we used a lower concentration of LCL161 (10 nM) and tested whether this would result in synergy with the modulators of innate immunity treatment. Treatment of Kym-1 cells with 10 nM LCL161 induced cytotoxicity by 15-fold when compared to DMSO control in BSA treated cells (Figure 6.2C). Although a modest increase in cytotoxicity was observed with co-treatment with CpG, the most striking increase in cytotoxicity occurred when Kym-1 cells

A


RH36
Fold Cytotoxicity
(# YOYO-1/total # of cells)
DMSO (0.1%)
LCL161 (100 nM)
BSA
poly(IC)
CpG
LPS
IFNα
IFNβ
IFNγ
sTWEAK
fTWEAK

B


RH41
Fold Cytotoxicity
(# YOYO-1/total # of cells)
DMSO (0.1%)
LCL161 (100 nM)
BSA
poly(IC)
CpG
LPS
IFNα
IFNβ
IFNγ
sTWEAK
fTWEAK

C

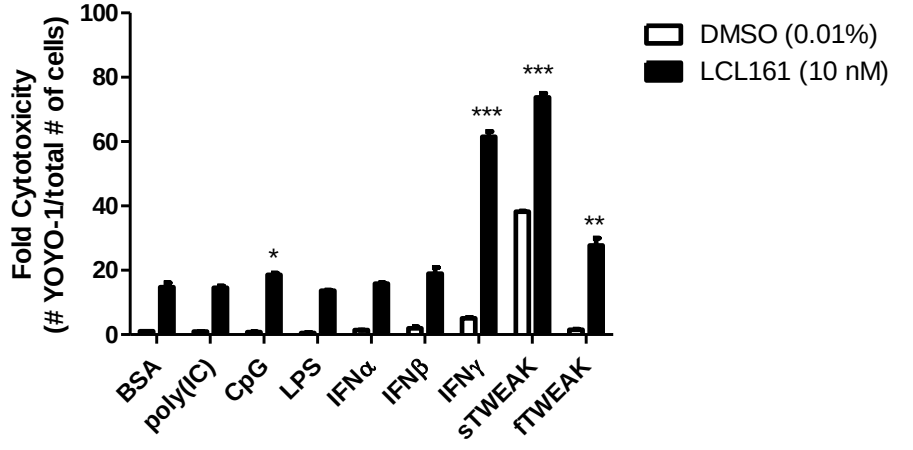

Kym-1
Fold Cytotoxicity
(# YOYO-1/total # of cells)
DMSO (0.01%)
LCL161 (10 nM)
BSA
poly(IC)
CpG
LPS
IFNα
IFNβ
IFNγ
sTWEAK
fTWEAK

Figure 6.2: Sensitivity of RMS cancer cells to a panel of immune modulators in the presence of LCL161

5,000 of RH36 **(A)**, RH41 **(B)** and Kym-1 **(C)** cells were treated with indicated amounts of LCL161 or DMSO control in combination with the following immune modulators: poly(IC) (1 ug/ml), CpG (3.33 ug/ml), LPS (1 ug/ml), IFNα (1000 U/ml), IFNβ (1000 U/ml), IFNγ (1000 U/ml), sTWEAK (100 ng/ml), fTWEAK (10 ng/ml) or BSA control (100 ng/ml). Cytotoxicity was determined on the Incucyte live cell imaging system by YOYO-1 dye incorporation over 48h and is expressed as fold cytotoxicity compared to BSA/DMSO control.

were co-treated with LCL161 and IFNγ (62-fold compared to BSA control), sTWEAK (74-fold) or fTWEAK (28-fold) (Figure 6.2C).

6.5.3 Determining the cause of RH36 and RH41 resistance to combined LCL161 and IFNγ or sTWEAK treatments.

We were intrigued by the fact that RH36 and RH41 were resistant to all the modulators of innate immunity tested, even in the presence of LCL61 as this suggest a blockage in signaling pathways that could be exploited for cancer treatment. Western blot analysis of the expression status of receptors of innate immunity modulators indicated that TNFR1 was invariably expressed in a panel of RMS cancer cell lines (Figure 6.3A). DR5, one of the TRAIL receptors was highly expressed in Kym-1 cells, whereas DR4 expression could not be detected in any cells (Figure 6.3A). INFR1, the receptor for type I interferons was detected in all cell lines except in Kym-1 cells, whereas TLR4, the receptor for LPS could not be detected in any cell lines (Figure 6.3A). Interestingly, INFγR1a, the alpha subunit of the IFNγ receptor, was expressed at comparable levels in all cell lines (Figure 6.3A). Expression of FN14, the receptor for TWEAK, could not be detected by western blot (Figure 6.3A); however, FN14 expression was previously reported in Kym-1 cells[29, 30] and qPCR analysis showed that FN14 mRNA was expressed in all the RMS cell lines tested (Figure 6.3B). Although RH36 cells had 50% less FN14 mRNA than Kym-1 cells, RH41 cells expressed 3-fold more FN14 mRNA; thus there exists no correlation between FN14 mRNA expression and responsiveness to sTWEAK treatment. Altogether, these data indicate that resistance of RH36 and RH41 cells to IFNγ or TWEAK treatment is not due to a defect in their receptor expression status or in TNFα or TRAIL receptors expression.

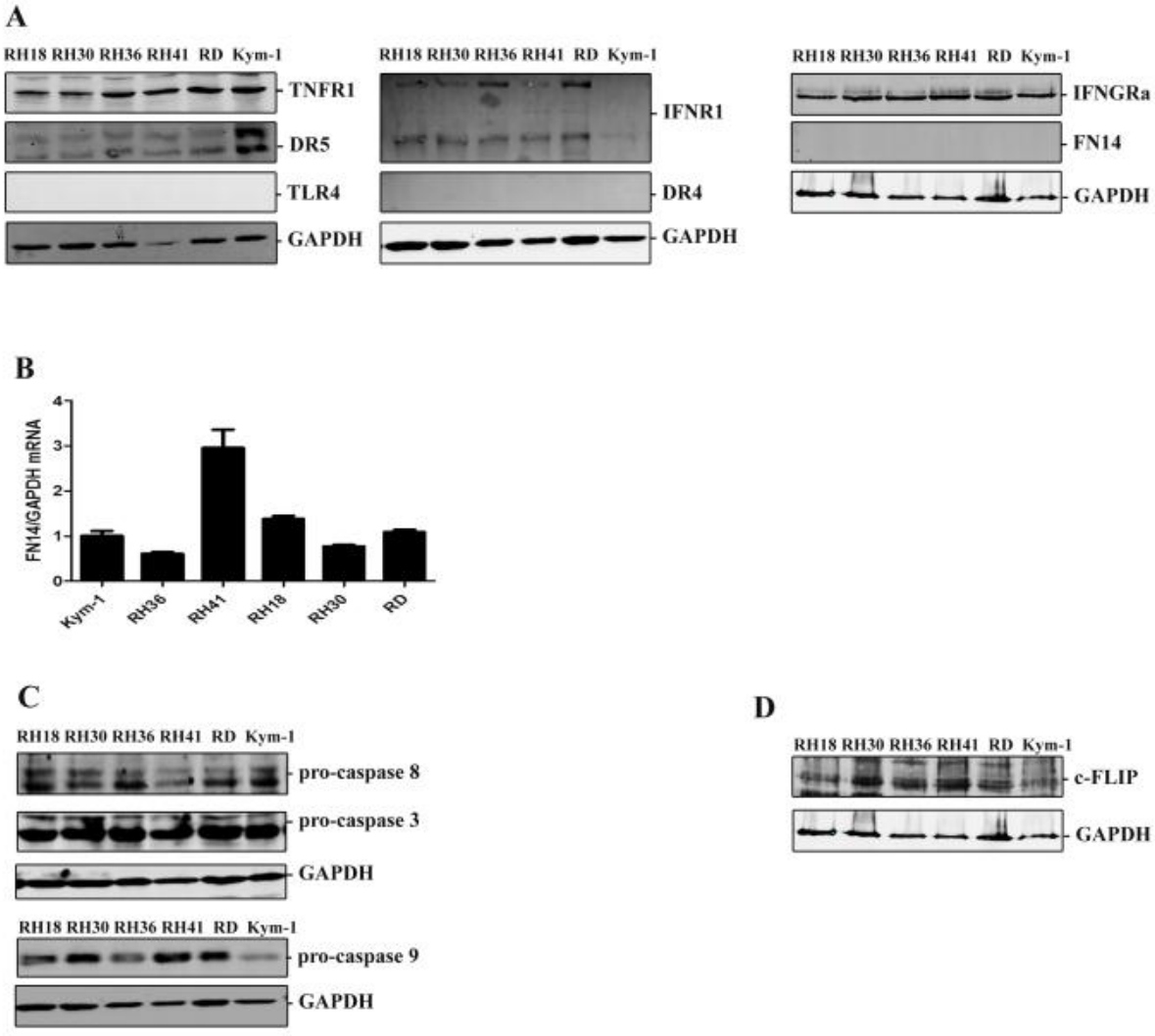

A
RH18 RH30 RH36 RH41 RD Kym-1
TNFR1
DR5
TLR4
GAPDH
RH18 RH30 RH36 RH41 RD Kym-1
IFNR1
DR4
GAPDH
RH18 RH30 RH36 RH41 RD Kym-1
IFNGRa
FN14
GAPDH
B
FN14/GAPDH mRNA
0
1
2
3
4
Kym-1
RH36
RH41
RH18
RH30
RD
C
RH18 RH30 RH36 RH41 RD Kym-1
pro-caspase 8
pro-caspase 3
GAPDH
RH18 RH30 RH36 RH41 RD Kym-1
pro-caspase 9
GAPDH
D
RH18 RH30 RH36 RH41 RD Kym-1
c-FLIP
GAPDH

Figure 6.3: Expression of immune modulators receptors and apoptosis pathway effectors in a panel of RMS cancer cells

A) A panel of RMS cancer cell lines was analysed by western blot for TNFR1, DR4, DR5, TLR4, IFNR1, IFNγRα, FN14 and GAPDH protein expression. **B)** FN14 mRNA expression was analysed by qRT-PCR in the same panel of RMS cancer cell lines, normalised to GAPDH mRNA and relative expression determined compared to levels in Kym-1 cells. **C)** Expression pro-caspases 3, 8 and 9 protein **(C)** as well as c-FLIP **(D)** was determined by western blot as in A.

Western blot analysis of pro-caspases 3, 8 and 9 expression also showed that they are invariably expressed across the RMS cell lines panel (Figure 6.3C).

These results show that there are no major defects in effector caspases expression between RH36, RH41 and Kym-1 cells that would explain the differences in the observed cell death resistance. Importantly, we have previously shown that both RH36 and RH41 undergo caspase 8-dependent apoptosis upon combined TNFα and LCL161 treatment, confirming that the extrinsic apoptotic pathway is intact in these cells.[5] The different threshold of apoptotic resistance in RH36, RH41 and Kym-1 cells could be also due to a differential expression of the anti-apoptotic protein c-FLIP an enzymatic dead homolog of caspase 8 which was shown to be a major factor in determining cancer cell lines resistance to SMC induced apoptosis.[31] However, western blot analysis of the RMS cell lines panel revealed that c-FLIP was expressed at comparable levels in all cell lines (Figure 6.3D).

To gain further insight into the difference in sensitivity to modulators of innate immunity treatment between RH36, RH41 and Kym-1, we next tested whether there was any difference in their signalling pathways in response to RFP-VSVΔ51, IFNγ or sTWEAK in the presence or absence of LCL161. We reasoned that such differences should be reflected in the expression of known downstream target genes of these signaling pathways. IFNγ signals through the JAK/STAT pathway upon binding to the IFNγ receptor whereas sTWEAK signals through the NFκB pathway. We therefore examined the mRNA expression of the interferon response factor IRF1 and of TNFα, two respective downstream targets of these pathways, in Kym-1, RH36 and RH41 cells. Treatment of Kym-1 cells with LCL161 greatly induced TNFα mRNA expression, even in PBS or BSA treated controls, consistent with these cells ability to produce autocrine TNFα (Figure 6.4A, black bars). VSVΔ51 treatment

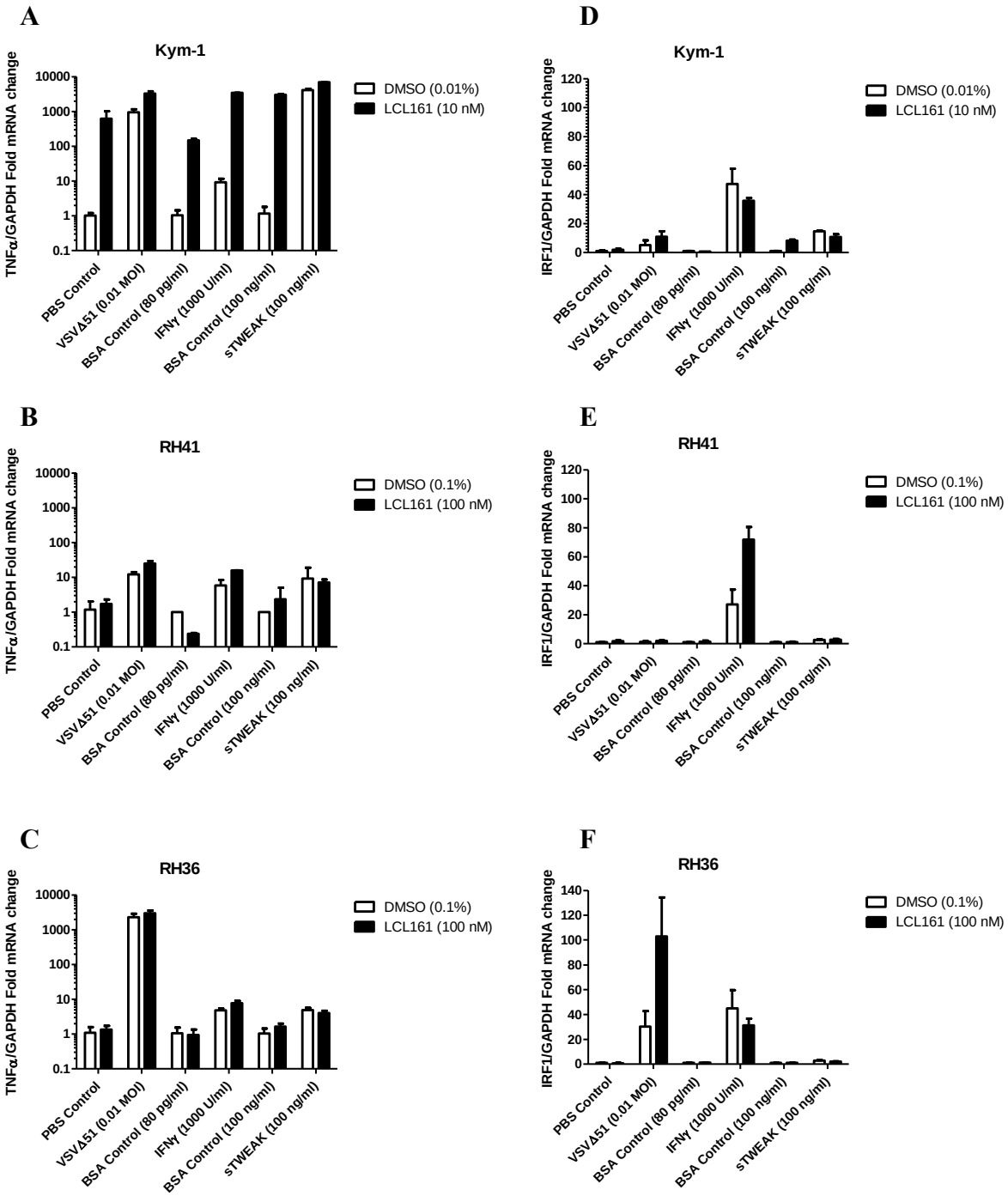

A
Kym-1
TNFα/GAPDH Fold mRNA change
10000
1000
100
10
1
0.1
DMSO (0.01%)
LCL161 (10 nM)
PBS Control
VSVΔ51 (0.01 MOI)
BSA Control (80 pg/ml)
IFNγ (1000 U/ml)
BSA Control (100 ng/ml)
sTWEAK (100 ng/ml)
D
Kym-1
IRF1/GAPDH Fold mRNA change
120
100
80
60
40
20
0
DMSO (0.01%)
LCL161 (10 nM)
PBS Control
VSVΔ51 (0.01 MOI)
BSA Control (80 pg/ml)
IFNγ (1000 U/ml)
BSA Control (100 ng/ml)
sTWEAK (100 ng/ml)
B
RH41
TNFα/GAPDH Fold mRNA change
10000
1000
100
10
1
0.1
DMSO (0.1%)
LCL161 (100 nM)
PBS Control
VSVΔ51 (0.01 MOI)
BSA Control (80 pg/ml)
IFNγ (1000 U/ml)
BSA Control (100 ng/ml)
sTWEAK (100 ng/ml)
E
RH41
IRF1/GAPDH Fold mRNA change
120
100
80
60
40
20
0
DMSO (0.1%)
LCL161 (100 nM)
PBS Control
VSVΔ51 (0.01 MOI)
BSA Control (80 pg/ml)
IFNγ (1000 U/ml)
BSA Control (100 ng/ml)
sTWEAK (100 ng/ml)
C
RH36
TNFα/GAPDH Fold mRNA change
10000
1000
100
10
1
0.1
DMSO (0.1%)
LCL161 (100 nM)
PBS Control
VSVΔ51 (0.01 MOI)
BSA Control (80 pg/ml)
IFNγ (1000 U/ml)
BSA Control (100 ng/ml)
sTWEAK (100 ng/ml)
F
RH36
IRF1/GAPDH Fold mRNA change
140
120
100
80
60
40
20
0
DMSO (0.1%)
LCL161 (100 nM)
PBS Control
VSVΔ51 (0.01 MOI)
BSA Control (80 pg/ml)
IFNγ (1000 U/ml)
BSA Control (100 ng/ml)
sTWEAK (100 ng/ml)

Figure 6.4: TNFα and IRF1 expression in RH36, RH41 and Kym-1 cells upon VSVΔ51, IFNγ or sTWEAK treatment in combination with LCL161.

Kym-1 **(A)**, RH41 **(B)** and RH36 **(C)** cells were treated with RFP-VSVΔ51 (10^{-1} MOI) or PBS control, IFNγ (1000 U/ml) or BSA control (80 pg/ml), sTWEAK (100 ng/ml) or BSA control (100 ng/ml) in the presence of indicated concentrations of LCL161 or DMSO control for 24h and TNFα mRNA expression determined by RT-qPCR, normalised to GAPDH mRNA. Cells were treated as in (A-C) and IRF1 mRNA expression determined by RT-qPCR amd normalised to GAPDH mRNA in Kym-1 **(D)**, RH41 **(E)** and RH36 **(F)** cells.

alone induced TNFα expression by ~1000 fold compared to a PBS control and combination with LCL161 induced expression by 3000 fold. IFNγ treatment induced TNFα expression by only ~10 fold compared to a BSA control (80 pg/ml) whereas combined treatment with LCL161 greatly induced TNFα expression by ~3500 fold (Figure 6.4A), thus explaining why Kym-1 cells are not sensitive to IFNγ treatment alone but are highly sensitive to combined IFNγ and LCL161 treatment (Figure 6.1C). sTWEAK treatment alone induced TNFα expression by ~4000 fold in Kym-1 cells compared to a BSA control (100 ng/ml), consistent with our own observations (Figure 6.1A) and other reports[29, 30, 32] that TWEAK induces Kym-1 cell death. Importantly, combined sTWEAK and LCL161 treatment induced TNFα expression by 7000 fold (Figure 6.4A), explaining the synergy in cell death induction observed.

However, VSVΔ51 induced TNFα production in RH41 only negligibly compared to Kym-1 cells (~10 fold vs 1000 fold) and although VSVΔ51 induced TNFα expression by 2000 fold in RH36, no synergy was observed in the presence of LCL161(Figures 6.4B, C). Similarly, IFNγ and sTWEAK induced TNFα expression only negligibly (less than 10 fold) in RH36 and RH41 and no synergy was observed in the presence of LCL161 (Figures 6.4B, C). These results possibly explain why LCL161 synergy with these modulators of innate immunity in inducing cell death was only observed in Kym-1 cells and not RH36 and RH41 (Figure 6.1). Interestingly, IFNγ treatment induced IRF1 mRNA expression to similar levels in all three cell lines and VSVΔ51 treatment induced IRF1 expression in RH36 more than it did in Kym-1 cells (Figures 6.4D-F). These results indicate that there are no defects in the IFN-JAK/STAT signalling pathways in RH36 and RH41 cells that would explain their resistance to IFNγ treatment.

Altogether, these results indicate that the resistance of RH36 and RH41 cells to combined LCL161 and IFNγ or sTWEAK treatments is mainly due to a defect in TNFα expression via the NF-κB signalling pathway, as there appears to be no difference in the JAK/STAT pathway activity and expression of receptors and apoptotic pathway modulators between RH36, RH41 and Kym-1 cells.

6.5.4 IFNγ synergizes with LCL161 to induce bystander, TNFα-mediated cell death in Kym-1 cells.

Next, we focused our study on better understanding the mechanism of IFNγ-induced cell death in Kym-1 cancer cells. First, we wanted to confirm whether IFNγ induces Kym-1 cell death mainly through its stimulation of TNFα production (bystander effect). To test this, we treated isolated Balb/c splenocytes with IFNγ for 24h, isolated the supernatant and treated Kym-1, RH41 and RH36 cells with dilutions of the conditioned media in the presence or absence of LCL161. IFNγ-conditioned media alone did not induce Kym-1 cell death even at high concentration but high cytotoxicity was observed when combined with LCL161 treatment (Figure 6.5A), indicating synergy between IFNγ signalling and LCL161 in inducing Kym-1 cell death. These results also suggest that treatment of immune cells with IFNγ treatment leads to the production of a soluble factor, likely TNFα, that is able to synergize with LCL161 to induce Kym-1 cell death. Interestingly, RH41 and RH36 remained resistant to combined LCL161 and IFNγ-conditioned media treatment, even at the highest concentrations (Figure 6.5B, C). This is consistent with the fact that higher concentrations of cytokines than that produced by the splenocytes in the context of this experiment are required to induce RH36 and RH41 cell death in the presence of LCL161.

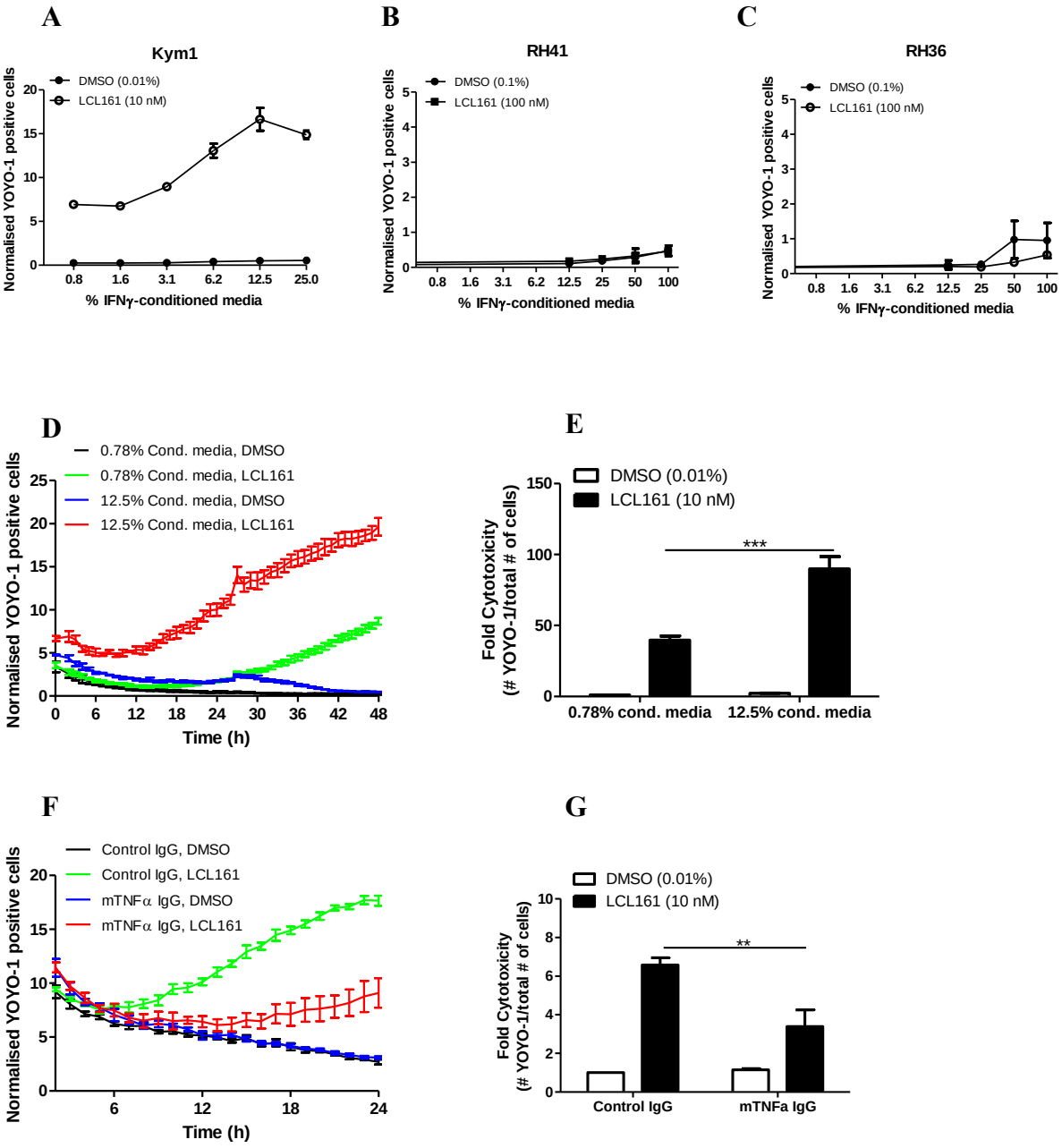

A
Kym1
DMSO (0.01%)
LCL161 (10 nM)
Normalised YOYO-1 positive cells
% IFNγ-conditioned media
B
RH41
DMSO (0.1%)
LCL161 (100 nM)
Normalised YOYO-1 positive cells
% IFNγ-conditioned media
C
RH36
DMSO (0.1%)
LCL161 (100 nM)
Normalised YOYO-1 positive cells
% IFNγ-conditioned media
D
0.78% Cond. media, DMSO
0.78% Cond. media, LCL161
12.5% Cond. media, DMSO
12.5% Cond. media, LCL161
Normalised YOYO-1 positive cells
Time (h)
E
DMSO (0.01%)
LCL161 (10 nM)
***
Fold Cytotoxicity
(# YOYO-1/total # of cells)
0.78% cond. media
12.5% cond. media
F
Control IgG, DMSO
Control IgG, LCL161
mTNFα IgG, DMSO
mTNFα IgG, LCL161
Normalised YOYO-1 positive cells
Time (h)
G
DMSO (0.01%)
LCL161 (10 nM)
**
Fold Cytotoxicity
(# YOYO-1/total # of cells)
Control IgG
mTNFa IgG

Figure 6.5: IFNγ synergizes with LCL161 to induce bystander, TNFα-mediated cell death of Kym-1

Conditioned media from Balb/c splenocytes treated with 1,000 U/ml IFNγ for 24h was applied in 2-fold dilution series to 5,000 Kym-1 **(A),** RH41 **(B)** or RH36 **(C)** cells in the presence of indicated concentrations of LCL161 or DMSO control and cytotoxicity determined by YOYO1 dye incorporation over 48h. **(D)** 5,000 Kym-1 cells were treated with 12.5 % or 0.78% of IFNγ conditioned media in the presence of 10 nM LCL161 or 0.01% DMSO control and cytotoxicity monitored over 48h by YOYO1 dye incorporation. **(B)** Fold cytotoxicity from (A) are expressed as the number of YOYO1 positive cells divided by total number of cells and normalised to DMSO/0.78% conditioned media control. **(C)** 5 000 Kym-1 cells were treated as in (A) but in the presence of 25 ug/ml of a mouse TNFα antibody or control IgG of the same isotype and cytotoxicity monitored over 24h. **(D)** Fold cytotoxicity from (C) are expressed as described in (B) and normalised to DMSO/IgG control

To confirm whether TNFα was the cytokine mediating conditioned media-induced Kym-1 cell death, Kym-1 cells were treated with 12.5% of IFNγ-conditioned media in the presence of LCL161 and a mouse TNFα antibody (mTNFα IgG) or control antibody (control IgG), and monitored cell death by YOYO1 dye incorporation. 12.5% IFNγ-conditioned media alone did not kill Kym-1 cells, however combination with LCL161 induced cytotoxicity by up to 100-fold when compared to a DMSO control (Figure 6.5D, E). Importantly, incubation with mTNFα antibodies significantly blunted combined LCL161 and IFNγ-conditioned media cytotoxicity compared to control IgG (Figure 6.5F, G). These results indicate that IFNγ-stimulated mouse splenocytes produce TNFα that in turn mediates, Kym-1 cell death. These findings are of potential clinical relevance as they confirm the hypothesis that a modulator of innate immunity such as IFNγ can stimulate immune cells to produce TNFα which in turn can synergize with SMC to induce RMS cancer cell death. Although RH36 and RH41 are resistant to combined IFNγ-conditioned media and LCL161 in this *ex-vivo* setting, it is likely that *in vivo*, IFNγ stimulation of the innate immune system will produce enough TNFα to induce, in combination with SMC, the death of this subset of resistant RMS cancer cells.

6.5.5 IFNγ and LCL161 induce Kym-1 cells apoptosis in a TNFR1-dependent manner

Since Kym-1 cells showed sensitivity to combined LCL161 and IFNγ treatment in the absence of immune cells mediators (Figure 6.1C), we wanted to characterise this direct mechanism of action as this could represent a therapeutically useful strategy. Co-treatment of Kym-1 cells with 1,000 U/ml of IFNγ reduced LCL161 IC_{50} by 3-fold (to ~5 nM) when

compared to a BSA control (Figure 6.6A). A dose response experiment showed that IFNγ strongly synergizes with 10 nM of LCL161, starting at 250 U/ml (Figure 6.6B). Co-treatment of Kym-1 cells with 10 nM LCL161 and 250 U/ml IFNγ induced cytotoxicity (Figures 6.7A, B) and caspase-3/7 (Figures 6.7C, D) activity by ~150 fold when compared to a DMSO/BSA control. To verify that IFNγ-induced Kym-1 apoptosis is mediated by TNFα production, Kym-1 cells were transfected with TNFR1 or control siRNA for 48h and treated with combined LCL161 and IFNγ for an additional 24h. As expected, TNFR1 knock-down reduced the IFNγ+LCL161-induced cytotoxicity (Figure 6.7E, F) and apoptosis (Figure 6.7G, H) by 2-fold when compared to the control siRNA. These results confirm that IFNγ and LCL161 induce Kym-1 apoptosis in a TNFα and TNFR1-dependent manner, and that combination of IFNγ and SMC is an attractive therapeutic strategy for RMS cancer cells with autocrine TNFα production.

6.5.6 LCL161 potentiates TWEAK killing of Kym-1 cells in a TNFR1-dependent manner.

TWEAK is a member of the TNF ligand family that is involved caspase-8 dependent apoptosis, NFκB signalling and muscle differentiation through binding to its receptor FN14.[32] TWEAK was identified in our earlier experiments as one of the immune modulators that, either in its soluble recombinant (sTWEAK) or its fused (fTWEAK) forms, triggers Kym-1 cell death (Figure 6.1). sTWEAK-induced apoptosis has been previously characterised and involves the recruitment of cIAP1/TRAF2 complexes to the FN14 receptor upon TWEAK binding, and their subsequent lysosomal degradation, which leads to

activation of caspase 8.[32] Interestingly, we found that 10 nM of LCL161 synergize with sTWEAK,

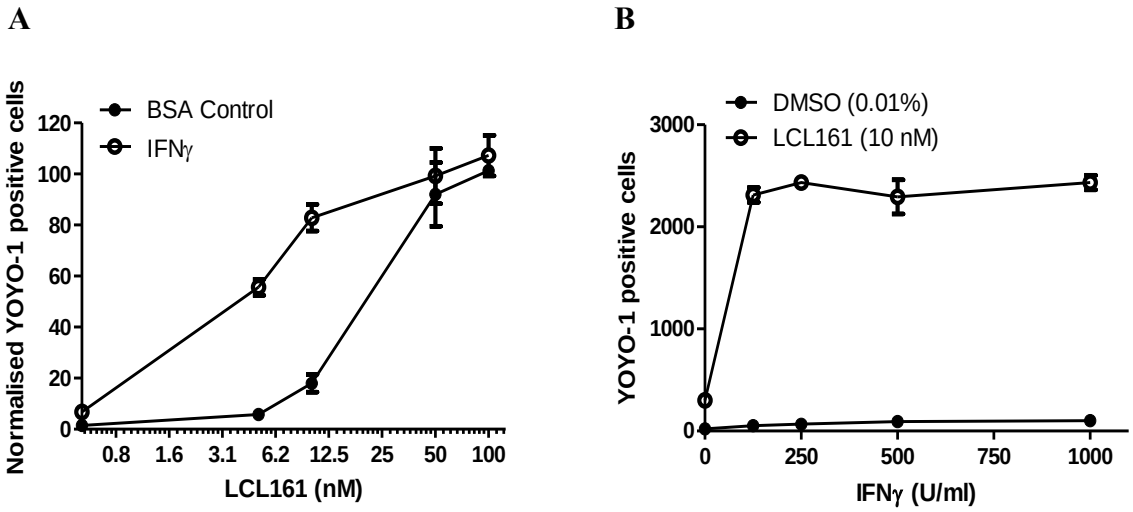

A
B
BSA Control
IFNγ
Normalised YOYO-1 positive cells
LCL161 (nM)
0.8
1.6
3.1
6.2
12.5
25
50
100
DMSO (0.01%)
LCL161 (10 nM)
YOYO-1 positive cells
IFNγ (U/ml)
0
250
500
750
1000
1000
2000
3000

Figure 6.6: Characteristics of the synergy between IFNγ and LCL161 in Kym-1 cells.

(A) 5,000 Kym-1 cells were treated with 1000 U/ml IFNγ or equivalent amount of BSA (80 pg/ml) in the presence of increasing LCL161 concentrations or DMSO control (0 nM LCL161) and cytotoxicity monitored by YOYO1 dye incorporation over 48h. **(B)** 5,000 Kym-1 cells were treated with 10 nM LCL161 or DMSO control in the presence of increasing concentrations of IFNγ or BSA control and cytotoxicity monitored as in (A).

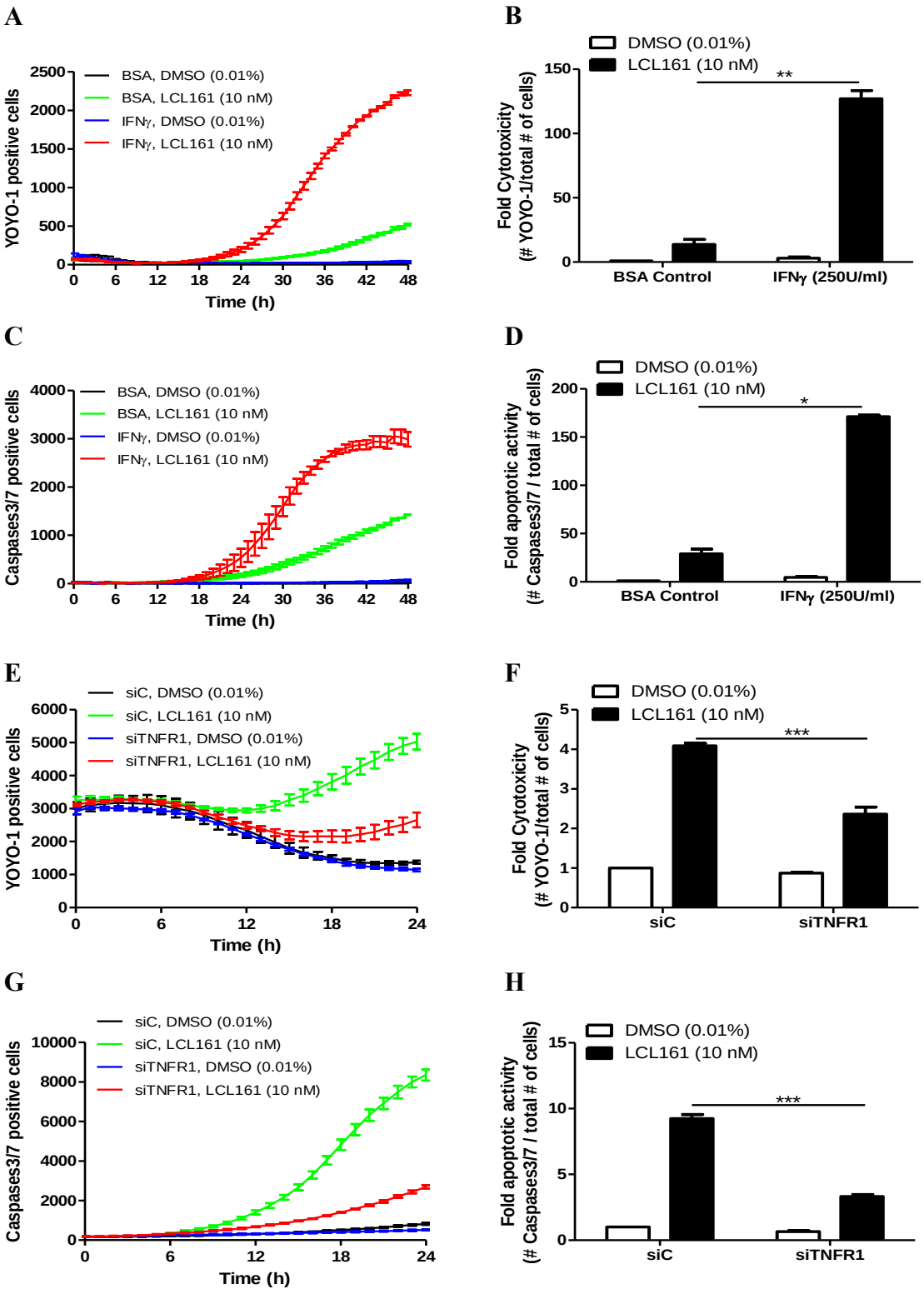

A
BSA, DMSO (0.01%)
BSA, LCL161 (10 nM)
IFNγ, DMSO (0.01%)
IFNγ, LCL161 (10 nM)
YOYO-1 positive cells
Time (h)
B
DMSO (0.01%)
LCL161 (10 nM)
Fold Cytotoxicity
(# YOYO-1/total # of cells)
BSA Control
IFNγ (250U/ml)
C
Caspases3/7 positive cells
D
Fold apoptotic activity
(# Caspases3/7 / total # of cells)
E
siC, DMSO (0.01%)
siC, LCL161 (10 nM)
siTNFR1, DMSO (0.01%)
siTNFR1, LCL161 (10 nM)
F
siC
siTNFR1
G
H

Figure 6.7: IFNγ and LCL161 induce Kym-1 cells apoptosis in a TNFR1-dependent manner

(A) 5,000 of Kym1 cells were treated with 250 U/ml IFNγ or BSA control (20 pg/ml) and 10 nM LCL161 or DMSO control and cytotoxicity monitored by YOYO1 dye incorporation over 48h. **(B)** Fold cytotoxicity from (A) was calculated as the number of fluorescent cells divided by the total number of cells and normalised to DMSO/BSA control at 48h. **(C)** Kym1 cells were treated as in (A) and caspases3/7 activity determined by fluorescent substrate cleavage over 48h. **(D)** Fold apoptotic activity from (C) was calculated as the number of fluorescent cells divided by the total number of cells and normalised to DMSO/BSA control at 48h. **(E)** 5,000 of Kym1 cells were transfected with 10 nM of control or TNFR1 siRNA for 48h, followed by treatment with 250 U/ml IFNγ and 10 nM LCL161 or DMSO control for an additional 24h and cytotoxicity was monitored by YOYO1 dye incorporation over 48h. **(F)** Fold cytotoxicity at 24h from (E) is shown. **(G)** Kym1 cells were treated as in (E) and caspases3/7 activity monitored over 24h. **(H)** Fold apoptototic activity from (G) is shown at 24h.

starting as low as 12.5 ng/ml when compared to a DMSO control (Figure 6.8A). Hence SMC treatment potentiates sTWEAK-induced apoptosis in Kym-1 cells, probably through further degradation of cIAP1 by autoubiquitination. This occurs in a TNFα-dependent manner as TNFR1 knock-down blunted by 2 fold LCL161 and sTWEAK induced cytotoxicity (Figures 6.8B, C) and apoptosis (Figures 6.8D, E).

6.6 Discussion

We have previously reported that combination of SMC and TNFα induced RMS cancer cells apoptosis and significant inhibition of tumour growth *in vivo*.[5] The goal of this study was to find alternative ways of inducing TNFα expression in order to potentiate SMC-induced cell death and to avoid the cytotoxicity associated with recombinant TNFα therapy. We report here that the modulators of innate immunity VSVΔ51, IFNγ and TWEAK potently synergize with the SMC LCL161 to induce apoptosis of Kym-1 RMS cancer cells. This synergy is dependent on induction of TNFα production by these modulators of innate immunity.

It was recently reported that oncolytic viruses can synergize with SMCs to induce cancer cell death through their bystander induction of TNFα expression.[22] Hence, we first wanted to see whether RMS cell lines were also sensitive to oncolytic virus treatment alone or in combination with LCL161. Oncolytic viruses are anti-cancer viruses that can infect and kill cancer cells without causing harm to normal cells.[33] Indeed, we found that three RMS cell lines RH36, RH41 and Kym-1 were all sensitive to treatment with the oncolytic rhabdovirus VSVΔ51. Unexpectedly, the synergy with the SMC LCL161 was only observed in the Kym-1 cell line (Figure 1). Oncolytic viruses can kill cancer cells by two major

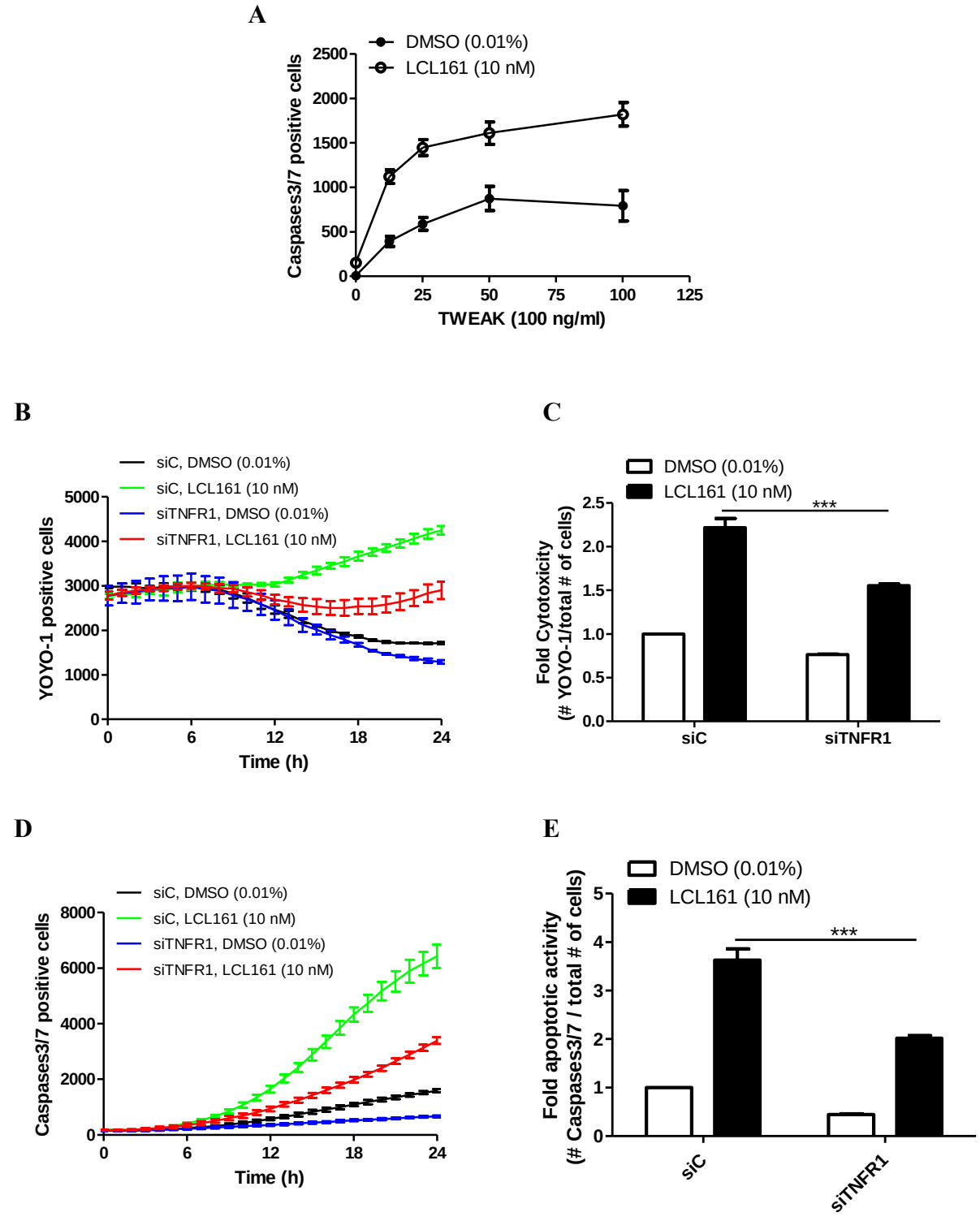

A
DMSO (0.01%)
LCL161 (10 nM)
Caspases3/7 positive cells
2500
2000
1500
1000
500
0
0
25
50
75
100
125
TWEAK (100 ng/ml)
B
siC, DMSO (0.01%)
siC, LCL161 (10 nM)
siTNFR1, DMSO (0.01%)
siTNFR1, LCL161 (10 nM)
YOYO-1 positive cells
5000
4000
3000
2000
1000
0
0
6
12
18
24
Time (h)
C
DMSO (0.01%)
LCL161 (10 nM)
***
Fold Cytotoxicity
(# YOYO-1/total # of cells)
2.5
2.0
1.5
1.0
0.5
0.0
siC
siTNFR1
D
siC, DMSO (0.01%)
siC, LCL161 (10 nM)
siTNFR1, DMSO (0.01%)
siTNFR1, LCL161 (10 nM)
Caspases3/7 positive cells
8000
6000
4000
2000
0
0
6
12
18
24
Time (h)
E
DMSO (0.01%)
LCL161 (10 nM)
***
Fold apoptotic activity
(# Caspases3/7 / total # of cells)
5
4
3
2
1
0
siC
siTNFR1

Figure 6.8: ***LCL161 potentiates TWEAK killing of Kym-1 cells in a TNFR1-dependent manner***

(A) 5,000 of Kym1 cells were treated with 10 nM LCL161 or DMSO control in the presence of increasing concentration of soluble recombinant TWEAK (sTWEAK) and capases3/7 activity was monitored by fluorescent substrate cleavage over 48h. **(B)** 5,000 of Kym1 cells were transfected with 10 nM of control or TNFR1 siRNA for 48h, followed by treatment with 100 ng/ml sTWEAK and 10 nM LCL161 or DMSO control for an additional 24h. Cytotoxicity was monitored over the 24h IFNγ/LCL161 treatment. **(C)** Fold cytotoxicity at 24h from (B) is shown. **(D)** Kym1 cells were treated as in (B) and caspases3/7 activity monitored over 24h. **(E)** Fold apoptototic activity from (D) is shown at 24h.

pathways; through a classic virus lytic cycle or through engagement of the host anti-cancer immune responses.[34] Our data suggest that VSVΔ51 induces cell death in RH36 and RH41 cells mainly through the virus lytic cycle. In contrast, in Kym-1 cells, the observed synergy with LCL161 suggest that cell death is occurring at least in part through death receptor-dependent apoptosis and production of cytokines such as TNFα, as was previously reported for breast and brain cancer cells.[22] Oncolytic viruses are showing great potential in the treatment of a broad range of cancers and are currently in phase II-II clinical trials (reviewed in [34]). However, like with any cancer monotherapy, there is the possibility that cancer cells will develop resistance to oncolytic viruses, chiefly by escaping immune surveillance through immunoediting.[17] We were therefore interested in exploring other immune modulators that could synergize better with SMC in order to induce RMS cancer cell death more effectively.

We identified IFNγ and TWEAK as two agents that showed great synergy with LCL161 (Figure 6.2). Interestingly, LCL161 and IFNγ or sTWEAK induced cytotoxicity was only observed in Kym-1 cells, but not in RH36 and RH41 cells. This and the VSVΔ51 data indicate that RH36 and RH41 are generally resistant to combined SMC and modulators of innate immunity, indicative of a defect in their signalling pathways. Comparative analysis between Kym-1, RH36 and RH41 revealed that there were no major differences in the expression of IFNγ (IFNγRα) or TWEAK (FN14) receptors or in the expression of pro-caspases 3 and 8 and c-FLIP (Figure 6.3). However, there was a difference in TNFα expression induction between these cells upon treatment with VSVΔ51, IFNγ or sTWEAK. Indeed, although VSVΔ51 greatly induced TNFα mRNA expression in RH36 to levels comparable to that in Kym-1 cells (~3000 fold), there was no further induction upon

combined treatment with LCL161 (Figure 6.4), thus explaining the lack of synergy between LCL161 and VSVΔ51 in inducing RH36 cell death. In the case of RH41, VSVΔ51 only weakly (10 fold) induced TNFα mRNA expression, confirming that the virus is probably killing these cells through other means than the production of TNFα. The difference in TNFα expression induction was even more striking upon IFNγ or sTWEAK treatment where combined treatment with LCL161 induced TNFα mRNA expression by 3000 to 4000 fold when compared to a BSA/DMSO control, whereas induction in RH36 or RH41 cells was less than 10 fold, with no further induction in the presence of LCL161 (Figure 6.4). These results indicate that RH36 and RH41 do not efficiently induce TNFα expression upon stimulation with modulators of innate immunity, which may be a reflection of a defect in the NF-κB signalling pathway. Normally, stimulation of the innate immune system leads to the production of the pro-inflammatory cytokines such as TNFα through activation of the NF-κB. Depletion of cIAPs by the use of SMCs activate the non-canonical NF-κB, thus contributing to increased TNFα production (reviewed in [19, 20, 23]). IFNγ was also shown to induce TNFα expression through the induction of the interferon response factors IRF1 and IRF8 and consequent transactivation of the TNFα promoter by these transcription factors.[35] However, we observed no significant difference in IFNγ signalling in the three RMS cell lines as the expression of IRF1 was efficiently induced in all three cell lines upon treatment with IFNγ or with VSVΔ51 (Figures 6.4D-F). Hence, it will be important to determine why RH36 and RH41 do not respond to these modulators of innate immunity and LCL161 by producing TNFα and in particular, which components of the canonical and non-canonical NF-κB signalling pathways are defective in these cell lines. Because RMS can arise from different tissues and at different points during muscle or even mesenchymal development,

there is a great heterogeneity in RMS subtypes.[1, 3] Hence it will be important to consider a genome wide approach to identify possible mutations in the NF-κB pathway that could contribute to the defects in TNFα expression observed upon treatment with modulators of innate immunity and SMCs.

It should also be noted that because Kym-1 cells have mechanisms of autocrine TNFα expression, they are ‘‘primed’’ for more TNFα production and TNFα-induced cell death, as is seen upon treatment with low dose of LCL161 alone, or with sTWEAK (Figure 6.1C, Figure 6.4A). It has been reported that binding of endogenous membrane-bound TNFα to TNFR2 in Kym-1 cells contributes to TRAF2-mediated depletion of cIAP1/2, further TNFα production and induction TNFR1-mediated apoptosis.[36, 37] Similarly, binding of soluble TWEAK to FN14 in Kym-1 cells induces lysosomal degradation of the cIAP1-TRAF2 complex, thus leading to activation of the non canonical NF-κB pathway, autocrine TNFα production and TNFR1-dependent apoptosis.[30] Therefore, co-treatment of Kym-1 cells with SMCs amplify this process and leads to more TNFα-induced cell death, thus explaining the great synergy observed between LCL161 and IFNγ (Figure 6.7) or sTWEAK (Figure 6.8) in inducing Kym-1 cells apoptosis in a TNFR1-dependent manner. Importantly, IFNγ treatment also induced TNFα expression in mouse splenocytes in the presence of LCL161, and in enough quantities to induce Kym-1 cells apoptosis, but not that of RH36 and RH41 cells (Figure 6.5). We have previously shown that RH36 and RH41 cells can undergo apoptosis when treated with SMCs and recombinant TNFα at high concentrations (from 10 to 100 ng/ml).[5] Therefore it is likely that RH36 and RH41 high resistance threshold to TNFα-induced apoptosis can be circumvented *in vivo*. Indeed, treatment with IFNγ would create a cytokine storm and production of sufficient TNFα by immune cells in the tumour

microenvironment, which in combination with cIAP1 depletion in the tumour, should lead to effective killing of these cells. However, this remains to be tested in an *in vivo* RMS mouse model and preferably a syngeneic model where full activation of the host innate immune system can be taken advantage of.

Apart from its role in sensitizing cancer cells to apoptosis, IFNγ can also inhibit proliferation, angiogenesis, stimulate MHC class I and II expression and stimulate antitumour immune activity (reviewed in [38, 39]). Importantly, the recombinant IFNγ protein Actimmune (IFNγ1b) has been used in the treatment of several diseases such as tuberculosis, hepatitis, osteoporosis and cancer, and treatment is relatively well tolerated with the most common adverse side effects being of a ''flu-like'' nature such as fever, chills, headache and myalgia.[38] However, it has been reported that IFNγ can have adverse effects in the treatment of certain cancers due to activation of detrimental interferon stimulated genes that promote tumour growth and metastasis, depending on the dosage and timing of treatment.[40] Hence, it will be important to assess the full spectrum of IFNγ effects in combination of LCL161 *in vivo*. However, our results present great potential since synergy in inducing Kym-1 cell death is observed at concentrations as low as 250 U/ml for IFNγ (~ 20 pg/ml) and 10 nM for LCL161. It still remains to be determined how these concentrations will translate to *in vivo* mouse models and eventually patients; however these results are promising as they would lead to reduced treatment associated side effects, less off-target effects, and a greater window of treatment opportunities, at least for patients with TNFα-expressing RMS tumours. TWEAK also presents great potential as a drug target and pre-clinical data show efficacy of treatment in disease models such as arthritis, cerebral ischemia and cancer-induced cachexia (reviewed in [32, 41]). Interestingly, most reported TWEAK/FN14 effects in

cancer are pro-tumoral, such as an increase in proliferation, cell migration, invasion and angiogenesis in different cancer models. However, like for other death-inducing ligand and receptor complex, the balance between NF-κB activation and TNFR1-dependent caspase-8 mediated apoptosis is important, but we foresee that in the presence of SMCs depleting cIAP1 and of autocrine TNFα production, treatment of RMS cancer cells with sTWEAK will lead to apoptosis and tumour regression *in vivo*.

In conclusion, we have identified in this study two modulators of innate immunity, IFNγ and sTWEAK, that promote TNFα production and synergize with LCL161 to induce TNFR1-mediated apoptosis of RMS cells. Hence, combined SMC and IFNγ or SMC and sTWEAK therapies show great potential for the treatment of RMS cancers, specifically of those with autocrine TNFα production, but also by taking advantage of the innate immune environment, potentially for those that don't produce endogenous TNFα.

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CHAPTER 7

General Discussion

The goal of this thesis dissertation was to present my recent findings on the regulation of cIAP1 IRES-mediated translation and how this relates to its role in carcinogenesis. In addition, I hope I was able to convey to the reader the greater message of how IRES-mediated translation of a given transcript is important to cellular homeostasis, and how understanding this regulation can be useful for targeted therapies of cancer. I will be revisiting this theme within this general discussion but first, I will start with a discussion of the mechanisms of cIAP1 IRES-mediated translation and the ITAFs that take part in it.

7.1 *In silico* prediction of an IRES trans-acting factor: the case of NF45

The focus of our laboratory has been to understand alternative mechanisms of translation control in conditions of stress and cancer, with a specific focus on the IRES-mediated regulation of anti-apoptotic proteins. Previous work from the laboratory identified four ITAFs that interact specifically with the cIAP1 IRES – NF45, NF90, IGF2BP1, RHA - and from which stemmed my PhD research project. Among these, the nuclear factor NF45 was shown to be an ITAF that is indispensable for the induction of cIAP1 IRES mediated translation during the unfolded protein.[1] Additional preliminary data at the time also suggested that NF45 regulation of IRES-mediated translation was not limited to that of the cIAP1 IRES, as it was observed that the activity of other AU-rich IRESes might be sensitive to NF45 knock-down in Hela cells whereas GC-rich IRESes did not respond to NF45 knock-down. This led us to hypothesize that IRES with a high AU content should be sensitive to regulation by NF45. Data presented in Chapter 4 clearly shows that indeed, NF45 regulates the IRES activity and translation of known cellular IRES with an AU content of more than 60%, including cIAP1, XIAP, NRF and ELG. To my knowledge, this work was the first

report that the regulation of IRES-containing mRNAs by a specific ITAF could be predicted and validated solely based on their nucleotide composition. Indeed, in the field of IRES-mediated translation, a lot of effort has been put in trying to categorize cellular IRES on the basis of their structure and the ITAFs that bind to them. However, unlike viral IRES, cellular IRES do not seem to have much structural/functional consensus and finding a way to better characterize them has been challenging.[2] Hence, our work was a first step towards not only finding cellular IRESes that are regulated by the same ITAF but also in trying to predict and discover new IRES-containing mRNAs based on their nucleotide composition. Importantly, the nucleotide composition of known cellular IRES to date do not deviate significantly from those of their 5'UTRs.[3] This allows for an easier prediction of ITAFs based only on the 5'UTR sequence, as more often than not the 5'UTR is known but not the exact IRES region.

In the study presented in Chapter 4, we used a candidate approach to identify the possible regulation of known cellular IRES (90 IRES at the time) by a single ITAF, NF45, using a CAT/βGal bicistronic reporter assay. This approach can be cumbersome if the functional relationship between several IRES and potential ITAFs were to be interrogated. Instead, a genome wide approach could be applied, where a library of RNA binding proteins specific siRNAs could be used to knock-down potential ITAFs and evaluate their effects on cellular IRES activity using an RFP/GFP fluorescence based bicistronic reporter assays. High throughput imaging and software based data analysis could then lead to broader prediction of new ITAFs and the IRES they regulate.

7.2 Common mechanisms of ITAF regulation: Is NF45 regulating an RNA operon ?

In Chapter 4, I have also shown that NF45 regulation of the cIAP1 and XIAP1 IRES-mediated translation leads respectively to deregulation of Survivin and Cyclin E protein

levels, contributing to the defects in mitosis and cytokinesis observed in NF45-depleted Hela cells. NF45 is then able to regulate the same important cellular process, mitosis, through the regulation of two distinct IRES containing mRNAs that have in common an AU-rich IRES. cIAP1 and XIAP1 also have in common the fact that they are anti-apoptotic proteins and can regulate NFκB signalling.[4] The NFκB repressing factor NRF[5] is another target of NF45 IRES-mediated translation, suggesting a role for NF45 in the NFκB signalling pathway. The fourth IRES regulated by NF45, the ELG IRES, regulates the expression of an uncharacterized protein whose function could not be uncovered due to a lack of experimental tools.

Altogether, one could postulate from these observations that NF45 regulates an RNA operon involved in the regulation of mitosis, NFκB signalling and apoptosis, three intimately linked functional pathways. Similar to a DNA operon, an RNA operon is a group of specific eukaryotic mRNAs that are co-regulated at the level of RNA export, stability, and/or translation by virtue of their cis-acting UTR elements and interactions with trans-acting factors.[6] The fact that NF45 knock-down in Hela cells leads to a multinucleated phenotype, in part due to the altered functions of cIAP1 and XIAP in regulating the protein levels of cyclin E and Survivin, further reiterates the plausibility of a functional NF45-modulated ‘‘IRES’’ regulon. We could not observe any difference in NFκB activity between NF45 knock-down (*d5*) and control (*c*) Hela cells; however, NF45 overexpression in both cell lines increased NFκB activity by more than 15-fold (unpublished observations from our laboratory). This suggests that NF45 might also have a role in the regulation of the NFκB signalling pathway though its modulation of the cIAP1, XIAP and possibly NRF IRES. It would be interesting to test if this NFκB arm of the NF45 RNA operon is functional in cells

with a normal, wild-type genetic background. Interestingly, while polyploidy is increased in *d5* cells, and IRES-mediated translation of cIAP1 and XIAP is impaired, cells are not undergoing apoptosis.[7] This lack of apoptosis is likely due to an inhibition of the intrinsic mitochondrial apoptotic pathway as a result of p53 degradation by the E6 protein of the human papilloma virus expressed in HeLa cells.[8]

Altogether, these observations argue for the possibility of an NF45 regulated RNA operon, specifically an IRES one. However more experiments need to be performed to prove the functionality and relevance of such a regulon in a normal cellular context and to identify additional transcripts modulated within this operon. For instance, it would be interesting to see if there exist NF45 regulated transcripts that are involved in DNA repair mechanisms. It was recently shown that NF45 is an important regulator of non-homologous end joining (NHEJ) DNA repair through its interaction with the DNA-activated protein kinase (DNA-PK), and that structured RNA may be important in this process.[9] Interestingly, the DNA repair protein SNM1 contains an AU-rich IRES[10] (59% AU-rich) although its activity was not significantly affected by NF45 knock-down in Hela cells (Figure 4.2A). It would, however, be interesting to revisit NF45 possible regulation of the SNM1 IRES and other target IRES in a different cellular context to see if they tie in to the DNA repair arm of the NF45 regulon (Figure 3.3).

7.3 cIAP1 IRES-mediated translation: a complex network of IRES trans-acting factors

Over the past several years, our laboratory has identified protein factors that regulate cIAP1 IRES mediated translation. DAP5/p86, the caspase-cleaved variant of the eIF4G homolog DAP5/p97, was the first ITAF identified to regulate the cIAP1 IRES.[11, 12]

DAP5/p86 is necessary for the caspase-mediated induction of cIAP1 IRES-mediated translation during ER stress. Indeed, RNAi-mediated knock-down of DAP5/p97 or inhibition of its caspase-mediated cleavage of p97 prevents induction of cIAP1 IRES activity and concomitant protein expression during the UPR.[11, 12] NF45 is also necessary for the induction of cIAP1 translation during ER stress, as NF45 overexpression rescues the defect in cIAP1 protein expression observed in NF45 depleted *d5* Hela cells during UPR.[1] Importantly, unpublished data from our laboratory indicates that induction of cIAP1 protein following overexpression of p86 is dependent on NF45 expression, suggesting that these two ITAFs work in concert to induce cIAP1 IRES-mediated translation during the UPR. Furthermore, the NF45 binding partner NF90, was found to interact with both the cIAP1 IRES (ref) and the cIAP1 3'UTR (unpublished observations from the laboratory), although it is not clear whether this interaction is direct or not. This further suggests that there is a complex of at least three ITAFs, namely DAP5/p86, NF45 and NF90, that ensures cIAP1 IRES-mediated translation in conditions of ER stress.

In Chapter 5, I have shown that IGF2BP1, another RNA binding protein identified to interact with the cIAP1 IRES, is also an ITAF that stimulates cIAP1 IRES mediated translation in steady-state conditions (HEK293, Figure 5.3) and in the context of cancer cells (RMS, Figure 5.1). Interestingly, IGF2BP1 knock-down decreases cIAP1 IRES activity and protein levels by about 50 % when compared to control cells (Figure 5.2 and 5.3), suggesting that other factor(s) contribute to cIAP1 IRES-mediated translation in this context. NF45 is a potential candidate for such co-regulation as it was found to interact with the cIAP1 IRES in the same complex as IGF2BP1[1] and both ITAFs stimulate cIAP1 IRES activity.[13, 14] Interestingly, NF45 binds to the 5'region of the cIAP1 IRES (stemloop I)[1], whereas

IGF2BP1 binds to the 3'region of the IRES (stemloop III, Figure 5.3), suggesting that these two ITAFs are not competing for binding to the cIAP1 IRES and instead could work in concert to stimulate cIAP1 IRES-mediated translation. It would be interesting to test the possibility of such cooperation first, by performing co-immunoprecipitation experiments in the presence or absence of the cIAP1 IRES to determine if the two ITAFs interact together. Second, cooperation between ITAFs can be tested by performing dual knock-down experiments to determine if there is an additive effect on IRES activity an protein levels regulation. Finally, the function of the fifth protein factor identified on the cIAP1 IRES, RHA, has not been established yet in the context of cIAP1 translation regulation. However, RHA has been previously found in complex with IGF2BP1[15-17] and NF45[15, 16, 18], and being an RNA helicase, may well serve as an ubiquitous accessory protein that unwinds the highly structured cIAP1 IRES during translation.

With the work presented in this dissertation and previous work from our group, we now have a better understanding of how cIAP1 IRES-mediated translation is regulated. However, more work needs to be done to have a clearer picture of the cIAP1 IRES pre-initiation complex formation and the different interactions that take place between ITAFs in normal and in stress conditions to ensure proper expression of the cIAP1 protein. The fact that IGF2BP1 is only expressed in embryonic and cancer cells[19] adds another level of complexity to this network and suggests that interactions between ITAFs on the cIAP1 IRES are tissue and context specific.

7.4 From the discovery of an ITAF to targeted cancer therapy: the case of IGF2BP1

We have identified IGF2BP1 as an ITAF that is overexpressed in RMS cancer cells and stimulates cIAP1 IRES-mediated translation, thus contributing to the apoptotic resistance of these cells (Chapter 5). Importantly, IGF2BP1 is an oncofetal protein that is only expressed during embryogenesis but becomes *de novo* expressed in cancer cells and it's important roles in promoting oncogenesis are now emerging (discussed in Chapter 3). To my knowledge, our study was the first report of IGF2BP1 overexpression in human rhabdomyosarcoma tumour-derived cancer cell lines and primary tumours. It also places IGF2BP1 and cIAP1 at the centre of the high apoptotic resistance observed in these cells. Indeed, IGF2BP1 and cIAP1 overexpression in RMS cancer cells protects them against TNFα or TRAIL mediated apoptosis, whereas IGF2BP1 siRNA-mediated knock-down sensitizes these cells to death ligands induced apoptosis in a cIAP1-dependent manner (Figure 5.4). Given the importance of cIAP1 in regulating caspase-8 dependent apoptosis and mediating RMS resistance to death ligand induced apoptosis[4, 14], we took advantage of Smac mimetic compounds (SMCs) that cause cIAP1 proteasomal degradation[20, 21], to sensitize RMS cells to TNFα-mediated apoptosis. Importantly, the SMC LCL161[22, 23] was efficient in inducing the apoptosis of TNFα producing Kym-1 RMS cells *in vivo*, thus leading to xenograft tumour regression and significantly extended survival of the mice (Figure 5.6). Hence, starting from the discovery of an RNA binding protein that interacts with the cIAP1 IRES, we were able to identify an important regulatory node of apoptotic resistance in RMS cancer cells and take advantage of a class of pharmaceutics that target cIAP1 protein for degradation, Smac mimetic compounds, as potential therapeutics for RMS cancer. Furthermore, IGF2BP1 could potentially be used as a biomarker to identify RMS cancers that would best respond to combined SMC and death ligands treatment.

The results presented in Chapter 5 further reiterate the importance of selective translation regulation, and in particular of IRES-mediated translation regulation in carcinogenesis. In the case of the cIAP1 IRES, we were fortunate that there was a drug already in advanced clinical trials that could target the output protein for degradation (reviewed in [4, 24]). Similarly, the Bcl-XL IRES for which identification of PDCD4 as an ITAF and its inhibition of Bcl-XL IRES mediated translation in glioblastoma led to the successful use of the small molecule compound ABT737 in combination with doxorubicin to trigger glioblastoma cancer cell death.[25, 26] However, in cases where there is no drug available to target the deregulated output protein, one could consider targeting the interaction between a positive ITAF regulator and its IRES. Indeed, small molecule library screens could be performed and IRES activity used as readout to identify compounds that disrupt the interaction and regulation between a given ITAF and its target IRES. Such screens were performed with success for the VEGF IRES and the Myc IRES and led to the identification of cardiac glycoside compounds that can inhibit translation from these IRES.[27]

However there are pros and cons to using such an approach to targeted therapy of cancer. One of the advantages of ‘‘drugging’’ the interaction between an IRES and its regulating ITAF is that it is more specific than targeting the translated protein or upstream regulatory pathways. Indeed, cellular IRES do not share much similarity in secondary structures or in the ITAFs that interact with them[28]. Therefore, a compound that disrupts the interaction between a given ITAF and structured IRES is likely to be specific and can be rendered even more specific by the addition of other chemical groups. However, because proteins usually share functional domains, it is more likely that a drug designed to target a specific protein will affect other proteins and have non-specific effects. This is the case for

SMCs that target several members of the IAP family such as XIAP, cIAP1, cIAP2 and Survivin[20, 21]; although in the context of TNFα-induced cell death in RMS, we showed that sensitisation to cell death is solely due to depletion of cIAP1 (Figure 5.4 and Suppl. Figure 5.5). Another advantage of targeting the interaction between an IRES and its regulating ITAF is that there is no or little ‘‘leakage’’ of remaining activity. Indeed, considering that the transcript is not translated in a cap-dependent manner, efficient blocking of the interaction between a positive ITAF regulator and its target IRES should inhibit any translation initiation from that IRES, resulting in no protein being produced. Instead, for a drug targeting the final protein product, leakage in activity can happen because the protein is already translated or because the dosage of the drug is not optimal.

There are also cons to the use of IRES-based therapies. One inconvenience is the fact that for most IRES, there is more than one ITAF regulating it and blocking the interaction with one of them does not preclude the IRES from functioning. For example in the case of the cIAP1 IRES, blocking the interaction between IGF2BP1 and the IRES would only inhibit cIAP1 protein translation by ~50 %, whereas the use of 100 nM of SMCs depletes more than 90% of cIAP1 protein in cells (Figure 5.1 and Suppl. Figure 5.2F). To address this limitation, one could design bulky compounds that will obstruct any binding to the IRES, thus blocking translation initiation. However this could be difficult for highly structured IRES. Moreover, for this to work, all the ITAFs that bind to the given IRES should be known and their binding sites mapped in order to design the best chemical compound inhibitors, adding to the complexity of such an approach to targeted therapy.

Finally, it is important to note that, like for any therapy, great caution should be taken to anticipate the undesirable effects of a given drug. Drugging the interaction between an

ITAF and its target IRES may have pleiotropic effects. In the case of cIAP1, blocking its IRES-mediated translation or depleting the protein directly by the use of SMCs will inhibit the canonical NFκB pathway, activate the non-canonical NFκB pathway and, in the presence of a death ligand, activate caspase-8 dependent apoptosis.[4] Hence, all of these considerations need to be taken into account when designing an IRES specific therapy of for any other type of drug design.

7.5 Combined therapies to improve the efficacy of treatment: The case of Smac mimetic compounds and modulators of innate immunity

In the last decade, cancer therapies have evolved from non-specific cytotoxic agents that kill rapidly dividing cells to specific, mechanism-based drugs. The use of chemotherapeutic agents to treat cancer has taught us that although they work well in certain types of cancer, they are often accompanied by a lot of cytotoxicity and drug-induced resistance to treatment.[29] One approach to overcome these problems has been to develop combined therapies in order to first avoid cytotoxicity by using lower doses of several drugs but most importantly, to ‘‘hit the cancer cells at different places’’ and avoid resistance to treatment. An example of such approach to therapy is to combine targeted therapy with immunotherapy.[29] Targeted therapies act by blocking essential biochemical pathways that are required for tumor cell growth and survival.[30] On the other hand, immunotherapy involves stimulation of the host immune responses to eliminate cancer cells recognised as ‘non-self’ entities.[31] Used separately, cancer cells can acquire resistance to these therapies respectively through mutations in a given pathway, or by escaping immune surveillance[29]. Instead, combined targeted and immune therapies have proven successful in some cases. For

instance, the use of SMCs was shown to greatly enhance T cell activity and combination of SMCs with tumour cell vaccines resulted in greater inhibition of melanoma tumour growth compared to either monotherapies.[32]

Hence, we wanted to take advantage of such an approach to cancer therapy in order to possibly improve the treatment of RMS cancers. In Chapter 6, I investigated the means of inducing TNFα production through the use of different modulators of innate immunity, in order to potentiate SMC-induced RMS cancer cell death, and to avoid the cytotoxicity associated with recombinant TNFα therapy. SMCs have been proven efficacious in inducing the apoptosis of several cancer types that have autocrine TNFα expression.[33, 34] However, about 90% of the cells in an NIH cancer cell lines panel were resistant to SMC treatment alone and when the cells were treated with a combination of SNC and TNFα, the sensitive portion increased to 48%.[33] This suggests that only a minority of cancer cells have autocrine TNFα production whereas about half of them are sensitive in the presence of an extrinsic death ligand is added. We observed the same phenomenon with RMS cancer cells where the Kym-1 cell line that has an autocrine TNFα production is highly sensitive to the SMC LCL161 alone, whereas RH36 and RH41 are only sensitive to LCL161 when high concentrations of TNFα (up to 100 ng/ml) are supplied to the cells (Figure 5.5). This provided the rationale to use modulators of innate immunity to induce the production of TNFα by RMS cancer cells or by immune cells, in order to synergize with SMCs. Importantly, this approach has been used with success to induce apoptosis and significant tumour regression of syngeneic models of breast and glioblastoma cancers.[34, 35]

In Chapter 6, we show that modulators of innate immunity such as VSVΔ51, IFNγ and TWEAK synergize with the SMC LCL161 to induce apoptosis of Kym-1 RMS cancer

cells and that this synergy is dependent on induction of TNFα production by these modulators of innate immunity. Unfortunately, RH36 and RH41 cells remained resistant to the combined treatment. We next showed that this resistance is due to failure of these RMS cancer cells to produce TNFα due to defects in IFNγ/TWEAK and downstream NFκB signalling. Efforts are underway in our laboratory to identify mutations and/or malfunctions in these signalling pathways in RH36 and RH41 cells. Once identified, the goal will then be to use additional targeted therapies in addition to SMC and IFNγ/TWEAK to overcome resistance to apoptosis.

The results obtained with Kym-1 cells are however encouraging because they show 1) that synergy can occur between SMCs and the modulators of innate immunity VSVΔ51, IFNγ or TWEAK; 2) that this synergy allows for the use of lower concentrations of the SMC LCL161 and of IFNγ/TWEAK, thus lowering the cytotoxicity associated with these treatments; and 3) that the resistance observed with RH36 and RH41 cells is a matter of these cells having not enough TNFα to induce apoptosis in the presence of SMCs, limitation that can potentially be overcome *in vivo* with the production of TNFα by immune cells present in the tumour microenvironment. Experiments are underway to test the efficacy of combined LCL161 and IFNγ or TWEAK treatments in syngeneic RMS cancer models.

7.6 Conclusions

In summary, my PhD research project aimed at studying the regulation of cIAP1 translation by the IRES trans-acting factors NF45 and IGF2BP1, and how this regulation is important in the context of cell biology and apoptosis in cancer. Through the different studies presented in this dissertation, it is evident that cIAP1 translation regulation by these

two ITAFs is highly important for maintaining proper functioning of the cell. Indeed, reduced NF45 expression leads to reduce cIAP1 and XIAP IRES-translation and subsequent defects in cytokinesis due to downstream deregulation of cyline E and Survivin protein levels. Furthermore, IGF2BP1 overexpression in RMS cancer cells leads to increased cIAP1 IRES-mediated translation and increased apoptotic resistance. The discovery that IGF2BP1 and cIAP1 are overexpressed in rhabdomyosarcoma provides a great potential for killing RMS cancer cells through the combined use of Smac mimetic compounds and of TNFα or TRAIL. Furthermore, immune modulators such as VSV-Δ51, IFNγ and TWEAK synergize with Smac mimetics to cause Kym-1 RMS cell death through TNFα production. Hence, our results advocate for the combined use of LCL161 and these immune modulators for RMS tumours that show autocrine TNFα production and possibly for those that are more refractory to TNFα production.

7.7 References

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APPENDIX A:

Assessment of selective mRNA translation in mammalian cells by polysome profiling.

PREAMBLE

‘‘Assessment of selective mRNA translation in mammalian cells by polysome profiling’’ is a methods paper is published in the peer-reviewed journal of visualized experiments (reference: e52295, doi:10.3791/52295, 2014) that describes the polysome profiling technique. Polysome profiling is a technique that allows the identification of specific mRNAs that are selectively translated under specifc conditions. For instance, it was used throughout my thesis to determine the state of cIAP1 translation regulation by the NF45 and IGF2BP1 ITAFs.

The protocol is accompanied by a video that gives step by step description of the technique and that can be accessed at http://www.jove.com/video/52295/assessment-selective-mrna-translation-mammalian-cells-polysome?status=a54301k

Author list

Mame Daro Faye, Tyson E. Graber and Martin Holcik

Author contributions

MDF wrote the manuscript and performed the experiments for videotaping. TEG wrote the first version of a polysome profiling from which this one was adapted. MH provided editorial support and made the figures and the illustrations for the video.

ABSTRACT

Regulation of protein synthesis represents a key control point in cellular response to stress. In particular, discreet RNA regulatory elements were shown to allow to selective translation of specific mRNAs, which typically encode for proteins required for a particular stress response. Identification of these mRNAs, as well as the characterization of regulatory mechanisms responsible for selective translation has been at the forefront of molecular biology for some time. Polysome profiling is a cornerstone method in these studies. The goal of polysome profiling is to capture mRNA translation by immobilizing actively translating ribosomes on different transcripts and separate the resulting polyribosomes by ultracentrifugation on a sucrose gradient, thus allowing for a distinction between highly translated transcripts and poorly translated ones. These can then be further characterized by traditional biochemical and molecular biology methods. Importantly, combining polysome profiling with high through-put genomic approaches allows for a large scale analysis of translational regulation.

INTRODUCTION

Regulation of protein synthesis (translation) is a key cellular process which is intimately linked to cellular survival. Given that translation consumes more than 50% of cell's energy, it is not surprising that translation is tightly regulated and that perturbations in translation are not well tolerated. Conversely, many aberrant cellular processes require that translation machinery and translation output (i.e. proteome) have to be modified. This is often the case in various stress response and disease states, and is probably best exemplified in cancer. A key advantage of translational control is the ability of cells to rapidly reprogram

the protein output in response to various external and internal triggers, thereby fine tuning mechanism that tips the balance between cell survival and death[1].

Two aspects of translational control are particularly interesting; understanding of the nature of the regulatory mechanism(s) and control elements that allow for specific translation, and identification of specific mRNAs and their protein products that participate in the cellular response to the particular trigger that elicited selective translation in the first place. Polysome profiling is a technique that allows effective study of both processes.

The overall goal of the polysome profiling technique is to study and quantify the translation state of specific mRNAs under different cellular conditions. The principle of the technique is to capture mRNA translation by ‘‘freezing’’ actively translating ribosomes on different transcripts and separate the resulting polyribosomes by ultracentrifugation on a sucrose gradient, thus allowing for a distinction between highly translated transcripts (bound by several ribosomes) and poorly translated ones (bound by one or two ribosomes). In this particular protocol, the antibiotic cycloheximide that binds to the 60S ribosomal subunit and blocks the release of deacetylated tRNA from the ribosome E site[2], is used to inhibit ribosome translocation and stall ribosomes on translating mRNAs. However, other blocking agents such as emetine that also blocks translation elongation[3], can be used.

Unlike the western blotting and ^{35}S-Methionine labeling techniques that measure the final output of the translation process, polysome profiling has the advantage of measuring ribosomes association with actively-translated mRNAs, thus allowing for a more detailed study of translation mechanisms under different conditions. Hence, the technique can be easily adapted to specifically study different modes of translation[4-6], proteins factors involved in translation regulation[7-9], stress conditions affecting protein synthesis[1,10,11] or the

effects of mRNA structures such as IRES or upstream open reading frames on protein synthesis[12-14]. Nowadays, polysome profiling applications are even broader with the use of DNA microarrays [15] or next-generation sequencing [16,17] to analyze polysomes-associated mRNAs.

PROTOCOL:

A note on working with RNA: Take **s**tandard precautions to protect against RNA degradation by RNases. Use gloves, barrier pipette tips, RNase-free plasticware and chemicals in all steps of the protocol. Use ultrapure or DEPC-treated water for all solutions. Decontaminate any suspected surfaces with RNase inactivation solution following the manufacturer's protocol.

1. Preparation of solutions.

1.1) Prepare 500 mL of Basic Solution (0.3 M NaCl; 15 mM $MgCl_2 \cdot 6H_2O$; 15 mM Tris-HCL, pH 7.4). Mix using a stir bar until the solution is clear and pass through a 0.45 μm filter. Store at room temperature.

1.2) Prepare 10 and 50% Sucrose Solutions and 60% Chase Solution as follows:

1.2.1) For 10% Sucrose Solution (w/v), in a 50 ml Centrifuge tube add 5 g sucrose and fill to 50 ml with Basic Solution. For 50% Sucrose Solution (w/v), in a 50 ml centrifuge tube add 25 g sucrose and fill to 50 ml with Basic Solution.

1.2.2) For 60% (w/v) Chase Solution, in a 50 ml centrifuge tube add 30 g sucrose and fill to 50 ml with Basic Solution. Add 100uL of saturated bromophenol blue solution (add

bromophenol blue to water until not more will dissolve; centrifuge to pellet the undissolved powder) to the 60% chase solution. Mix using a vortex and verify complete dissolution.

1.2.3) Store solutions at 4 °C until needed.

1.3) Prepare RNA lysis buffer. Prepare this buffer fresh on the day of the experiment. Prepare 500 µL of RNA lysis buffer for each sample. To 1 ml of Basic Solution add 10 µL Triton X-100 (1% [v/v] final), 1 µL of 100 mg/ml cycloheximide in DMSO (CHX, 0.1 mg/ml final) and 3.5 µL RNasin (140 U/ml). Mix using a vortex. Keep on ice.

CAUTION! Cycloheximide is highly toxic and may cause mutations. Avoid skin contact and inhalation. To avoid handling the powder form too often, prepare a larger quantity of 100 mg/ml stock in DMSO, aliquot and keep at -80 °C for long term storage. Keep working stock at -20 °C.

1.4) The day of the experiment, prepare Sucrose+CHX Solutions by adding CHX (final concentration of 0.1 mg/ml) to the desired volume of 10% and 50% sucrose solution (~7 ml of each solution per gradient).

2. Preparation of sucrose gradient using an automated gradient maker

2.1) Turn the gradient maker ‘‘ON’’ and level the plate that will hold the gradients (critical step!) Click on ‘‘Done’’ when plate is leveled.

2.2) Select on the menu the gradient program to be run:

2.2.1) Press ‘‘GRAD’’ menu and select ‘‘LIST’’.

2.2.2) Select “SW41”.

2.2.3) Scroll through the list and select the ‘‘10-50% gradient - 11 steps’’ program by pressing the ‘‘RUN’’ button.

2.3) Place a conical ultracentrifuge tube (SW-41 tube, 14x89 mm) into the Marker Block and use the provided fine tube marker to trace a mark on the tube along the upper step of the Marker Block. Place tubes in a fitting rack on a steady surface.

Note: This mark will be the filling guide for layering the 10 and 50% sucrose solutions.

2.4) Attach the two provided cannula to two 10 ml syringes and wash by pipetting up and down with warm distilled water containing RNase inactivation solution. Make sure to remove all traces of water afterwards.

2.5) Fill one of the syringes with 10% sucrose+CHX and insert the cannula at the bottom of the ultracentrifuge tube. Gently dispense 10% sucrose solution until it goes just past the mark made on the tube. Note: Avoid making bubbles. If bubbles form, gently tap tube to displace them.

2.6) Fill the other syringe with 50% sucrose+CHX, wipe extra liquid off with a wipe and gently insert cannula at the bottom of the ultracentrifuge tube. Gently dispense the 50% sucrose solution until it reaches exactly the mark. Quickly and smoothly remove the cannula.

Note: The 10% sucrose should rise in the tube and form a meniscus at the top. If not, add more 10% sucrose solution at the surface.

2.7) Insert the provided cap by tilting the ultracentrifuge tube slightly and displacing all air bubbles. Remove excess liquid in the cap's reservoir with a micropipet.

2.8) Wipe excess liquid along the tube wall and place on the gradient maker plate.

2.9) Press the ''RUN'' button. **Note:** The plate will tilt at the specified angle, pause for 5 sec and the rotations to form the gradient will begin.

2.10) When gradient formation is complete (about 2 mins, the machine will beep), gently remove the ultracentrifuge(s), place on rack and swiftly remove cap in an upward movement

to avoid disturbing the gradient.

2.11) Keep gradient(s) on ice. Don't disturb! Alternatively, gradients keep overnight at 4 °C.

2.12) Alternatively, use a two-chamber gradient maker to make gradients as follows:

2.12.1) Rinse tubing and gradient maker with RNase inactivation solution and RNase-free water.

2.12.2) Add 5 ml of 50% sucrose+CHX to proximal chamber of gradient maker and ensure that any air between the two chambers is removed.

2.12.3) Add 5 ml of 10% sucrose+CHX to distal chamber of gradient maker.

2.12.4) Add 0.5 ml of 50% sucrose+CHX to bottom of a conical centrifuge tube (SW41 tube, 14x89 mm) and place in tube holder.

2.12.5) Turn on stirrer placed in proximal chamber, open stop-cocks and deposit gradient at speed set to "3" (about 1ml/min).

2.12.6) Place gradient(s) on ice. Don't disturb! Note: Gradients can be kept overnight at 4 °C.

3. Cell lysis and ultracentrifugation.

3.1) Place SW-41 rotor and buckets at 4 °C and set centrifuge to 4 °C.

Note: The cell lysis procedure is optimized for two 150 mm sub-confluent (~70-80% confluent) plates of HEK293 cells per gradient and the volumes used may need to be adjusted for different cell lines.

3.2) Plate cells at the appropriate cell density in the growth media at least 24 hours prior to lysis.

3.3) Prepare an aliquot (20 ml per 150 mm plate) of growth media containing 0.1 mg/ml

CHX.

3.4) Incubate cells in CHX + media (20ml per 150mm plate) for 3 minutes at 37 °C/5% CO_2 to arrest and stabilize polysomes.

3.5) Working quickly, aspirate media from plates, place plates on ice and wash cells once with 10 ml ice-cold phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4) supplemented with CHX (final concentration of 0.1 mg/ml) being careful to pipette slowly down the side of the dish wall to minimize lifting of the cell monolayer.

3.6) Aspirate PBS and add an additional 10 ml PBS+CHX to each plate, scrape cells with a cell lifter and transfer the total volume from both plates to a 50ml Centrifuge tube on ice. Retain 1 ml of cell suspension in a microfuge tube on ice for downstream protein analysis.

3.7) Centrifuge the 50 ml tube of cells at 300 x g at 4 ^{o}C for 10 minutes.

3.8) Remove all PBS and resuspend pellet in 500 µl of ice-cold RNA lysis buffer.

3.9) Transfer cell suspension to a 2 ml microcentrifuge tube.

3.10) Pipette up and down to lyse cells and incubate on ice for 10 mins with occasional vortexing.

3.11) Centrifuge at 2,000 x g at 4 ^{o}C for 5 min to pellet nuclei.

3.12) Transfer supernatant to a new 2 ml microcentrifuge tube.

3.13) Centrifuge at 17,000 x g at 4 ^{o}C for 5 min to pellet cell debris.

3.14) Transfer supernatant to a new 2 ml microcentrifuge tube and use 2 □l to measure optical density at 260 nm (use lysis buffer as blank).

3.15) Remove 50 □l (10% of total) from each sample for analysis of total RNA. **Note:** Lysate can be stored at -80 ^{o}C until needed.

3.16) Gently load equal A260 units onto each sucrose gradients (min. 10 OD, optimal 25 OD, in a maximum volume of 500µl; dilute concentrated lysate with additional lysis buffer if needed).

3.17) Ensure gradients are within 0.1 g of each other prior to ultracentrifugation.

3.18) Centrifuge gradients at 39,000 rpm (260,343 x g) for 1.5 hours at 4 ^{o}C.

3.19) During spindown keep rotor brake on and turn it off during deceleration when it reaches 1,000 rpm. **Note:** This step minimizes any disturbance of the gradients caused by abrupt changes in acceleration as the brush heads contact the rotor during braking.

4. Gradient fractionation system instrument set-up.

4.1) Set-up the instrument and blank the spectrophotometer with water as follows:

4.1.1) Turn on the components and allow the lamp to warm-up for at least 15 minutes.

4.1.2) Ensure fraction collector is set to the "polysome" method (press folder icon twice; return arrow twice to return to home screen).

4.1.3) Ensure the valve on the fraction collector is set to waste (arrow pointing to trash bin icon). Place a 250 ml Erlenmeyer flask containing distilled water under the waste collection tube.

4.1.4) Select the following settings on the chart recorder: Set Sensitivity dial to "SET LAMP AND OPTICS"; set Noise filter switch to "1.5"; set Peak separator dial to "OFF"; set Chart speed dial to "30 cm/hour" (5 cm/10 minutes); set Baseline Adjust dial (on top of the spectrophotometer unit) to "MAX OPEN".

Note: Optional: A digital recorder software can be used instead of the chart recorder. On the computer, click on "start". An acquisition window will open: under the Options menu,

uncheck Pause graphics. Under Scaling, set the graph's limits to -10 and 100 (can be changed on-the-fly during run). Click on the Save button to create a new file and record the run.

4.1.6) Place the syringe and barrel in the pump and tighten into place (use provided screws and Allen key). **CAUTION!** Do not over-tighten.

4.1.7) Fill the syringe with Chase Solution by using the ''REV'' command at RAPID speed. Place the pump in upright position and chase air through the tubing using the ''FORWARD'' command. Only use RAPID for this function and washes.

4.1.8) Install an ultracentrifuge tube filled with RNase-free water.

4.1.9) Pierce the ultracentrifuge tube with the cannula until the two black marks are visible (the dispensing hole is located between these two marks) and start syringe pump on ''Manual'' at 6.0 ml/min.

4.1.10) As the water passes through the flow cell (when chase solution fills 1/3 of the tube), switch speed to variable and set to 1.0 ml/min.

4.1.11) At a flow rate of 1.0 ml/min, adjust the Baseline Adjust dial on the spectrophotometer until the voltage on the chart is at zero (+/- 0.5 units). **Note:** Observe water exit from the waste tube into the Erlenmeyer flask.

4.1.12) Set the desired Sensitivity to "0.5" or "1.0" AU. **Note:** 1.0 AU works best for 10 OD units in a 10 ml gradient. If OD is lower than 10, "0.5" sensitivity can be used to enhance signal.

4.1.13) Push the Auto Baseline button and adjust the baseline setting to the 10% mark on the chart recorder by turning the Recorder offset dial (optional if using digital recorder).

4.11.14) Once a stable baseline is achieved, turn the pump switch to "OFF" and the Chart

Speed dial to 60 cm/hour if using analogue chart recorder or "OFF" if using digital recorder.

4.1.15) Recover the Chase Solution by switching pump to "REV" position (if needed one can increase flow rate back to 6.0ml/min...DO NOT USE RAPID) until it reaches the first mark on the piercing cannula.

4.1.16) Remove centrifuge tube and chase any air bubbles within the cannula by running a little bit of the Chase Solution through it. Wipe the piercing stage clean. **Note:** Some residual water may leak from the flow cell.

5. Gradient fractionation and collection of samples.

5.1) Install and pierce the centrifuge tube containing the sucrose gradient.

5.2) Place 11 2 ml microcentrifuge tubes in the fraction collector tray positions 1-11 such that the caps do not protrude upwards. Replace ‘‘tube #1’’ for now with a ‘‘waste tube’’ to collect any liquid left in the collector tubing.

5.3) Set the pump control dial to "Manual" and flow rate on the syringe pump to "6.0 ml/min"

5.4) Press the "Play" icon on the fraction collector. As soon as the arm reaches the first tube, press the ‘‘pause’’ button (this will position the arm and open the fraction-dispensing valve).

5.5) Start the pump by using the ‘‘FORWARD’’ command and monitor Chart recorder pen. When it starts deflecting upwards (indicating that sample is passing through the flow cell of the spectrophotometer), quickly replace ‘’waste tube’’ with ‘‘tube #1’’.

5.6) When the first drop is collected, quickly switch commands to "Remote start/stop", ‘‘Variable (1.0 ml/min)’’ and press ‘‘Play’’ icon. Note: The pump is now controlled by the fraction collector interface and 1 ml fractions will be collected every minute.

5.7) From this point on, A260 readings will appear on the digital recorder interface (or the analogue chart recorder). Make sure the ‘‘Record’’ button on the software is pressed!

5.8) After the blue Chase Solution enters the collecting tube or the last tube (usually tube 11), press the "Stop" icon. Note: The dispensing valve should switch to the waste valve.

5.9) Keep fractions on ice until all gradients have been fractionated. At the end of the day fractions can be stored at -80 ^{o}C prior to protein or RNA isolation. If protein *and* RNA analysis is required, split each fraction in half (500ul each for protein and RNA analysis).

6. Wash (this is an optional step to be performed only if multiple gradients are to be collected).

6.1) Submerge the waste-tube into the Erlenmeyer flask containing ~50 ml of ultrapure water and reverse the pump using a 6 ml/min flow rate.

6.2) Stop the pump when the Chase Solution reaches the first mark on the piercing cannula.

6.3) Remove the centrifuge tube, chase any air out of the cannula and clean the piercing stage. Note: Some residual water may leak from the flow cell.

6.4) Repeat the fractionation procedure starting at step 5.1.

7. Final clean-up.

7.1) Disconnect the pump tubing from the bottom of the piercer and pump the Chase Solution into a 50 ml centrifuge tube using the rapid flow setting. Store the Chase Solution at 4 ^{o}C as it can be reused.

7.2) Connect pump tube to a 3-way tubing system and close the valve leading to the syringe. Connect one tube to a 50 ml syringe filled with distilled water and the other tube to the base

of the piercer.

7.3) Install the ultracentrifuge tube used for the blanking step and pass at least 50 ml of distilled water through the spectrophotometer and collection tube (switch to collection menu on the interface by clicking the ‘‘Start/Pause’’ icon).

7.4) Remove tube, syringe (use Allen key to remove screws) and all other removable components and wash thoroughly using warm tap water and then rinse with ultrapure water. Note: Take special care when washing the plunger, barrel, tubing and piercing cannula. **CAUTION!** Handle the glass syringe barrel with care! Store all components away from dust.

7.5) Wipe bottom of flow cell with a soft tissue moistened with distilled water. Wipe-down the fraction collector tray and any other areas where the sucrose may have been spilled.

8. Isolation of RNA from sucrose fractions.

8.1) Prepare 50 µl of Proteinase K solution for every 1 mL of sucrose fraction (37.5 µl 10% SDS, 7.5 µl 0.5M EDTA, 1 µl Glycoblue, 4 µl of 20 mg/ml Proteinase K).

8.2) In 2 ml flat bottom tube, incubate 1 ml sucrose fraction with 50 □l of Proteinase K solution at 55 ^{o}C for 1 hour (if using 500 µl fractions adjust volume of Proteinase K solution accordingly).

8.3) Add an equal volume of phenol:chloroform:Isoamyl alcohol (125:24:1, preferably acidic (pH4.5) to minimize DNA contamination) to the sucrose fractions. **Note:** Add an extra 200 µl of chloroform to fractions 7-10 because they are heavier and phases could get inverted. CAUTION: phenol/chloroform can cause burns on contact and inhalation. Wear lab coat, safety goggles, and use a fumehood.

8.4) Vortex ~30 sec and centrifuge at maximum speed for 5 min at room temperature.

8.5) Remove ~80-90% of aqueous phase (do not touch interphase which could contain genomic DNA) and place in new tube.

8.6) Add an equal volume of chloroform and repeat step 8.4.

8.7) Remove aqueous phase being careful to avoid interphase.

8.8) Add 1:10 volume of 3 M sodium acetate, pH 5.2 (alternatively 5 M ammonium acetate can be used).

8.9) Add 1.5 volumes of chilled, absolute ethanol and vortex for 15 seconds.

8.10) Precipitate overnight at -20 ^{o}C (or 1 h at -80 ^{o}C if in a rush). Note: The samples can be left at -20^{o}C for longer if needed.

8.11) Centrifuge at maximum speed for 30 minutes at 4 ^{o}C.

8.12) Wash the pellet with 1 ml of chilled RNase-free 70% ethanol.

8.13) Air-dry the pellet and resuspend in 20 □l of RNase-free water.

8.14) Quantify total RNA by spectrophotometry to ensure adequate yield and purity (based on A260/280 ratio) and proceed to standard RT-qPCR analysis[14] using equal volumes of each fraction and PCR primers specific to the mRNA(s) of interest.

9. Isolation of proteins from sucrose fractions.

9.1 In addition to RNA, proteins can be isolated and identified in individual polysome fractions as well. Use one of many excellent protocols available for protein isolation, e.g [18].

REPRESENTATIVE RESULTS:

We have recently described a novel role for the tumour suppressor PDCD4 as a selective translation inhibitor of IRES-harboring mRNAs encoding apoptotic inhibitors XIAP and Bcl-xL[12]. Polysome profiling was a key technique to demonstrate the specific involvement of PDCD4 in IRES-mediated translation. HEK293 cells were transiently transfected to deplete endogenous levels of PDCD4 (Figure 1A) and then subjected to polysome profile analysis. We observed that reducing levels of PDCD4 did not impair global cellular translation, as judged by the lack of changes in the polysome profile when comparing siCTRL and siPDCD4 treated cells (Figure 1B). Similarly, polysome distribution of non-IRES variant of XIAP [19] was unchanged between treated and untreated cells (Figure 1C). In contrast, polysome distribution of the two IRES-harboring mRNAs, XIAP and Bcl-xL, was significantly altered. In the absence of PDCD4 the distribution of these two mRNAs is shifted into heavy ribopolysomes (Figure 1D, E). Since this is not accompanied by changes in steady-state levels of XIAP and Bcl-xL mRNA (Figure 1F) these results demonstrate enhanced translation of XIAP and Bcl-xL in cells with reduced PDCD4 levels, which was further confirmed by Western blotting (Figure 1G).

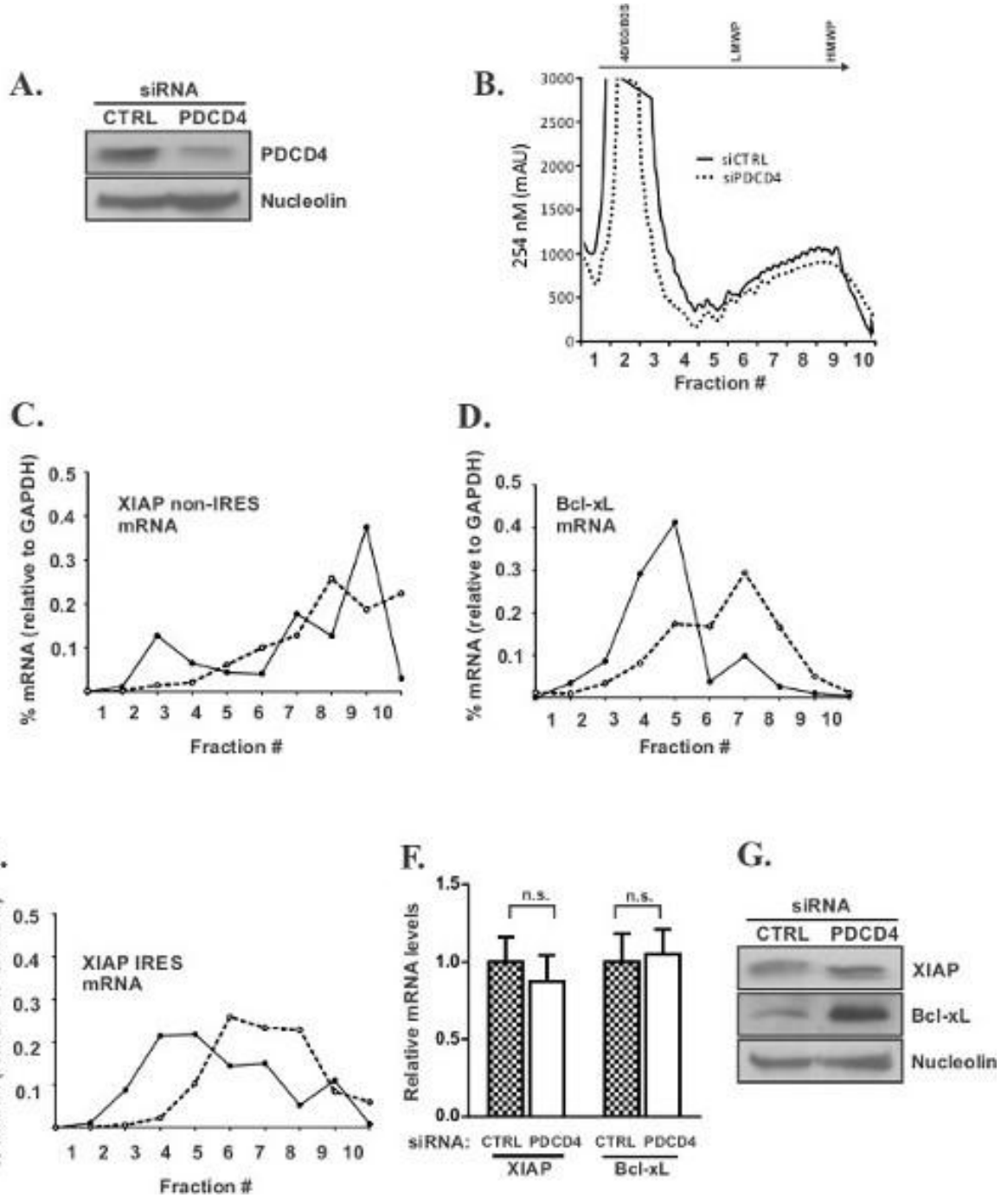
A.
siRNA
CTRL PDCD4
PDCD4
Nucleolin
B.
40S/60S
LMWP
HMWP
254 nM (mAU)
siCTRL
siPDCD4
Fraction #
C.
XIAP non-IRES mRNA
% mRNA (relative to GAPDH)
Fraction #
D.
Bcl-xL mRNA
% mRNA (relative to GAPDH)
Fraction #
E.
XIAP IRES mRNA
% mRNA (relative to GAPDH)
Fraction #
F.
Relative mRNA levels
n.s.
n.s.
siRNA: CTRL PDCD4 CTRL PDCD4
XIAP
Bcl-xL
G.
siRNA
CTRL PDCD4
XIAP
Bcl-xL
Nucleolin

Figure 1: Polysome profiling identifies PDCD4 as selective inhibitor of XIAP and Bcl-xL translation. (A) HEK293 cells were treated with PDCD4 siRNA or control (CTRL), non-targeting siRNA, lysed and subjected to Western blot analysis with anti-PDCD4 and anti-Nucleolin antibodies to verify the extent of PDCD4 knock-down. (B) PDCD4 was knocked down as in (A) and cell lysates were subjected to polysome profiling. Representative polysome profile is shown in the top panel; distribution of the XIAP non-IRES (C), Bcl-xL (D), and XIAP IRES (E) mRNAs relative to that of GAPDH is shown as the percent of total mRNA (% mRNA) in each fraction. (F) Steady-state mRNA levels were measured by RT-qPCR in PDCD4 siRNA or control (CTRL) siRNA treated cells. (G) The lysates from (A) were also subjected to western blot analysis with anti-XIAP, anti-Bcl-xL, and anti-Nucleolin antibodies. (NB. Data presented in Figure 1 were originally published in [12])

DISCUSSION:

Polysomal profiling is a powerful technique that allows for the quantification of the state of translation of specific transcripts under different treatment conditions. It is a very simple technique in principle yet it requires several steps of execution, some of which are critical for producing good polysome profiles. These key steps are: 1) using RNase-free chemicals and plasticware, 2) preparing good sucrose gradients (in our experience a 10-50% sucrose gradients work best for efficiently resolving 40/60S subunits and mRNAs with bound ribosomes), and 3) using cells that are exponentially growing and no more than 80% confluent in order to capture the highest rates of translation. This latter step should be taken into consideration when doing long cell treatments, such as gene knockdown or overexpression, so that the amount of cells to plate will be a function of how much the cells will be confluent at endpoint.

In the results presented here, polysome profile analysis was an important tool for assessing PDCD4 role in the translation regulation of the IRES-harboring mRNAs XIAP and Bcl-xL[12]. The fact that we did not observe any change in the general polysome profiles upon PDCD4 knock-down (Figure 1B) allowed us to conclude that PDCD4 did not impair global cellular translation under the conditions of the experiment. We next used RT-qPCR analysis to determine the distribution of the XIAP and Bcl-xL mRNAs in cells treated with PDCD4 or control siRNAs. qPCR is a method of choice for analyzing polysome profiles, as opposed to standard RT-PCR or Northern blotting, because it allows for a quantitative rather than semi-quantitative analysis of mRNA distribution and for the detection of subtle shifts in translational efficiency between treatments. Using this technique, we were able to show that the distribution of the XIAP and Bcl-xL mRNAs (expressed as a percent of total target

mRNA across the gradient) was significantly shifted into heavy polyribosomes (mRNAs with a large number of ribosomes associated with them) after PDCD4 knock-down (Figure 1D, E), thus indicating an increase in translation of these mRNAs. This was further confirmed by an increase in XIAP and Bcl-xL protein levels but not in their steady-state RNA levels (Figure 1F, G). Importantly, the polysome distribution of the non-IRES variant of XIAP was unchanged between treated and untreated cells (Figure 1C). Alternatively, translational efficiency could be presented as a ratio of mRNA content in polysomes (usually fractions 5 to 10) versus monosomes (usually fractions 2 to 4)[14].

The use of controls throughout the protocol is important in validating any polysome profiling result, as peaks observed from absorbance readings at 254 nm cannot on their own be attributed to ribosomal RNA (detergents, such as Triton X-100 strongly absorb in this region of the spectrum and may obscure 40/60S peaks at the top of the gradient). Blank gradients without lysate and/or with lysate buffer need to be run to determine the relative contribution of the buffers to the absorbance at 254 nm. Artefactual higher order structures can also lead to erroneous interpretations of polysomes where there are in fact none (e.g. "pseudo-polysomes"[20]). Sequestering magnesium (used throughout the procedure to stabilize 80S ribosomes) with the divalent cation chelator EDTA added to lysates and to the gradient buffer (20 mM for 15 min) will cause separation of the ribosome into its component small (40S) and large (60S) subunits. Thus, if absorbance peaks recorded at 260 nm are indeed polysomes, EDTA treatment will collapse the profile such that a single maximum will be observed near the top of gradient that corresponds to free mRNA and ribosomal units (data not shown). Coincident with EDTA-induced collapse of the profile, all of the specific targets analyzed by qPCR should now be found at the top of the gradient.

The number of ribosomes associated with a mRNA is not always an indicator of how efficiently that particular mRNA is being translated. Indeed, there are specific contexts in which active translation on polysomes becomes stalled [21,22] which the reader should be aware of. Determining whether or not transcripts are actively engaged with the translation machinery can be done by a) treating the sample with puromycin, which unlike EDTA, causes "run-off" of ribosomes that are actively translocating across a mRNA, or b) treating the sample with homoharringtonine which inhibits translocation of only the first ribosome at the start codon, again causing "run-off" of any downstream translocating ribosomes.

The use of control transcripts for qPCR analysis is also critical. The selection of transcripts that are not affected by different treatment conditions such as some housekeeping genes (GAPDH, Actin, Tubulin, Nucleolin), ribosomal mRNAs (18S, RPL13A) or in this case the non-IRES variant of the XIAP IRES, is important in verifying if the treatment's effect on translation is specific or generalized. These control transcripts can be used to normalize the distribution of the analyzed mRNAs as was done here (XIAP and Bcl-xL mRNAs were normalized to GAPDH mRNA and expressed as a percentage of the total mRNA content represented by the sum of mRNA content in every fraction) or alternatively, target and control mRNAs can be expressed as absolute values and shown separately. qPCR analysis of the input lysates (usually 10% of total volume) is another step that will control for the distribution of specific mRNAs. Ideally, the mRNA content of a specific transcript in the input lysate should be comparable to the sum of mRNA content in all 10 fractions analyzed. Finally, an optional step to control for the phenol:chloroform extraction step is to spike each polysome fraction with an *in vitro* transcribed reporter mRNA (such as CAT,

chloramphenicol acetyl transferase) that will be subsequently quantified by qPCR and can even be used to normalize the data [14].

As with any technique, the polysome profiling presents some limitations. The fact that usually a minimum of 10 fractions (more subtle shifts in translation efficiency may require 20 or more) need to be collected in order to have nice polysome distributions makes this step limiting, in the sense that only a maximum of 4 samples can be analyzed at a time to be able to comfortably handle RNA extraction and qPCR analysis of 40 samples and 4 input controls at once. The sample size can easily add up with how many transcripts are to be analyzed and how many qPCR replicates to do, and this can be costly. Another limitation of a qPCR-based polysomal profiling analysis is that it can only be done on a candidate-based approach (where the transcripts to be analyzed are known beforehand) and cannot be applied for genome-wide analysis of translation. However, at the beginning of the 21st century, investigators started to interrogate their polysomal RNA at a genomic level using DNA microarrays [15] and more recently with next-generation sequencing [16,17]. In this powerful approach, polysomal profiling is performed as described in this protocol except that rather than performing qPCR analysis of individual fractions, monosomes fractions (usually fraction 2-4) and polysomes fractions (usually fractions) are pooled together and variations in global translation analyzed by DNA microarray or whole-genome RNA sequencing. Hence with this approach, it is possible to assess all the mRNAs whose distribution shifts from polysomes to monosomes (decrease in translation) or vice-versa (increase in translation) upon a specific treatment. Validation and quantification of specific mRNA polysomes distribution can then be done by qPCR. An even more powerful use of the polysomal profiling principle is ribosome profiling. Further high throughput extension of

this technique was reported recently that allows for simultaneous monitoring of hundreds of polysome fractions [23]. This provides a clear advantage when probing translational efficiencies of mutant collections or in a large-scale RNAi screens. Ribosome profiling is a technique that takes advantage of next-generation sequencing to map RNA fragments protected by ribosomes (ribosome footprints) engaged in protein synthesis[24,25]. In this technique, ribosome translocation can be inhibited with cycloheximide (reversible) or emetine (irreversible) followed by cell lysis and RNase digestion to yield a population of ribosome footprints. Ribosomes are enriched, total RNA is isolated, and contaminating ribosomal and mitochondrial ribosomal RNA is removed. RNA footprints of approximately 26-28 nucleotides are isolated, reverse transcribed, converted into a cDNA library and sequenced[26]. Unlike standard polysome profiling, this approach not only identifies specific mRNAs that are translationally regulated but also yields mRNA-specific information at codon resolution such as the exact occupation and density of ribosomes along a specific transcript. This is particularly of interest for mRNAs with specific modes of translation such as IRES-harboring mRNAs, as it could give more information on where on the transcript ribosome-recruitment is occurring.

DISCLOSURES:

The authors have nothing to disclose.

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25 Ingolia, N. T. Ribosome profiling: new views of translation, from single codons to genome scale. *Nat Rev Genet.* **15** (3), 205-213, doi:10.1038/nrg3645, (2014).

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APPENDIX B:

Curriculum vitae

Mame Daro Faye

EDUCATION AND DIPLOMAS

2014 – present	Enrolled in the Doctor of Medicine and Master of Surgery (MDCM) program. McGill Medical School. Montreal, QC.
2010 – present	Doctorate in Biochemistry (Ph.D). University of Ottawa and Apoptosis Research Center, Children's Hospital of Eastern Ontario, Ottawa, ON. Thesis: *Regulation of the Cellular Inhibitor of Apoptosis 1 (cIAP1) translation* Supervisor: Dr Martin Holcik
2007 - 2009	Masters in Biochemistry (M. Sc). Université de Moncton and Atlantic Cancer Research Institute, Moncton, NB. Thesis: *Study of the regulation of Pax-5 expression in breast cancer – Role of estrogen receptors ERα and ERβ* Supervisor: Dr Rodney Ouellette
2003 – 2007	Bachelor in Biochemistry, specialisation, COOP option. Université de Moncton, Moncton, NB.

RESEARCH EXPERIENCE

PhD Candidate in Biochemistry, University of Ottawa and Apoptosis Research Center, Children's Hospital of Eastern Ontario, Ottawa, ON. (2010 – present)

My research project aims at characterising the translation control of the cellular inhibitor of apoptosis cIAP1 and how it contributes to apoptosis resistance of rhabdomyosarcoma cancer cells. As such, I have the following laboratory and research skills:

- Specific translation assays: polysome profiling, ^{35}S de novo protein synthesis assay, IRES reporter assay
- Specific RNA assays: RNA immunoprecipitation, RNA pull-down, ^{32}P UV-crosslinking RNA binding assay. REMSA, quantitative PCR.
- Diverse cell physiology assays: cell viability, cytotoxicity, apoptosis assays
- Other laboratory skills: establishment of stable cell lines, lentivirus production, Luciferase assay, ELISA, immunofluorescence, immunohistochemistry, site-directed

mutagenesis

- Research skills: critical thinking, experiments design, data analysis, peer-reviewed publications, grant writing.

Masters in Biochemistry, Université de Moncton and Atlantic Cancer Research Institute, Moncton, NB. (September 2007 – December 2009)

My project aimed at characterising the expression of Pax-5, a known oncogene involved in lymphomas, in breast cancer tumors. I have shown that Pax-5 expression in breast cancer cells was regulated by the estrogen receptors alpha and beta.

Skills developed: cloning techniques, luciferase reporter assays, EMSA, cell culture, western blot, immunoprecipitation, immunofluorescence, mmunohistochemistry of tissue microarrays, scientific writing (paper manuscript and Masters thesis).

Research assistant as a COOP student, Atlantic Cancer Research Institute, Moncton, NB. (June to August 2007)

My project consisted in characterising the cellular distribution of Pax-5 isoforms in B cell lymphomas by immunohistochemistry. Another aspect of my project was to determine the subcellular localisation of Pax-5 isforms by Differential Detergent Extraction followed by Free-Solution Isoelectric Focusing (DD-FS-IEF)

Laboratory technician in clinical biochemistry as a COOP student, University/Teaching Hospital Aristide Le Dantec, Dakar, Senegal. (June to August 2006)

My tasks consisted in sampling blood or urine from patients and analysing for various markers: blood/urine sugar levels, cholesterol and other lipids, uremia, reproductive hormone levels, APS levels, etc.

Laboratory assistant for high-throughput DNA plasmid sequencing, Biotechnology laboratory, Université de Moncton, Moncton, NB. (January to April 2006).

I was responsible for preparing bacterial DNA plasmids for sequencing, thus helping in a screen for the identification of antibiotics-producing bacteria that could be used as an alternative to chemical pesticides. My tasks consisted in tagging and amplifiying the bacterial DNA by PCR, then purify the products for sequencing

Research assistant as a COOP student, Biotechnology laboratory, biology department, Université de Moncton, Moncton, NB (May to August 2005)

My tasks consisted in doing bacteria isolates from soil, culturing bacteria in optimised media, extracting DNA and screening by PCR for potential genes responsible for antibiotic resistance. Once hits were found, I would follow up by confirming the bacteria antibioc potential in the presence of pathogenic fungi. I was also responsible for compiling results of the screen in a database and keeping it up to date.

TEACHING EXPERIENCE

Teaching assistant: basic classes in biochemistry and immunology – Chemistry and biochemistry department, Université de Moncton (September 2008 to April 2009)

I was responsible for marking assignments and exams for 3rd year classes in biochemistry and immunology as well as answering students' questions

Teaching assistant: advanced laboratories in biochemistry - Chemistry and biochemistry department, Université de Moncton (January to April 2008 and September to December 2008)

I was responsible for preparing 4th year biochemistry laboratories, monitoring them and marking students' performance.

PUBLICATIONS

1. Faye, M. D. and Holcik, M. ***The role of IRES trans-acting factors in apoptosis and cancer.*** Submitted to Biophysica Biochimica Acta – Gene Regulatory Mechanisms (Manuscript # BBAGRM-14-202).
2. Faye, M. D., Graber, T. E. and Holcik, M. (2014). ***Assessment of selective mRNA translation in mammalian cells by polysome profiling.*** doi:10.3791/52295. [Epub ahead of print].
3. Faye, M. D., Beug, S. T., Graber, T. E., Earl, N., Xiang, X,, Wild, B., Langlois, S., Michaud, J., Cowan, K. N., Korneluk, R. G. and Holcik, M. (2014). ***IGF2BP1 controls cell death and drug resistance in rhabdomyosarcomas by regulating translation of cIAP1.*** doi: 10.1038/onc.2014.90. [Epub ahead of print].
4. Faye, M. D., Graber, T. E., Liu, P., Thakor, N., Baird, S. D., Durie, D. and Holcik, M. (2014). ***Nucleotide composition of cellular IRES defines dependence on NF45 and predicts a post transcriptional mitotic regulon.*** *Mol. Cell. Biol.* 33(2):307-18.
5. Faye, M. D*., Liwak, U*. and Holcik, M. (2012). ***Translation Control in Apoptosis.*** *Exp. Oncol*. 34(3):218-30. * First co-authors.

ORAL PRESENTATIONS and CONFERENCE ABSTRACTS

1. Faye, M. D., Beug, S. T., Graber, T. E., Earl, N., Xiang, X,, Wild, B., Langlois, S., Michaud, J., Cowan, K. N., Korneluk, R. G. and Holcik, M. (2014). ***Characterization of the cellular inhibitor of apoptosis 1 (cIAP1) IRES trans-acting factors and their contribution to apoptotic resistance in Rhabdomyosarcomas.*** American Association of Cancer Research (AACR) 2014. San Diego, CA (Poster).
2. Faye, M. D. and Holcik, M. (2013). ***Role of IRES trans-acting factors in cytokinesis***

and cancer. Dr Robert Schneider's laboratory, Breast Cancer Program, NYU Cancer Institute. New York, NY. (Invited oral presentation)

3. Faye, M. D., Beug, S., Graber, T. E., Earl, N., Langlois, S., Cowan, K. N., Korneluk, R. G. and Holcik, M (2013). ***IGF2BP1 controls cell death and drug resistance in rhabdomyosarcomas by regulating the translation of the cellular inhibitor of apoptosis 1 (cIAP1)***. Children's Hospital of Eastern Ontario Research Day 2013. (Oral presentation)

4. Faye, M. D., Beug, S., Graber, T. E., Earl, N., Langlois, S., Cowan, K. N., Korneluk, R. G. and Holcik, M (2013). ***Identification of the insulin-like growth factor 2 mRNA binding protein (IGF2BP1) as an important regulator of cIAP1 translation and apoptosis in rhabdomyosarcomas.*** American Association of Cancer Research 2013, Washington, DC. (Poster)

5. Faye, M. D., Graber, T. E. and Holcik, M. (2012). ***Regulation of cIAP1 translation by the Insulin-Like Growth Factor 2 mRNA-Binding Protein 1 (IGF2BP1) in Rhabdomyosarcoma.*** Children's Hospital of Eastern Ontario Research Day 2012. (Poster)

6. Faye, M. D., Graber, T. E. and Holcik, M. (2011). ***Regulation of cIAP1 translation by the Insulin-Like Growth Factor 2 mRNA-Binding Protein 1 (IGF2BP1)***. Sixteenth Annual Meeting of the RNA Society RNA2011, Kyoto, Japan. (Poster)

7. Faye, M. D., Graber, T. E. and Holcik, M. (2010). ***Translational regulation of cIAP1 by the IRES trans-acting factor IGF2BP1***. Children's Hospital of Eastern Ontario Research Day 2010. (Poster)

8. Faye, M. D., Robichaud, G. and Ouellette, R. (2009). ***Studying the role of Pax-5 in breast cancer: Implication of oestrogen receptors α and β.*** Cancer Research Training Program 2009
Symposium, Halifax, NS. (Poster)

9. Faye, M. D. and Ouellette, R. (2009). ***Study of Pax-5 expression regulation in breast cancer: Implication of oestrogen receptors α and β.*** Armand-Frappier Congress 2009, Laval, Quebec.
(Oral presentation)

10. Faye, M. D. and Ouellette, R. (2009). ***Study of the activity of Estrogen Response Elements present on Pax-5 promoter and their potential role on regulating Pax-5 expression in breast cancer.*** Experimental Biology 2009, New Orleans, Louisiana, USA. (Poster)

11. Faye, M.D. and Ouellette, R. (2008). ***Study of Pax-5 isoforms expression in breast cancer.*** 20th contest of young researchers, Faculty of Graduate Studies and Research, Université de Moncton, Moncton, NB. ***3rd prize winner.*** (Poster)

12. Faye, M.D. and Ouellette, R. (2008). ***A study of Pax-5 isoforms expression in breast cancer.*** Atlantic Omics Symposium and Expo 2008, Moncton, NB. (Poster)

13. Faye, M. D., Griffiths, S. and Ouellette, R. ***Subcellular Fractionation and Protein Analysis of a Leukemia Cell-Line by Differential Detergent Extraction Followed by Free-Solution Isoelectric Focusing (DD-FS-IEF): Subcellular Immunoreactivity of Antisera Raised Against Peptides from Pax-5 isoforms.*** Atlantic Omics Symposium and Expo 2007, Moncton, NB. (Poster)

ACADEMIC TRAINING and OTHER SKILLS

Training	Graduate courses in RNA metabolism and molecular biology of diseases. Basic and graduate courses in biochemistry: molecular biology, inter- and intracellular mechanisms, proteins, lipids, metabolism, immunology, cellular biology, genetics and microbiology. Basic courses in chemistry: organic, inorganic, physical and analytical. Basic statistics. Advanced laboratories in biochemical techniques. Basic laboratories in analytical chemistry and organic synthesis. Scientific papers and grants writing.
Computer skills	Microsoft Office, BioEdit, RefWorks, EndNote, GraphPad Prism, Photoshop.
Languages	French: excellent, English: excellent, Wolof: mother tongue
Other skills	Methodical, polyvalent, collaborative and leadership

SCHOLARSHIPS and AWARDS

- University of Ottawa Admission Scholarship (September 2013 to August 2014) - $4,671
- Ontario Graduate Scholarship, University of Ottawa (May 2013 to April 2014) - $15,000
- Conference travel grant for the AACR 2013, Faculty of Graduate and Post-doctoral Studies, University of Ottawa (April 2013) - $300
- Institute Community Support Travel award – Canadian Institute of Health Research (CIHR, April 2013) - $1,000
- Vanier Canada Graduate Scholarship/Award, CIHR (May 2010 to April 2013) - $150,000
- University of Ottawa Excellence Scholarship (May 2010 to August 2014) – $42,861
- MITACS ACCELERATE Research award (August to November 2009) - $15,000

- Assomption Life Excellence Scholarship (April 2009) – $5,000
- Travel grant for the Experimental Biology 2009 Conference, Faculty of Graduate Studies and Research, Université de Moncton (April 2009) - $700
- Excellence Scholarship in Graduate Studies, Université de Moncton (September 2007 to April 2009)- $7,000
- ‘‘Programme Canadien de Bourses de la francophonie (PCBF)’’ scholarship, Canadian International Development Agency (September 2003 to April 2007) - $100,000

VOLUNTEERING ACTIVITIES

- Kitchen and Housekeeping Volunteer, Roger’s House, Ottawa, ON (March to July 2014)
- BMI Graduate Students Association, representing CHEO Research Institute (May 2012 to present)
- Fundraising and participation: Children’s Hospital of Eastern Ontario 2013 Telethon, Ottawa, ON
- Fundraising and participation: Children’s Hospital of Eastern Ontario 2012 Telethon, Ottawa, ON
- Participation: ‘‘ASBMB 5K Fun Run’’, 2009, New Orleans, Louisiana
- Fund raising and participation: ‘‘Assomption Life Run 2008’’, Dieppe, NB
- Fund raising and participation: ‘‘Curl for Cancer 2008’’, Moncton, NB
- Socio-cultural vice president at the Université de Moncton international students organisation (AEIUM), Moncton, NB (January to September 2005)
- Information and communication secretary at the students newspaper “Sans Frontières”, Université de Moncton, Moncton, NB (October 2003 to April 2005)
- Editor of Health section at the students newspaper “Sans Frontières”, Université de Moncton, Moncton, NB (October 2003 to April 2005)
- Reproductive health and HIV/AIDS peers advisor, ‘‘Fondation pour l’Enfance’’youth foundation, Bamako, Mali (1999 to 2002)
- Volunteer in public awareness campaigns during national vaccination days against poliomyelitis, Fondation pour l’Enfance, Bamako, Mali (1999-2001)
- Administrative secretary and project development at the “Groupe Amadou Toumani Touré (GATT)” youth foundation, Bamako, Mali (1999-2001)