CHARACTERIZATION OF THE PRO-APOPTOTIC FUNCTION OF eIF5A IN HUMAN CANCER CELL LINES

by

Zhong Sun

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Biology

Waterloo, Ontario, Canada, 2007

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis.

This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Eukaryotic translation initiation factor 5A (eIF5A) is the only known protein containing the unique amino acid, hypusine. eIF5A is present in all eukaryotic cells and is highly conserved, but its function is not well understood. The present investigation was undertaken to study the regulatory role of eIF5A in the induction of apoptosis in human cancer cell lines.

Suppression of eIF5A1 using specific siRNA was shown to have no effect on the growth and proliferation of mammalian cells, although inhibition of the post-translational hypusination of eIF5A1 resulted in G1 phase cell cycle arrest. Treatment of HT-29 human colon adenocarcinoma cells with eIF5A1 siRNA did, however, reduce their sensitivity to pro-apoptotic stimuli including nitric oxide, Actinomycin D, proteaosome inhibition and serum starvation. Furthermore, over-expression of eIF5A1 in HT-29 cells or Hela S3 human cervical carcinoma cells using adenovirus constructs strongly induced apoptosis in a time-dependent manner.

The pro-apoptotic effect of eIF5A1 appears to reflect at least in part its ability to activate the mitochondrial pathway of apoptosis in that its up-regulation resulted in dissipation of mitochondrial $\Delta\Psi$m, release of cytochrome c, activation of caspase 9 and caspase 3 and translocation of Bax from the cytosol to mitochondria. Similar effects were observed following treatment with eIF5A2, a second isoform of human eIF5A, and in addition eIF5A2 induced up-regulation of cleaved Bcl-2 which is thought to be pro-apoptotic. A mutant of eIF5A1 in which the conserved lysine, lysine$_{50}$, that is post-translationally modified to hyusine was switched to alanine [eIF5A1(K50A)] also proved capable of inducing apoptosis by activating the mitochondrial pathway. As well, eIF5A1 and eIF5A1(K50A) both induced strong up-regulation of p73, a homolog of the tumor suppressor p53, in Hela S3 cells containing null p53. The finding that up-regulation of eIF5A1 also resulted in activation of caspase 8 indicates that it may be involved in regulation of the death receptor pathway of apoptosis as well. This contention is further supported by confocal microscopy studies indicating that, following its up-regulation, eIF5A1 localizes to the inner surface of the plasma membrane in a time-dependent manner that correlates temporally with the induction of apoptosis.

eIF5A1 and its post-translationally modified forms were isolated by 2-dimensional Western blotting and sequenced by mass spectrometry. These analyses indicated that eIF5A1 containing unmodified lysine$_{50}$ is the form of the protein that accumulates coincident with induction of apoptosis either by up-regulation of eIF5A1 or treatment with NO. These observations, together with the

finding that eIF5A1(K50A) is capable of inducing apoptosis, indicate that it is the unhypusinated form of eIF5A1 that is apoptogenic.

Acknowledgements

Many people have given me their generous support and advice throughout the completion of my thesis in many different ways. Without their help, it would not have been possible for me to finish my project.

First of all, I would like to thank my supervisor Dr. John Thompson for giving me an opportunity to study and work in his laboratory, and providing me with tireless guidance, dedicated assistance as well as financial support. Your enormous knowledge and expertise were the most valuable resources for me.

I wish to thank members of my Ph.D. committee, Dr. Matt Vijayan and Dr. Bernard Duncker, for all their valuable suggestions, time, and effort towards this thesis. Especially, I would like to thank Dr. Vijayan for allowing me to use the fluorescence microscope and microplate reader in his lab.

I am also very grateful to Dr. Brendan McConkey. Without his valuable sights and suggestions as well as permission to use the 2DE equipment in his lab, my work on the different post-translationally modified forms of eIF5A would not have been possible.

I would also like to express my gratitude to my present and past lab members including Catherine Taylor, Mike Wang, Hong Ming, Songmu Jin, Marianne Hopkins, Bin Ye, Eva Adam, Bithi Eshaque, Dominic Cliché, Adrienne Boone, Boun Thai, Linda Nowack, Fengshan Ma, Zhongda Liu, Qifa Zheng, Chunguang Zhang, Ruth Tshin, Yulia Lampi, Jeremy Duguay, Greg Perry, Hsiu Yue Chen and Wendy Wu as well as Richard Dondero from Senesco. Working with them has been a truly wonderful experience, and I have learned a lot from everyone. Thank you all for your encouragement, and for making our lab an excellent and supportive working environment.

I would like to thank Mishi Savulescu and Dale Weber from the Department of Biology and Ju Guan from Health Studies & Gerontology for their invaluable technical support with flow cytometry and confocal microscopy.

Table of Contents

List of Figures

List of Tables

Abbreviations

AbeAdo	5'-([(Z)-4-aminobut-2-enyl]methylamino)-5'-deoxyadenosine
ACN	Acetonitrile
AG	Aminoguanidine
AIF	Apoptosis inducing factor
aIF5A	Archaeal initiation factor 5A
AMP	Apoptogenic mitochondrial protein
ANT	Adenine nucleotide translocator
Apaf-1	Apoptotic protease activating factor 1
ATP	Adenosine triphosphate
BCA	Bicinchoninic Acid
Bcl-2	B cell leukaemia/lymphoma-2
BH	Bcl-2 homology
BIR	Baculovirus IAP repeat
Bp	Base pair
BSA	Bovine serum albumin
CAD	Caspase-activated deoxyribonuclease
DAO	Diamine oxidase
DBD	DNA binding domain
DD	Death domain
DED	Death effector domain
DFMO	α-difluoromethylornithine
DHH	Deoxyhypusine hydroxylase
DHS	Deoxyhypusine synthase
DISC	Death-inducing signaling complex
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EF-P	Elongation factor P
eIF5A	Eukaryotic initiation factor 5A
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FA	Formic acid
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HIV-1	Human immunodeficiency virus type 1
HPV	Human papillomavirus
HTLV-1	Human T-cell leukemia virus type I

Htra2	HtrA serine peptidase 2
IAA	Iodoacetamide
IAP	Inhibitor of apoptosis
ICAD	Inhibitor of caspase-activated deoxyribonuclease
IFNα	Interferon α
IMS	Intermembrane space
IP	Immunoprecipitation
JNK	Jun N-terminal protein kinase
kDa	Kilodalton
MAP	Methionine amino peptidase
MEM	Minimum essential medium Eagle
MIM	Mitochondrial inner membrane
MOM	Mitochondrial outer membrane
MOMP	Mitochondrial outer membrane permeabilization
MW	Molecular weight
NAA	N-α-acetylation
NES	Nuclear export signal
NK	Natural killer
NLS	Nuclear localization signal
NMD	Nonsense-mediated decay
NME	N-terminal methionine excision
OB	Oligonucleotide/oligosaccharide binding
OD	Ornithine decarboxylase
ODC	Oligomerization domain
OPG	Osteoprotegerin
PBMC	Peripheral blood mononuclear cell
PBR	Peripheral benzodiazepine receptor
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
Pfu/cell	Plaque forming unit per cell
PI	Propidium iodide
POD	Peroxidase
PS	Phosphatidylserine
PT	Permeability transition
PTPC	Permeability transition pore complex
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRE	Rev response element
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SELEX	Systematic evolution of ligands by exponential enrichment
siRNA	Small interfering RNA
Smac	Second mitochondria-derived activator of caspases
SNP	Sodium nitroprusside
SSB	Single strand break
SV40	Simian virus 40
tBid	Truncated Bid
TAD	Transactivation domain
TBS	Tris buffered saline
TBS-T	Tris buffered saline with Tween 20
TdT	Terminal deoxynucleotidyl transferase
TMB	Transmembrane
TNF	Tumor necrosis factor
TNFα	Tumor necrosis factorα
TNFR	Tumor necrosis factor receptor
TRADD	TNF receptor-associated death domain
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TNF-related apoptosis-inducing ligand Receptor
TUNEL	TdT-mediated dUTP Nick-End Labeling
VDAC	Voltage-dependent anion channel
$\Delta\Psi m$	Mitochondrial transmembrane potential
2D	2-dimensional

Chapter 1 Introduction

1.1 Apoptosis

Apoptosis, also known as programmed cell death, is responsible for the deletion of unwanted cells and is critical for organ development, tissue homeostasis and elimination of defective or potentially dangerous cells in complex organisms (Reviewed by Arends and Wyllie, 1991). Apoptosis was originally observed and characterized by Kerr et al. (1972) when they were studying ischemic liver injury using electron microscopy. They observed a novel, controlled mechanism of cell death that is distinct from uncontrolled necrotic cell death. Apoptosis exhibits a characteristic morphology which includes cell shrinkage, plasma membrane blebbing, chromatin condensation, nuclear fragmentation, formation of apoptotic bodies and phagocytosis of apoptotic bodies by macrophages (Reviewed by Arends and Wyllie, 1991; Häcker, 2000). Apoptotic cells exhibit distinct biochemical characteristics such as activation of caspases, protein cleavage by caspases, phosphatidylserine (PS) exposure on the surface of plasma membrane and DNA fragmentation into 200 bp fragments by endonucleases (Hengartner, 2000).

Apoptosis can be triggered by various stimuli including developmental signals, DNA damage, lack of survival signals, viral infection, disruption of the cell cycle and cellular stresses including chemotherapeutic agents, cytokines, ionizing radiation, osmotic stress and starvation (Martin, 1993a; Dragovich et al., 1998). It is considered to be a process that eliminates superfluous, ectopic, damaged or mutated cells without a concomitant inflammatory response in the surrounding tissues. In contrast to necrosis, which is a form of cell death that results from acute cellular injury and happens in an uncontrolled manner, apoptosis is highly regulated and generally confers advantages during the life cycle of an organism (Dragovich et al., 1998; Hengartner, 2000). It starts with an initiation phase that is largely dependent on cell type and apoptotic stimulus, followed by integration and amplification of these signals by second messengers. The death effector proteases and nucleases, which are activated by systematic biochemical events during the amplification phase (Dragovich et al., 1998), participate in the degradation phase by cleaving target proteins and DNA (Arends et al., 1990; Ferri and Kroemer, 2000; Hengartner, 2000). Defects in the control of apoptotic pathways may contribute to a variety of diseases including cancer, autoimmune and neurodegenerative conditions and AIDS (Arends and Wyllie, 1991; Martin, 1993b; Thompson, 1995).

1.1.1 Caspases

Apoptosis is mediated in part by a family of proteins known as caspases, which are cysteine proteases that cleave at conserved aspartic acids (Reviewed by Earnshaw et al., 1999). In mammals, 14 caspases have been identified so far, although not all of them participate in apoptosis. Members of the caspase family can be divided into three groups based on their functions: (1) the initiators of apoptosis, including caspase 2, 8, 9, 10 and 12; (2) the effectors/executioners of apoptosis, including caspase 3, 6 and 7; and (3) participants in cytokine activation, including caspase 1, 4, 5, 11, 13 and 14 (Reviewed by Cohen, 1997; Thornberry, 1997; Nicholson, 1999; Degterev et al., 2003). Different initiator caspases are involved in the various types of stimuli-induced cell death. For example, caspase 8 and 10 and their adaptor Fas-associated death domain (FADD) are involved in death receptor-induced apoptosis. Caspase 9 and its adaptor Apaf-1 are involved in cellular stress (e.g., DNA damage, oncogene activation) -induced apoptosis (Cohen, 1997; Nicholson, 1999), whereas caspase 12 is involved in endoplasmic reticulum (ER) stress-induced apoptosis (Nakagawa et al., 2000).

Caspases produced as catalytically inactive zymogens are activated by proteolysis (Reviewed by Nicholson, 1999; Boatright and Salvesen, 2003). In detail, the initiator caspases, which exist in the cytosol as inactive monomers, have a protease domain and a long N-terminal prodomain that contains specific protein-protein interaction motifs to mediate its association with the adaptor proteins. Recruitment of the initiator caspase zymogens to the adaptor induces clustering and aggregation of the zymogens, stimulating their activation through self-proteolysis (Boatright et al., 2003). In contrast, the effector caspases, which also have a protease domain and a short prodomain, pre-exist as dimers in healthy cells. Their activation requires direct proteolytic cleavage of the protease domain by activated initiator caspases (Reviewed by Shi, 2002). Thus, initiators and executioners form a proteolytic caspase cascade.

Active executioner caspases mediate the degradation of various cellular substrates (Reviewed by Fischer et al., 2003). For example, caspases cleave the inhibitor of caspase-activated deoxyribonuclease (ICAD), thereby activating caspase-activated deoxyribonuclease (CAD) which is a cytoplasmic endonuclease that, upon activation, enters the nucleus and degrades chromosomal DNA (Enari et al., 1998). The cleavage of DNA, which is a signature of apoptosis, has a protective function for the whole organism in that it reduces the likelihood of genes in a potentially active site being transferred from dying cells to the nucleus of viable neighboring cells (Arends et al., 1990). Caspases are also responsible for the proteolytic cleavage of a diverse array of structural and regulatory

proteins which are crucial for the maintenance of DNA repair, translation, cell cycle control, the cellular cytoskeleton, the nuclear scaffold and signal transduction (Kaufmann et al., 1993; Martin et al., 1995; Mashima et al., 1995; Song et al., 1996; Widmann et al., 1998). The action of these caspases gives rise to the apoptotic phenotype.

Activated caspases are also thought to create a positive feedback loop that contributes to amplification of the death signal by processing and activating the zymogens of other members of the caspase family (Slee et al., 1999; Van de Craen et al., 1999; Viswanath et al., 2001) and by altering or reversing the function of certain cellular target proteins. For example, Bid, which is cleaved by caspase 8 in the extrinsic pathway, can activate the mitochondrial pathway, thus amplifying the apoptotic signal (Li et al., 1998; Luo et al., 1998). Bcl-2 and Bcl-xL, on the other hand, can be cleaved by effector caspases, which cause them to switch from anti- to pro-apoptotic molecules (Cheng et al., 1997; Clem et al., 1998; Fujita et al., 1998).

1.1.2 Extrinsic apoptotic pathway

Two pathways of caspase-mediated cell death have been described for mammals. The extrinsic or death receptor-mediated pathway is critical to immune homeostasis, whereas the intrinsic, mitochondria-dependent pathway is used extensively in response to extracellular cues and internal insults such as DNA damage, heat shock and oncogene activation (Reviewed by Danial and Korsmeyer, 2004).

Initiation of the extrinsic apoptotic pathway requires the action of extracellular messengers, termed death ligands. The binding of death ligands to members of the membrane-bound tumor necrosis factor receptor (TNFR) family (e.g., TNFR1, Fas, and TRAIL receptor 1 and 2) is followed by the formation of the death-inducing signaling complex (DISC), which in turn results in the activation of procaspase 8 and 10 (Reviewed by Nagata, 1997; Ashkenazi and Dixit, 1998, 1999; Peter and Krammer, 2003). Caspase 8 is necessary for TNFα-, FasL- or TRAIL-induced apoptosis (Varfolomeev et al., 1998). On the other hand, although caspase 10 is activated in the signaling complexes induced by death ligand treatment, it does not play a critical role in the extrinsic pathway (Sprick et al., 2002; Jin and El-Deiry, 2006).

1.1.2.1 FasL

Expression of Fas Ligand (FasL) is restricted mainly to activated T cells and natural killer (NK) cells and to immune-privileged sites (Reviewed by Nagata, 1997). In response to Fas signaling, Fas receptors self-assemble into trimers upon binding with three FasL molecules at the cell surface. The clustering of Fas induces the recruitment of the adapter protein FADD, which bears both death domains (DD) and death effector domains (DED). FADD binds to Fas via the DD and recruits procaspase 8 via the DED (Chinnaiyan et al., 1995; Boldin et al., 1996). A high local concentration of procaspase 8 at DISC causes its autocatalytic activation and subsequent activation of the downstream effector caspases (Muzio et al., 1998). Activation of the Fas signaling pathway by the addition of FasL, or even receptor agonist antibodies, rapidly triggers cell death (Nagata, 1999; Yonehara, 2002; Adachi et al., 2003).

1.1.2.2 TNFα

Tumor necrosis factor α (TNFα) is produced mainly by activated macrophages and T cells in response to infection (Reviewed by Liu, 2005). Ligation of TNFα to TNFR1 recruits the TNF receptor-associated death domain (TRADD) to the receptor (Hsu et al., 1995). TRADD then triggers the recruitment and oligomerization of FADD, forming DISC. Oligomerized FADD then recruits procaspase 8 via its DED domain, a process that leads to the activation of caspase 8 (Chinnaiyan et al., 1996; Varfolomeev et al., 1996). However, unlike FasL, TNFα is not cytotoxic to most cells because TRADD also recruits receptor-interacting protein (RIP) and TNFR-associated factor 2 (TRAF2) resulting in activation of the NF-κB-mediated survival pathway and Jun N-terminal protein kinase (JNK) (Rothe et al., 1995; Hsu et al., 1996). Although activated JNK is thought to play a pro-apoptotic role in TNFα-induced signaling (Deng et al., 2003), the activation of NF-κB can block the activation of caspase 8 by activating a group of gene products that function cooperatively to suppress TNFα-mediated apoptosis (Beg and Baltimore, 1996; Wang et al., 1998). Therefore, the destiny of the cell depends on the balance between the apoptosis and survival signaling pathways (Reviewed by Wallach et al., 1999). TNFα-induced apoptosis requires NF-κB inhibition, which can be achieved by blocking RNA or protein synthesis (Wang et al., 1998).

1.1.2.3 TRAIL

Another set of death receptors has been shown to respond to a different death ligand known as TNF-related apoptosis-inducing ligand (TRAIL). Unlike FasL and TNF, both of which are only transiently expressed in activated cells, TRAIL mRNA has been detected in a wide variety of tissues, most predominantly spleen, lung and prostate (Wiley et al., 1995). In humans, TRAIL may interact with any one of five membrane receptors (Reviewed by Bouralexis et al., 2005). The primary function of TRAIL receptor 1 (TRAIL-R1) and TRAIL receptor 2 (TRAIL-R2), both of which have a cytoplasmic DD, is to elicit an apoptotic death response upon ligation of TRAIL by inducing DISC formation and caspase activation, events that are similar to those of the FasL and TNFα pathways (Chaudhary et al., 1997; MacFarlane et al., 1997). The other three receptors, TRAIL-R3/DcR1, TRAIL-R4/DcR2, and osteoprotegerin (OPG), have truncated, nonfunctional DDs (Degli-Esposti et al., 1997; Sheridan et al., 1997; Marsters et al., 1997; Pan et al., 1997; Emery et al., 1998). Since these receptors are not capable of activating the caspase cascade but may rather activate NF-κB and block apoptosis, they are considered as antagonistic "decoy" receptors for the DD-containing TRAIL-R1 and R2. Transient over-expression of the decoy receptors confers resistance to TRAIL-induced apoptosis (Degli-Esposti et al., 1997; Sheridan et al., 1997).

Among members of the TNF family, TRAIL is unique for its potent anticancer effect and low toxicity toward normal tissues *in vivo* (Ashkenazi et al., 1999; Walczak et al., 1999). Indeed, while cancer cells are normally sensitive to TRAIL-mediated apoptosis, normal cells display relativly high resistance to TRAIL due to the expression of decoy receptors (Reviewed by LeBlanc and Ashkenazi, 2003).

1.1.3 Intrinsic apoptotic pathway

In addition to their involvement in metabolism and energy generation, mitochondria also play an important role in the initiation of the mitochondrial apoptotic pathway, also known as the intrinsic pathway, by inducing the release of apoptogenic mitochondrial proteins (AMP) (Reviewed by Harris and Thompson, 2000; Lemasters et al., 1999; Zamzami and Kroemer, 2001; McBride et al., 2006). In detail, in response to cellular stress such as DNA damage, mitochondrial outer membrane permeabilization (MOMP) occurs, allowing the release of soluble proteins retained in the intermembrane space (IMS) of mitochondria (Reviewed by van Gurp et al., 2003). The first indication of the importance of mitochondria in apoptosis arose from the finding that cytochrome *c* is released

from the IMS into the cytoplasm in response to apoptotic stimuli (Liu et al., 1996), and that cytochrome *c* facilitates apoptosis by associating with the apoptotic protease activating factor 1 (Apaf-1) in the presence of dATP or ATP. Apaf-1, which pre-exists in the cytoplasm as a monomer, contains a caspase recruitment domain (CARD) and an ATPase domain. The hydrolysis of dATP/ATP and the binding of cytochrome *c* promote Apaf-1 oligomerization, inducing the formation of a large multimeric Apaf-1:cytochrome *c* complex, termed apoptosome (Li et al., 1997; Hu et al., 1999). The apoptosome recruits procaspase 9 via the respective N-terminal CARD, inducing auto-proteolytic cleavage and activation of the caspase 9 zymogen (Li et al., 1997; Srinivasula et al., 1998; Zou et al., 1999).

In addition to cytochrome *c*, second mitochondria-derived activator of caspases (Smac; also known as DIABLO) (Du et al., 2000; Verhagen et al., 2000), HtrA serine peptidase 2 (Htra2; also known as Omi) (Suzuki et al., 2001), endonulease G (Li et al., 2001) and apoptosis inducing factor (AIF) (Susin et al., 1999; Lorenzo et al., 1999) are also released from mitochondria of cells which are exposed to apoptotic stimuli. Smac and Htra2 enhance and accelerate caspase-dependent apoptosis by binding and neutralizing inhibitors of apoptosis (IAP) (Du et al., 2000; Verhagen et al., 2000; Suzuki et al., 2001). IAPs inhibit the activation of caspases through their baculovirus IAP repeat (BIR) domains (Takahashi et al., 1998). Endonuclease G and AIF, two other IMS proteins released after MOMP, accelerate the apoptosis process by facilitating DNA fragmentation (Susin et al., 1999; Lorenzo et al., 1999; Li et al., 2001; Candé et al., 2004).

1.1.4 Cross-talk between the extrinsic and intrinsic pathways

Although the death receptor pathway and the mitochondrial pathway are capable of operating independently, accumulating evidence suggests that the signaling from these two pathways for caspase activation can sometimes be interconnected (Li et al., 1998; Luo et al., 1998; Deng et al., 2003). Bid, a pro-apoptotic BH3-only Bcl-2 family protein, is cleaved by caspase 8 in response to death receptor signaling, generating a truncated C-terminal fragment named tBid. tBid translocates from the cytosol to the mitochondrial outer membrane (MOM) to induce the release of cytochrome *c*, which in turn activates caspase 9 (Li et al., 1998; Luo et al., 1998). As well, in response to TNFα signaling, Bid can be cleaved by the activated JNK pathway, generating jBid (JNK-mediated cleaved Bid) (Deng et al., 2003). jBid translocates to mitochondria to induce the selective release of Smac, which functions to relieve the inhibition of caspase 8 activation (Du et al., 2000). In fact, cells have

been classified according to their response to apoptotic stimuli (Scaffidi et al., 1998). In type I cells, induction of apoptosis is accompanied by activation of large amounts of caspase 8 in the DISC, without the necessity for amplification of the apoptotic signal by proteolysis of tBid. In type II cells, the apoptosis signal generated from the activation of caspase 8 within the DISC is not strong enough, and therefore the mitochondrial amplification loop involving the generation of tBid and formation of the apoptosome is required in order to generate sufficient caspase activity to kill the cell.

1.1.5 Caspase-independent apoptosis

Recent studies have demonstrated that some types of cell death can occur in the absence of caspase activation. Indeed, in most, if not all, models of stress-induced apoptosis, and even death receptor-induced apoptosis, specific caspase inhibitors are unable to inhibit apoptosis (Xiang et al., 1996; McCarthy et al., 1997; Deas et al., 1998; Pastorino et al., 1998; Dumont et al., 2000; Eby et al., 2000; Cheng et al., 2001). This suggests that there are factors other than caspases that mediate apoptosis. Since mitochondrial permeability transition (PT) occurs in most of these cases, it is thought that some of these factors may reside normally in mitochondria, with AIF and endonuclease G being candidates (Susin et al., 1999; Li et al., 2001).

1.1.5.1 AIF and chromatin condensation

AIF is a highly conserved mitochondrial flavoprotein that is released from mitochondria after mitochondrial membrane depolarization and is thought to be capable of inducing apoptosis independently of caspases (Susin et al., 1999). Released AIF redistributes to the nucleus, where it induces chromatin condensation and large-scale DNA fragmentation (more than 50 kilo base pairs), events that resemble those observed in cells in which apoptosis is induced under conditions of caspase inhibition (Xiang et al., 1996; McCarthy et al., 1997; Deas et al., 1998; Pastorino et al., 1998; Dumont et al., 2000; Eby et al., 2000). Therefore, it has been suggested that AIF is responsible for at least some of the caspase-independent features of apoptosis (Candé et al., 2004). The mechanism by which AIF mediates chromatin condensation and DNA fragmentation is still not clear. It has been proposed that it might have a concealed nuclease activity, or that it might bind to DNA and recruit proteases and nucleases that cause chromatin condensation (Hong et al., 2004).

1.1.5.2 Endonuclease G

Endonuclease G is another mitochondrial protein that translocates to the nucleus during apoptosis (Li et al., 2001; Parrish et al., 2001). It belongs to a family of DNA/RNA non-specific nucleases (Rangarajan and Shankar, 2001), which can act on all types of nucleic acid including double-stranded DNA, single-stranded DNA, single-stranded RNA and RNA/DNA duplexes (Gerschenson et al., 1995). Upon apoptosis induction, endonuclease G translocates to the nucleus and induces oligonucleosomal fragmentation independently of caspases and CAD (Li et al., 2001; Parrish et al., 2001; van Loo et al., 2001; Hahn et al., 2004).

Thus, there are two pathways leading to chromatin condensation and degradation during apoptosis (Reviewed by Zhang and Xu, 2002). One pathway involves caspases, ICAD and CAD, and leads to enhanced chromatin condensation and oligonucleosomal DNA fragmentation (Liu et al. 1997c; Zhang et al., 1999). The other pathway is caspase-independent and involves AIF and endonuclease G, which lead to large-scale DNA fragmentation and nucleosomal fragmentation, respectively (Susin et al., 1999; Li et al., 2001).

1.1.5.3 Role of mitochondria in caspase-independent apoptosis

After MOMP, loss of large quantities of IMS proteins, many of which participate directly in the maintenance of mitochondrial homeostasis (Reviewed by Garrido and Kroemer, 2004), impacts mitochondrial respiration, ATP production and free radical production, resulting in an organelle dysfunction program (Cai and Jones, 1998; Luetjens et al., 2000; Mootha et al., 2001). It is thought that this is part of the reason some cells die in the presence of caspase inhibitors since these caspase inhibitors do not prevent MOMP (Munoz-Pinedo et al., 2006). Furthermore, from the perspective of being a factory for ATP generation mitochondria are also important for apoptosis, since the level of cellular ATP determines in part whether cell death occurs by necrosis or apoptosis (Reviewed by Chiarugi, 2005). If ATP levels fall profoundly, cells die via necrosis. If ATP levels are at least partially maintained, caspase activation and apoptosis follow mitochondrial PT (Eguchi et al., 1997; Ferrari et al., 1998; Nicotera et al., 1998; Lemasters et al., 1999).

1.1.6 Bcl-2 family proteins

B cell leukaemia/lymphoma-2 (Bcl-2) family proteins are the major regulators of the mitochondrial pathway. Their death-regulating activity depends on their ability to modulate MOMP and to regulate the release of AMP (Reviewed by Adams and Cory, 2001). Bcl-2 family proteins not only function at the point of convergence of multiple apoptotic signals, but also at the critical decision point immediately upstream of an irreversible commitment to apoptosis since MOMP is suggested to be a "point of no return" in the pathway to cell death (Reviewed by Newmeyer and Ferguson-Miller, 2003).

1.1.6.1 Homology of Bcl-2 family proteins

Although the overall amino acid sequence homology among Bcl-2 family members is low, the homology is relatively high within four small segments, which are designated Bcl-2 homology (BH) regions. Based on their structural and functional characteristics, Bcl-2 family proteins are subdivided into three groups (Reviewed by Borner, 2003; Scorrano and Korsmeyer et al., 2003; Kuwana and Newmeyer, 2003; Danial and Korsmeyer, 2004): (1) multi-domain anti-apoptotic proteins such as Bcl-2, Bcl-xL, Mcl-1, Bcl-w and A1 which contain all four BH domains (BH1, BH2, BH3 and BH4); (2) multi-domain pro-apoptotic Bcl-2 family proteins such as Bax and Bak which contain only BH1, BH2 and BH3; (3) BH3-only proteins such as Bid, Bim, Bad, Bik, Bmf, Puma and Noxa that exhibit the most structural diversity since they only share the BH3 domain. Because some members promote apoptosis and some promote survival, it has been suggested that the relative amounts of the pro- and anti-apoptotic Bcl-2 family proteins help determine the susceptibility of cells to a death signal (Oltvai et al., 1993).

1.1.6.2 BH1, BH2, BH3 and BH4 domains

One of the unique features of Bcl-2 family proteins is the formation of heterodimers between anti- and pro-apoptotic members (Oltvai et al., 1993; Yang et al., 1995). This heterodimerization process is mediated by the insertion of the BH3-domain of a pro-apoptotic protein such as Bax or Bak into an elongated hydrophobic cleft composed of BH1, BH2 and BH3 on an anti-apoptotic protein such as Bcl-2 or Bcl-xL (Yin et al., 1994; Muchmore et al., 1996; Sattler et al., 1997). Since heterodimerization inhibits the activity of the partner proteins, BH1, BH2 and BH3 are crucial for the

functions of anti-apoptotic Bcl-2 family members (Muchmore et al., 1996; Sattler et al., 1997). In contrast, the BH3 domain of the pro-apoptotic proteins is essential for their protein binding and pro-apoptotic activity (Chittenden et al., 1995). Replacement of the BH3 region of Bcl-2 with the BH3 domain of Bax converts Bcl-2 from an inhibitor to an activator of cell death, indicating there are critical differences between the BH3 domains of anti- and pro-apoptotic Bcl-2 family members (Hunter and Parslow, 1996).

The N-terminal BH4 domain, which is restricted to anti-apoptotic Bcl-2 family members, is also indispensable for their ability to suppress cell death (Huang et al., 1998; Shimizu et al., 2000). However, the exact mechanism of the anti-apoptotic function of the BH4 domain is still uncertain. It has been suggested that it regulates apoptosis by interacting with apoptosis regulatory proteins, such as Bad, Raf-1, Apaf-1 and Calcineurin (Wang et al., 1996; Yang and Korsmeyer, 1996; Shibasaki et al., 1997; Huang et al., 1998), as well as by facilitating heterodimerization with Bax, thereby inhibiting apoptosis (Hirotani et al., 1999).

1.1.6.3 TMB

Most of the Bcl-2 family proteins also have a C-terminal hydrophobic region that is proposed to be a transmembrane domain (TMB) due to its homology to the pore-forming subunits of the ion-channel domain of the bacterial proteins, diphtheria toxin and colicins (Reviewed by Lazebnik, 2001). This has led to the suggestion that some Bcl-2 family proteins have pore- or channel-forming ability, with the TMB domain being responsible for the insertion of these molecules into the MOM, ER and outer nuclear membrane (Muchmore et al., 1996). Indeed, Bcl-2, Bcl-xL, Bax and Bid have all been demonstrated to form ion-channels in synthetic lipid membranes (Schendel et al., 1997; Minn et al., 1997; Antonsson et al., 1997; Schendel et al., 1999). However it still needs to be determined if these proteins actually form ion channels *in vivo* and if this activity directly regulates apoptosis.

1.1.6.4 Anti-apoptotic Bcl-2 family proteins

Bcl-2 family proteins play an essential role in the regulation of MOMP and the release of IMS proteins. Bcl-2 and Bcl-xL are redundant in their capacity to protect cells from apoptosis (Chao et al., 1995). They mainly reside on the MOM where they bind to, and neutralize, pro-apoptotic Bcl-2 family proteins, thus preventing the allosteric activation of Bax and Bak, subsequent MOMP and the

release of cytochrome *c* and AIF (Sedlak et al., 1995; Hunter and Parslow, 1996; Yang et al., 1997; Susin et al., 1999; Cheng et al., 2001).

1.1.6.5 Pro-apoptotic multi-domain Bcl-2 family proteins

1.1.6.5.1 Bax

In healthy cells, inactive monomeric Bax is either located in the cytosol with its TMB inserted into the hydrophobic pocket formed by its BH1, BH2 and BH3 domains or loosely attached to the MOM (Suzuki et al., 2000; Schinzel et al., 2004). In response to stress signals, Bax undergoes a conformational change involving N-terminal exposure as well as the release of its C-terminal TMB (Schinzel et al., 2004). Bax is subsequently inserted into mitochondria as a homo-oligomer to trigger an apoptotic program of mitochondrial dysfunction that results in decrease in mitochondrial transmembrane potential, release of cytochrome *c* and, ultimately, apoptosis (Wolter et al., 1997; Jurgensmeie et al., 1998; Rosse et al., 1998; Finucane et al., 1999; Murphy et al., 1999; Putcha et al., 1999; Antonsson et al., 2001; Shou et al., 2003).

1.1.6.5.2 Bak

Although Bax and Bak are functionally equivalent under most circumstances (Lindsten et al., 2000; Wei et al. 2001), Bak localizes differently from Bax in healthy cells. Under normal conditions, monomeric inactive Bak resides on the MOM and the ER via association with Bcl-xL or Mcl-1 (Griffiths et al., 1999; Willis et al., 2005), both of which negatively regulate the activation of Bak by masking its BH3 domain which is necessary for its oligomerization and pro-apoptotic function (Chittenden et al., 1995; Willis et al., 2005). In the event of death stimuli, Bak dissociates from Bcl-xL and Mcl-1, exposing its N-terminal BH3 domain (Griffiths et al., 1999; Cuconati et al., 2003). Released Bak then either associates into homo-oligomers or forms higher-order complexes that contain both Bax and Bak (Wei et al., 2001; Mikhailov et al., 2003). Formation of these complexes is important for the induction of MOMP, and thus is critical for the killing activity of Bak and Bax (Mikhailov et al., 2003).

1.1.6.5.3 Bax, Bak and apoptosis

Bax and Bak are essential for the activation of caspases and for the intrinsic apoptotic pathway (Ruiz-Vela et al., 2005). Indeed, cells in which the genes encoding these protein are disrupted are resistant to multiple apoptotic stimuli including protein kinase c inhibitor, UV light, growth factor deprivation, TNFα and oncogene activation (Wei et al., 2001; Degenhardt et al., 2002a&b; Ruiz-Vela et al., 2005). In addition, all the BH3-only molecules absolutely require Bax and Bak to induce apoptosis, regardless of their selective binding affinity, and a deficiency in Bax and Bak renders the pro-apoptotic function of BH3-only proteins inactive (Cheng et al., 2001; Zong et al., 2001). Thus, Bax and Bak are critical for the induction of apoptosis, and the definitive commitment to cell death occurs at the step of Bax and/or Bak activation.

1.1.6.5.4 Bax, Bak and the ER

A number of studies have indicated that Bax and Bak also function as a gateway to the intrinsic death pathway by functioning at the ER (Nutt et al., 2002; Zong et al., 2002). In response to ER stress, a small fraction of total cellular Bak and Bax translocates to the ER, where they undergo conformational changes and form oligomers. Oligomerization of Bax and Bak on the ER leads to damage of the ER membrane, thereby inducing the release of Ca^{2+} sequestered in the ER and Ca^{2+} accumulation in mitochondria (Scorrano et al., 2003). This transfer of Ca^{2+} from the ER to the mitochondria is in turn thought to initiate certain apoptotic signals (Reviewed by Demaurex and Distelhorst, 2003; Scorrano, 2003; Szabadkai and Rizzuto, 2004).

1.1.6.6 BH3-only proteins

BH3-only proteins, which act as sensors of cellular stress, require the cooperation of their multi-domain relatives to induce apoptosis. They interact with the hydrophobic groove on either anti- or pro- apoptotic multidomain Bcl-2 family members, with their BH3 domain, which is an amphipathic α-helix, serving as the binding motif. BH3-only molecules may exert their pro-apoptotic action via two mechanisms (Letai et al., 2002; Kuwana et al., 2005; Fletcher and Huang, 2006): (1) Bid, Bim and Puma have been termed "activators" since they can directly induce the activation of Bax and Bak, either by stimulating the translocation of Bax to the MOM or by local effects on Bak, resulting in MOMP and efflux of cytochrome *c* (Eskes et al., 2000; Wei et al., 2000; Kuwana et al., 2002; Cartron et al., 2004); (2) The other BH3-only proteins, which include Bad, Bik and Noxa, are

termed "sensitizers" since they can directly interact with Bcl-2, Mcl-1 or Bcl-xL, occupying the pocket of these anti-apoptotic molecules and dissociating them from other BH3-only proteins or from Bax and Bak, thereby promoting MOMP and efflux of IMS molecules (Kuwana et al., 2005; Willis et al., 2005). The simple inhibitory effect of the "sensitizers" alone is insufficient to induce apoptosis, and hence they need to work in collaboration with the direct "activators" (Kuwana et al., 2005).

1.1.6.6.1 Specificity of BH3-only proteins

BH3-only proteins display selective binding activity to specific pro- and anti- apoptotic Bcl-2 family members (Reviewed by Gélinas and White, 2005). In detail, Bim and Puma can potently engage all the prosurvival proteins (Letai et al., 2002; Chen et al., 2005). Bid and Bim can bind to Bax and Bak to promote their oligomerization (Cheng et al., 2001; Chen et al., 2005). In contrast, Bad binds tightly to Bcl-2, Bcl-xL and Bcl-W, but only weakly to A1 and not to Mcl-1. Noxa binds to Mcl-1 and A1, but not to Bcl-2 and Bcl-xL. Thus, apoptosis relies on the selective interactions between particular subsets of Bcl-2 family proteins. Indeed, release of Bak from Bcl-xL and Mcl-1 and its subsequent activation require over-expression of both Bad and Noxa (Cheng et al., 2001; Willis et al., 2005), whereas over-expression of Puma alone is sufficient to induce Bak-mediated cell death (Willis et al.s, 2005). It has, therefore, been proposed that, depending on the death stimuli, more than one BH3-only protein may be activated to guarantee tight control over apoptosis and that the ability of these BH3-only proteins to associate with their anti-apoptotic partners is essential for their cell-killing function (Gustafsson and Gottlieb, 2007).

1.1.6.6.2 Activation of BH3-only proteins

Most BH3-only proteins are localized outside the mitochondria in living cells. They respond to selected death signals and are activated by transcriptional up-regulation, translocation and/or posttranslational modifications such as phosphorylation, ubiquitination and proteolytic cleavage (Reviewed by Puthalakath and Strasser, 2002). In detail, Bid is activated via proteolytic cleavage by caspase 8 in the death receptor signaling pathways (Li et al., 1998; Lou et al., 1998), and after cleavage the potency of Bid to induce apoptosis can be further enhanced by N-myristoylation (Zha et al., 2000). In response to TNFα signaling, Bid also can be cleaved by a protease activated by JNK, generating jBid which translocates to mitochondria and results in preferential release of Smac without

affecting cytochrome *c* (Deng et al., 2003). Bid also has been shown to be cleaved by other proteases, including granzyme B, calpain and lysozyme (Heibein et al., 2000; Sutton et al., 2000; Cheng et al., 2001; Stoka et al., 2001). These mofications may trigger molecular rearrangement and exposure of the BH3 domain, allowing Bid to bind to, and inactivate, anti-apoptotic molecules.

Bad, which is phosphorylated at multiple sites and sequestered by 14-3-3 in living cells, is rapidly dephosphorylated and released from 14-3-3 in response to growth factor deprivation (Zha et al., 1996). Released Bad translocates to the MOM where it associates with Bcl-xL and Bcl-2 to exert its cell-killing activity (Yang et al., 1995).

In healthy cells, Bmf and Bim are localized to the actin cytoskeleton and the microtubules, respectively. Various types of intracellular damage, such as that induced by anoikis or treatment with taxol, cause their dissociation from the cytoskeleton and microtubules and their migration to the MOM, the site at which they perform pro-apoptotic functions (O'Connor et al., 1998; Puthalakath et al., 1999 and 2001). However, recent studies have indicated that Bim is also localized on the MOM via association with Mcl-1, Bcl-2 or Bcl-xL in healthy cells. In response to UV, granzyme B, Melphalan, TRAIL or oncogene-induced apoptotic stimuli, Bim is released from the anti-apoptotic proteins to directly activate Bax (Nijhawan et al., 2003; Opferman et al., 2003; Zong et al., 2003; Chen and Zhou, 2004; Gomez-Bougie et al., 2005; Han et al., 2004, 2005 & 2006).

Puma, Noxa and Bik are transcriptionally activated by p53 in the event of DNA damage- or oncogenic stress- induced apoptosis (Oda et al., 2000; Nakano and Vousden, 2001; Yu et al., 2001; Villunger et al., 2003; Shore and Viallet, 2005). While Puma directly activates Bax, Noxa synergistically activates Bax with Puma by inactivating Mcl-1 and Bcl-xL (Nakajima and Tanaka, 2007). On the other hand, Bik, which is newly synthesized in response to genotoxic stress, but not to ER stress, is integrated almost exclusively into ER membrane (Germain et al., 2002; Mathai et al., 2002) where it initiates Bax and Bak-dependent release of Ca^{2+} from ER stores (Mathai et al., 2005), leading ultimately to the release of cytochrome *c* and apoptosis (Germain et al., 2002).

1.1.7 Permeabilization of mitochondrial outer membrane

Mitochondria are circumscribed by two membranes: the outer membrane, which is permeable to molecules up to 6 kDa due to the presence of the abundant voltage-dependent anion channel (VDAC), and the inner membrane which is virtually impermeable (Zalman et al., 1980; Narita et al., 1998). The limited permeability of the inner membrane not only allows the existence of a matrix that

is distinct from the cytoplasm, but also permits the generation of the inner mitochondrial transmembrane potential (ΔΨm) and pH gradient, both of which arise from the respiratory chain (Reviewed by Mayerand Oberbauer, 2003; Ohta, 2003; Kroemer et al., 2007; Schwarz et al., 2007).

Apoptogenic mitochondrial proteins (AMP), which include cytochrome *c*, Smac/DIABLO, Htra2/Omi, endonulease G and AIF, are mainly localized in the IMS between the outer and inner membranes (Ohsato et al., 2002). Therefore, MOMP is essential for the release of AMP and the subsequent progression of the mitochondrial apoptotic pathway (Kroemer, 2003; van Gurp et al., 2003). Although it has been shown that Bax and Bak are required for MOMP, not only in purified mitochondria but also in living cells (Wei et al., 2001; Ruiz-Vela et al., 2005), the nature and mechanism of MOMP still remain unknown. Several models have been proposed (Reviewed by Newmeyer and Ferguson-Miller, 2003).

One possibility is that the release of cytochrome *c* is mediated by mitochondrial permeability transition (PT), an event that results in physical rupture of the MOM (Vander Heiden et al., 1997). PT, which consistutes an abrupt increase in mitochondrial inner membrane (MIM) permeability to solutes with molecular masses of <1,500 Da, is mediated by opening of the PT pore complex (PTPC), a multi-protein channel formed at the contact site between the MIM and MOM (Rviewed by Bernardi et al., 1994; Petit et al., 1996). PTPC is composed of VDAC and peripheral benzodiazepine receptor (PBR) on the MOM, adenine nucleotide translocator (ANT) on the MIM, and the matrix protein, cyclophilin D (Zamzami et al., 1998; Kroemer et al., 2007). PT can be triggered by various agents, including toxins, anoxia, reactive oxygen species (ROS) and Ca^{2+} overload (Reviewed by Zoratti and Szabo, 1995). Long lasting PT leads to the influx of solutes, causing expansion of the mitochondrial matrix and dissipation of ΔΨm and the pH gradient. The expansion of the matrix results in sufficient swelling to physically rupture the MOM, triggering the release of IMS proteins and subsequent caspase activation (Reviewed by Bernardi, 1999). PT inhibitors specifically targeting ANT, cyclophilin D or VDAC have been shown to efficiently suppress apoptosis (Marchetti et al., 1996; Zamzami et al., 1996), supporting the notion that PT may mediate the mitochondrial pathway. It is thought that Bcl-2 family proteins are involved in this process (Reviewed by Harris and Thompson, 2003; Breckenridge and Xue, 2004). It has been shown that Bax-mediated release of cytochrome *c* is dependent on PT pore opening (Marzo et al., 1998; Narita et al., 1998; Shimizu et al., 1999) and that Bax and Bak oligomers induce PT by directly interacting with components of the PTPC, such as ANT (Marzo et al., 1998) and VDAC (Narita et al., 1998; Shimizu et al., 1999). In contrast, the anti-apoptotic proteins, Bcl-2 and Bcl-xL, which are antagonists of Bax and Bak, inhibit PT pore opening

induced by various reagents, including Ca^{2+}, H_2O_2 and tert-butyl hydroperoxide, by directly binding to, and closing, VDAC (Shimizu et al., 1998 and 1999). Therefore, both pro- and anti-apoptotic members of the Bcl-2 family may regulate $\Delta\Psi m$ and the release of cytochrome *c* by interacting with the components of PTPC, and PTPC serves as a crossroad of apoptosis regulation by members of the Bcl-2 family.

In addition, there is evidence that Bax and Bak may induce MOMP independently of PT. Specifically, Bax and some other Bcl-2 family members share structural similarities with certain pore-forming proteins and are able to form pores in artificial lipid membranes (Muchmore et al., 1996; Schendel et al., 1997; Minn et al., 1997; Antonsson et al., 1997; Schendel et al., 1999). It is therefore conceivable that, upon apoptotic stimulation, Bax and Bak undergo conformational changes to form a transmembrane pore across the MOM, causing loss of $\Delta\Psi m$ and efflux of IMS proteins without simultaneously inducing swelling of mitochondria and PTPC opening (Jurgensmeier et al., 1998). Furthermore, the insertion of Bax and Bak oligomers into the MOM may simply destabilize the mitochondrial membrane, thereby triggering the release of AMP without the necessity to form specific pores (Reviewed by Kroemer et al., 2007; Schwarz et al., 2007).

In summary, several models, all of which eventually lead to the permeabilization of both the MIM and the MOM, release of AMP, mitochondrial dysfunction and apoptosis, have been proposed. The precise mechanism utilized may vary depending on cell type and the nature of the apoptotic stimulus (Reviewed by Kroemer et al., 2007).

1.1.8 p53 and apoptosis

The tumor suppressor, p53, coordinates the cellular response to different kinds of stresses including DNA damage, hypoxia, oncogene activation, anoikis and redox stress (Reviewed by Fuster et al., 1997; Sionov and Haupt, 1999; Haupt et al., 2003; Helton and Chen, 2007). Under normal conditions, the cellular level of p53 protein is relatively low due to its tightly regulated degradation through the ubiquitin pathway (Chowdary et al., 1994; Honda et al., 1997). Various stimuli activate p53 by increasing its stability and/or inducing posttranslational modifications that enhance its activity (Reviewed by Lavin and Gueven, 2006). Activated p53 induces either reversible cell cycle arrest and DNA repair or permanent withdrawal from cell proliferation followed by the induction of apoptosis or senescence (Reviewed by Sionov and Haupt, 1999; Roos and Kaina, 2006; Helton and Chen, 2007).

Cell type, stimulus type, the extent of DNA damage as well as the absolute level of p53 are factors that decide the particular cell fate (Midgley et al., 1995; Chen et al., 1996; Fei et al., 2002).

p53 regulates cell cycle arrest and DNA repair by binding with specific DNA sequences and activating the transcription of target genes encoding proteins involved in cell cycle control including p21, 14-3-3σ, Gadd45, Mdm2, PCNA and BTG2 (Sax and El-Deiry, 2003; Vousden et al., 2006). In addition, p53 regulates apoptosis by stimulating the expression of various apoptosis-related genes including Bax, Puma, Noxa, Apaf-1, Fas and TRAIL-R2 as well as redox-related p53-induced genes such as PIG2, PIG3, PIG6, PIG7, PIG8, PIG11 and the p85 regulatory subunit of phosphatidyl-3-OH kinase (Reviewed by Levine, 1997; El-Deiry, 1998; Vousden and Lu, 2002; Sax and El-Deiry, 2003). p53 also transcriptionally represses the expression of key anti-apoptotic proteins including Bcl-2, Bcl-xL and survivin of the IAP family (Miyashita et al., 1994; Hoffman et al., 2002). In addition, however, p53 can induce apoptosis independently of its transcription regulatory activity (Caelles et al., 1994; Kokontis et al., 2001). Several reports have shown that p53 translocates to mitochondria in response to cell stress-induced apoptosis, both *in vivo* and *in vitro* (Marchenko et al., 2000; Sansome et al., 2001; Mihara and Moll, 2003; Erster et al., 2004; Endo et al., 2006). Once reaching mitochondria, p53 either associates with anti-apoptotic Bcl-2 and Bcl-xL to inhibit their protective function (Mihara et al., 2003), or complexes with pro-apoptotic Bak or Bad to directly induce Bak oligomerization and MOMP (Leu et al., 2004; Jiang et al., 2006).

p53 is the most intensively studied apoptosis factor contributing to cancer. Indeed, it is inactivated in more than 50% of human cancers (Hainaut and Hollstein, 2000). The mechanisms responsible for the inactivation of p53 include gene deletion or mutation, over-expression of Mdm2, which is a negative regulator of p53, and inactivation by oncoproteins encoded by DNA tumor viruses such as the E6 protein of human papillomavirus (HPV) and the Simian virus 40 (SV40) large T antigen (Mietz et al., 1992; Leach et al., 1993; Patterson et al., 1994; Marchetti et al., 1995; Haitel et al., 2000; Khor et al., 2005).

1.2 eIF5A

1.2.1 Hypusine

Hypusine, an unusual amino acid, was originally isolated from bovine brain and identified as N6-(4-amino-2-hydroxybutyl)lysine in 1971 (Shiba, et al., 1971). The name "hypusine" reflects the fact that it comprises moieties of hydroxyputrescine and lysine. eIF5A (eukaryotic initiation factor 5A) is the only cellular protein known to contain hypusine (Cooper et al., 1983). Formation of hypusine on the eIF5A precursor is one of the most specific polyamine-dependent biochemical reactions known, and there are two enzymes, deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DHH), involved in this two-step posttranslational modification (Park et al., 1982). In the first step, the aminobutyl moiety of the polyamine, spermidine, is transferred by DHS in an NAD^+-dependent reaction to the ε-amino group of a specific lysine residue in eIF5A to form deoxyhypusine (Imaoka and Nakajima, 1973; Park et al., 1981, 1982 & 1988; Wolff et al., 1990). This intermediate does not accumulate and is immediately subjected to the second step of the modification (Abbruzzese et al., 1986; Park, et al., 1991) in which DHH catalyzes the hydroxylation of the deoxyhypusine residue to hypusine (Park et al., 1982 & 1988; Abbruzzese et al., 1986 & 1988). Hypusine persists in this protein without further modification or reversion to lysine (Gordon et al., 1987b; Park et al., 2003). There is a recent report of efficient reversal of deoxyhypusine synthesis in a cell-free system (Park et al., 2003). However the deoxyhypusine hydroxylation is thought to be irreversible (Park et al., 2003). The fact that nature has committed two enzymes to produce one hypusine residue on a single protein underscores the importance of this modification (Xu and Chen, 2001) and raises the possibility that different post-translationally modified eIF5A proteins may have different functions.

1.2.2 Properities of eIF5A

eIF5A, which was previously named eIF4D, was originally isolated as a small protein factor (18 kDa in size) from the ribosomal fraction of rabbit reticulocyte lysates (Kemper et al., 1976; Benne et al., 1978). Hypusine-containing eIF5A is thought to be ubiquitous in eukaryotes (Gordon et al., 1987; Park, 1988; Hu et al., 2005), and cells typically contain at least two eIF5A genes, one DHS gene and one DHH gene (Park et al., 2006). In fact, proteins closely related to eIF5A have been found in all types of living organisms, including archaea, also known as archaebacteria (Bartig et al., 1992),

and eubacteria, also known as bacteria (Glick and Ganoza, 1975 & 1976). In archaea, eIF5A is named archaeal initiation factor 5A (aIF5A). While both aIF5A and archaeal DHS have been identified, DHH or its homolog was not detected in archaea (Park et al 2006). However, various archaea are reported to contain hypusine- or deoxyhypusine-containing eIF5A1, or a mixture of both (Bartig et al., 1990). The mechanism as to how deoxyhypusine is hydroxylated in archaea is still unclear. Eubacterial eIF5A, which is named elongation factor P (EF-P), appears to be ubiquitous in eubacteria (Lipowsky et al., 2000) and is essential for viability and protein synthesis in *Escherichia coli* (Aoki et al., 1997). Although eubacteria lack a hypusine-containing eIF5A equivalent as well as DHS and DHH enzymes (Liao et al., 1998; Gerloff et al., 1998), the sequence similarity among eIF5A, aIF5A and EF-P suggestes that they are homologous proteins (Kyrpides and Woese, 1998). Thus, eIF5A/aIF5A/EF-P is considered a universally conserved protein.

The gene encoding eIF5A has been cloned from several eukaryotes including yeast *Saccharomyces cerevisiae* (Mehta et al., 1990; Schnier et al., 1991), slime mould *Dictyostelium discoideum* (Sandholzer et al., 1989), dinoflagellate *Crypthecodinium cohnii* (Chan et al., 2002), Arabidopsis (Wang et al., 2001), corn (Dresselhaus et al., 1999), tobacco (Chamot and Kuhlemeier, 1992), alfalfa (Pay et al., 1991), carnation (Wang et al., 2001), *Caenorhabditis elegans* (Wilson et al., 1994), zebrafish *Danio rerio* (Woods et al., 2005), frog *Xenopus tropicalis* (Klein et al., 2002), *Drosophila melanogaster* (Harvey et al., 1997), silkworm *Bombyx mori* (Hu et al., 2005), chicken (Rinaudo et al., 1993), rabbit (Smit-McBride et al., 1989a), cattle (Moore et al., 2005) and human (Smit-McBride et al., 1989a). eIF5A is highly conserved from yeast to human. Indeed, eIF5A from various eukaryotes shares an overall amino acid sequence homology of 50-95% (Si et al., 1996; Jenkins et al., 2001; Molitor et al., 2004). Most of the conserved regions are clustered in the N-terminus of the protein, especially around the hypusine site. A sequence of 12 amino acids around the hypusine residue, Ser-Thr-Ser-Lys-Thr-Gly-**Hpu**-His-Gly-His-Ala-Lys, not only is completely conserved among all eukaryotes (Chamot and Kuhlemeier, 1992), but also cannot be found in any other protein in the database (Park et al., 1993), suggesting an important role for hypusination of eIF5A. Furthermore, eIF5A from slime mould, alfalfa, rat or human can substitute functionally for yeast eIF5A in strains where yeast eIF5A genes are disrupted (Magdolen et al., 1994; Koettnitz et al., 1995; Maiti and Maitra, 1997). On the contrary, archaeal aIF5A when expressed in yeast is neither modified to contain hypusine, nor does it allow growth of cells deficient for yeast eIF5A (Magdolen et al., 1994), suggesting the conservation between eIF5A and aIF5A is limited.

19

1.2.3 Different isoforms of eIF5A

At least two isoforms of eIF5A encoded by separate genes are present in eukaryotic cells (Park et al., 2006). Five isoforms of eIF5A were found in potato, exhibiting differential expression patterns in developing tuber and fruit (In et al., 1997). In tomato, four different isoforms that exhibit remarkable sequence identity (89%-92% at the amino acid level and 70%-80% at the nucleotide level) have been identified (Wang et al., 2001). In addition, two isoforms of eIF5A were found in tobacco, with one constitutively expressed and the other preferentially expressed in photosynthetic tissues (Chamot and Kuhlemeier, 1992).

1.2.3.1 Yeast eIF5A

Yeast has two isoforms of eIF5A, which are encoded by two separate genes, *TIF51A* (also known as *HYP2*) and *TIF51B* (also known as *HYP1*) (Schnier et al., 1991). These two eIF5A proteins not only share 92% identity, but also are more than 60% identical to human eIF5A (Schnier et al., 1991). Although *TIF51A* and *TIF51B* fulfill very similar functions (Schwelberger et al., 1993), they are expressed under different oxygen conditions (Mehta et al., 1990). Under aerobic growth conditions, only *TIF51A* is transcriptionally active, leading to the expression of eIF5A1; whereas under anaerobic condition, *TIF51A* is turned off and eIF5A1 mRNA becomes very unstable, and thus yeast cells only express mRNA transcribed from *TIF51B* (Mehta et al., 1990). Interestingly, *TIF51B* will express aerobically only when *TIF51A* is disrupted, and *TIF51A* can substitute for *TIF51B* under anaerobic conditions when the latter is disrupted (Wohl et al., 1993). Expression of either *TIF51A* or *TIF51B* alone is sufficient for the normal growth of yeast under any metabolic conditions (Schwelberger et al., 1993; Magdolen et al., 1994).

1.2.3.2 Human eIF5A

There are two isoforms of eIF5A in all vertebrates examined so far, including human, fish, chicken and amphibians (Jenkins et al., 2001; Rinaudo et al., 1993; Wolff et al., 1992; Woods et al., 2005). In *Homo sapiens*, eIF5A proteins are encoded by two different genes, although three intronless pseudogenes have also been reported and mapped to 10q23.3, 17q25 and 19q13.2 (Steinkasserer et al., 1995).

Human eIF5A1 cDNA was first isolated in 1989 (Smit-McBride et al., 1989a). The genomic sequence, roughly 5 kb, was determined (Koettnitz et al., 1995) and mapped to 17p12-p13 in 1995 (Steinkasserer et al., 1995). However, eIF5A2, which is 84% identical and 94% similar to eIF5A1, was not described in human or mammalian cells until 2001, and then by two separate groups (Clement et al., 2001; Jenkins et al., 2001). Therefore, most of the published literature uses the general name, eIF5A, to stand for the eIF5A1 isoform. Although both genes are able to complement disrupted yeast eIF5A (Clement et al., 2003), it has been suggested that in human cells they bind to/interact with different mRNAs or proteins due to the differences in their C-terminal putative RNA binding domains and protein structures (Guan et al., 2001; Jenkins et al., 2001; Clement et al., 2003). In addition, their gene-expression patterns are distinct. eIF5A1 mRNA and protein appear to be present in all human tissues and cell types. In contrast, although eIF5A2 mRNA is detectable in most cell lines, it appears to be inefficiently translated (Clement et al., 2006). Thus, eIF5A2 protein is expressed only in certain tissues such as testis and brain, but it is over-expressed in certain cancers and cancer cell lines (Jenkins et al., 2001; Clement et al., 2003; Clement et al., 2006).

The finding that there are several isoforms of eIF5A in cells not only prompts the proposal that different isoforms may facilitate the translation of different subsets of mRNAs required for specific physiological functions, but also implies the importance of this protein, since it has been suggested that having more than one copy for essential proteins might be a way to ensure survival of an organism (Sidow, 1996).

1.2.4 Cellular localization of eIF5A

Shi and colleagues (1996a) using indirect immunofluorescence and confocal microscopy in conjunction with subcellular fractionation demonstrated that eIF5A exists in the cytoplasm of mammalian cells in two forms: a soluble fraction and a fraction bound to the ER. The subcellular distribution pattern of eIF5A is not significantly altered in different stages of the cell cycle; nor is it changed by viral oncogene transformation or by treatment with RNA synthesis inhibitors (Shi et al., 1997). Interestingly, treatment with puromycin, a protein synthesis inhibitor known to dissociate ribosomes, dramatically altered the distribution of eIF5A "from an ER network-like perinuclear structure to a patched dotted pattern dispersed throughout the cytoplasm" (Shi et al., 1997). This observation indicates that eIF5A may associate with ribosomes and thus is consistent with its putative function in protein synthesis. *In situ* staining of human tissue as well as localization studies in yeast

cells have both confirmed the cytoplasmic localization of hypusine-containing eIF5A (Valentini et al., 2002; Cracchiolo et al., 2004).

Although hypusinated eIF5A appears to be localized in the cytoplasm (Shi et al., 1997; Valentini et al., 2002; Cracchiolo et al., 2004), inhibition of hypusination using MG-132 or GC7 or by point mutation of the specific lysine residue of eIF5A that becomes hypusinated appears to change the cytoplasmic localization of eIF5A to whole-cell distribution (Jin et al., 2003b). This observation was interpreted as indicating that inhibition of hypusination prevents eIF5A from achieving its proper site in the cytoplasm, possibly polyribosomes (Jin et al., 2003b). There are also reports that eIF5A is localized in the nucleus as well as in the cytoplasm in untreated cells (Ruhl et al., 2003; Jao and Chen, 2002). Moreover, this whole-cell distribution pattern was not changed by energy depletion, heat shock or inhibition of transcription, translation, polyamine synthesis and Crm1-dependent nuclear export (Jao and Chen, 2002). Even though eIF5A is thought to enter the nucleus by passive diffusion (Jao and Chen, 2002), the observation that it is present in both the nucleus and the cytoplasm is consistent with the notion that eIF5A may function as an RNA-binding protein, since many RNA-binding proteins are distributed between the cytoplasm and nucleus (Shen et al., 2000).

1.2.5 Structure of eIF5A

There have been several studies of the crystal structure of eIF5A from different archaeal species including *Methanococcus jannaschii* (Kim et al., 1998), *Pyrobaculum aerophilum* (Peat et al., 1998) and *Pyrococcus horikoshii* OT3 (Yao et al., 2003). The data suggest that eIF5A is comprised of two distinct anti-parallel β-sheet domains that are arranged in an elongated fashion and connected by a flexible peptide hinge. In the N-terminal domain, there is a protruding hairpin loop bearing the conserved lysine hypusination site that is pointing away from the main structure. This renders the hypusination site accessible to spermidine as well as DHS and DHH (Kim et al., 1998; Peat et al., 1998; Yao et al., 2003). There is also a stretch of basic amino acids clustered around the hypusine residue, making it a possible nucleic acid-binding site (Xu and Chen, 2000). In addition, the C-terminal domain contains a leucine-rich oligonucleotide/oligosaccharide binding (OB) fold, which is a common RNA-binding module that exists in prokaryotic cold shock protein A (CspA) as well as prokaryotic IF1 and eukaryotic eIF2α translation initiation factors (Wistow, 1990; Schnuchel et al., 1993). These observations collectively suggest that eIF5A is capable of binding with nucleic acid, particularly RNA, with both the hypusine site and the OB domain being potential binding sites (Kim

et al., 1998; Peat et al., 1998; Yao et al., 2003). The 3D structure of human eIF5A is highly similar to that of aIF5A (Facchiano et al., 2001).

Using exclusion chromatography under neutral isotonic conditions, Chung et al. (1991) demonstrated that native eIF5A largely exists in a tightly associated dimeric form under physiological conditions, and has the potential to undergo reversible association to form higher polymers. However, in another study it was shown that although eIF5A has the tendency to form dimers, the dimer is only loosely associated and will dissociate in the presence of DHS (Peat et al., 1998).

1.2.6 Proposed functions of eIF5A

The true function of eIF5A still remains unknown even after nearly three decades of intensive study. Recent studies have suggested that eIF5A is involved in translation, nucleocytoplasmic shuttling, mRNA turnover, cell proliferation and apoptosis (Kemper et al., 1976; Benne and Hershey, 1978; Benne et al., 1978; Ruhl et al. 1993; Park et al., 1993b & 1997; Hanauske-Abel, 1995; Wang et al., 2001).

1.2.6.1 Protein translation

Based on its purification from the ribosomes of reticulocyte lysates and its stimulatory effect on the formation of the dipeptide analogue, methionyl-puromycin, in a cell free system, eIF5A1 was originally deemed to be a translation initiation factor that stimulates the initiation phase of protein synthesis via transient association with ribosomes (Kemper et al., 1976; Benne and Hershey, 1978; Benne et al., 1978). It was subsequently proposed that eIF5A facilitates ribosomal subunit joining and the positioning of the initiator tRNA for the synthesis of the first peptide bond (Schnier, et al., 1991). Hypusine modification was thought to be required for this biological activity of eIF5A, since the unmodified eIF5A precursor failed to stimulate methionyl-puromycin synthesis (Park, 1989; Smit-McBride et al., 1989b; Park et al., 1991). The deoxyhypusine-containing intermediate form of eIF5A provided a degree of methionyl-puromycin synthesis stimulatory effect, but not as effectively as the hypusine-containing form (Park et al., 1991).

More recently, this proposed function of eIF5A has been questioned due to lack of a clear correlation between eIF5A and general protein synthesis as well as the demonstration that complete and rapid intracellular depletion or inactivation of eIF5A in yeast cells does not significantly affect

protein synthesis or polyribosome profiles (Kang and Hershey, 1994; Zuk and Jacobson, 1998). In addition, eIF5A exhibits different expression and location patterns from those of other translation initiation factors. First, it is not only more abundant than the ribosomes in the cell (Hershey, 1994), but also ten times more abundant than the common initiation factors (Thomas et al., 1979). Second, it mainly localizes in the cytoplasm and associates with the ER-bound polyribosomes (Shi et al., 1997), whereas genuine translation factors only bind with ribosomes during initiation (Lockwood et al., 1972; Trachsel and Staehelin, 1978; Unbehaun et al., 2004). However, two recent studies have demonstrated hypusine-dependent and translation-dependent interaction of eIF5A with the structural components of the 80S ribosome complex as well as the translation elongation factors, eEF1A and eEF2 (Jao and Chen, 2006; Zanelli et al., 2006). In light of these findings, it has been proposed that eIF5A may participate in ribosome-related events such as protein folding rather than the synthesis of polypeptide chains *per se* (Jao and Chen, 2006).

Although hypusine-containing eIF5A may not be required for global protein synthesis, it is thought to facilitate the translation of some specific subsets of mRNAs (Kang and Hershey, 1994). Treatment of mammalian cells with inhibitors of DHS or DHH induces cell cycle arrest at the G1/S boundary as well as inhibition of cell proliferation (Park et al., 1993b; Hanauske-Abel et al., 1994; Chen et al., 1996; Shi et al., 1996b; Tome and Gerner, 1997; Jin et al., 2003a). In addition, expression of eIF5A appears to be a cell cycle-sensitive event. For example, expression of eIF5A peaks at early G1 phase, but decreases dramatically upon entry into S phase and is very low at the S and G2/M phases (Dresselhaus et al., 1999; Chan et al., 2002; Jin et al., 2003a). Thus, the transition from G1 to S phase may be a hypusine-sensitive event, and eIF5A may be involved in the translation of proteins regulating this process.

1.2.6.2 Nucleocytoplasmic shuttle protein

There are also indications that eIF5A functions as an mRNA nuclear exporter. This hypothesis was initially proposed based on the finding that eIF5A appears to serve as a cellular cofactor binding to the human immunodeficiency virus type 1 (HIV-1) Rev transactivator protein (Reviewed by Bevec and Hauber, 1997).

1.2.6.2.1 Rev protein

Rev is a viral auxiliary protein involved in the nuclear export of unspliced and partially spliced HIV-1 viral mRNAs, which serve as templates for the translation of viral structural protein or as viral genomes in the formation of progeny virus (Reviewed by Emerman, 1989; Cullen and Malim, 1991). In the absence of Rev, only fully spliced HIV-1 mRNA can be exported to the cytoplasm (Malim and Cullen, 1991; Malim and Cullen, 1993). Rev, which mainly localizes in the nucleus, binds specifically to the Rev response element (RRE) sequence in its target viral mRNAs as well as to a number of cellular cofactors (Daly et al., 1989; Zapp and Green, 1989). Through the protein-protein interaction with its cofactors, Rev shuttles constantly between the nucleus and cytoplasm of host cells, coupling HIV-1 mRNAs to the cytoplasm (Cochrane et al., 1990; Meyer and Malim, 1994; Wolff et al., 1995). This bi-directional transport is mediated by two specific Rev sequences: a nuclear localization signal (NLS) that overlaps the RRE-binding domain, and a leucine-rich C-terminal activation domain that not only acts as a nuclear export signal (NES), but is also required for the interaction with cellular cofactors (Malim et al., 1989; Cochrane et al., 1990).

1.2.6.2.2 Rev, Rex and eIF5A

eIF5A has been identified as a cellular cofactor binding specifically to the C-terminal activation domain of Rev (Ruhl et al. 1993). In fact, in the absence of eIF5A or in eIF5A mutant constitutively expressed human T cells, viral replication and the function of Rev are blocked (Ruhl et al. 1993; Bevec et al., 1996; Junker et al., 1996; Hofmann et al., 2001). In addition, eIF5A gene expression is highly up-regulated not only in human peripheral blood mononuclear cells (PBMCs) of healthy individuals in response to T-lymphocyte-stimulating agents, but also in the PBMCs of HIV-1 patients who have activated T lymphocytes (Bevec et al., 1994). Thus, up-regulation of eIF5A in activated T-cells has been suggested to contribute to efficient viral replication in these cells (Bevec et al., 1994).

It has also been suggested that eIF5A is a cofactor for Rex (Katahira et al., 1995), which is the transactivator protein of human T-cell leukemia virus type I (HTLV-1) and is responsible for nuclear export and cytoplasmic expression of incompletely spliced viral mRNAs (Hidaka et al., 1988). In somatic cells microinjected with export substrates, specific inhibitors of eIF5A were shown to block nuclear export of NESs derived from HIV-1 Rev and HTLV-1 Rex, supporting the contention that eIF5A serves as an essential cofactor for the nuclear export of Rev and Rex (Elfgang

et al. 1999). Consistent with this, by direct visualization of green fluorescent protein (GFP)-tagged eIF5A Jin and his colleagues (2000) found that eIF5A localizes in both the nucleus and the cytoplasm for a short time after expression, then steadily redistributes to the cytoplasm only. This observation is indicative of the putative nuclear exporting ability of eIF5A (Jin et al., 2003).

1.2.6.2.3 eIF5A in anti-HIV therapy

As a putative cellular cofactor of Rev, eIF5A is a novel target in anti-HIV therapeutic strategies. Several inhibitors of DHS and DHH enzymes have been developed and used in antiretroviral therapy. CNI-1493, which is a potent inhibitor of human DHS, inhibits the replication of macrophage- and T cell-tropic lab strains, clinical isolates as well as HIV-1 strains with multi-drug resistance by inhibiting the transactivation ability of Rev and does not have any effect on cell proliferation or cell viability (Hauber et al., 2005). Mimosine and deferiprone, both of which are iron chelators and hence inhibitors of DHH (Csonga et al., 1995; Caraglia et al., 1999; Kim et al., 2006; Park et al., 2006), also interfere with the translation of incompletely spliced retroviral mRNAs, thus suppressing replication of HIV-1 (Andrus et al., 1998). In addition, they trigger extensive apoptosis, particularly in cells that actively produce HIV-1 (Andrus et al., 1998). 1,8-Diaminooctane, an inhibitor of DHS, significantly reduces the replication of lentivirus feline immunodeficiency virus (FIV), which also depends on the Rev regulatory system, by decreasing the levels of unspliced and singly spliced retroviral mRNAs in the cytoplasm in a dose-dependent manner (Hart et al., 2002).

The results of other studies, however, argue against the hypothesis that eIF5A is involved in Rev-dependent nuclear export. First, interaction between Rev (either free or RNA-bound) and eIF5A was not detected (Henderson and Percipalle, 1997; Lipowsky et al., 2000). Second, transient expression of Rev does not alter the cytoplasmic distribution of endogenous eIF5A (Shi et al., 1996; Valentini et al., 2002). Indeed, the function of eIF5A as a nucleocytoplasmic shuttle protein has also been questioned based on the observation that eIF5A does not shuttle between the nucleus and the cytoplasm (Shi et al., 1996; Shi et al., 1997; Valentini et al., 2002). Also, eIF5A appears to gain nuclear entry via passive diffusion instead of active shuttling (Lipowsky et al., 2000; Jao and Chen, 2002). Moreover, since hypusinated aIF5A is essential for growth of archaea (Jansson et al., 2000) and archaea do not have nuclei, it has been suggested that the role of eIF5A in the nucleocytoplasmic trafficking, "if any, is unlikely to be its conserved essential function" (Jao and Chen, 2006).

1.2.6.3 eIF5A as an RNA binding protein

It has been suggested that eIF5A serves as an RNA-binding protein (Liu et al., 1997), with both the hypusine residue and the C-terminal domain contributing to the interaction (Kim et al., 1998; Peat et al., 1998; Yao et al., 2003). Several studies indicated that hypusine-containing eIF5A is essential for transporting specific mRNAs to the cytoplasm. Intracellular depletion of eIF5A in yeast results in a marked accumulation of a specific set of mRNAs in the nuclear compartment (Liu and Tartakoff, 1997). Hanauske-Abel et al (1995) observed the disappearance of several specific sets of mRNAs, including those encoding enzymes critical for proliferation, from polyribosomes after the inhibition of hypusination, as well as the reappearance of these mRNAs after removal of the inhibition. Furthermore, inhibition of hypusine modification induces inhibition of S phase entry (Park et al., 1993b; Hanauske-Abel et al., 1994; Chen et al., 1996; Shi et al., 1996b; Tome and Gerner, 1997; Jin et al., 2003a). It has, therefore, been proposed that hypusine-containing eIF5A may be required for nucleocytoplasmic export of a subset of specific mRNAs encoding proteins that are involved in proliferation (Hanauske-Abel, 1995). Based on these findings, together with the discovery of various isoforms of eIF5A, it has been suggested that each isoform of eIF5A is responsible for transporting a unique subset of mRNAs related to a specific stage of development (Park et al., 1993b & 1997; Hanauske-Abel, 1995; Wang et al., 2001).

The ability of eIF5A to bind to specific RNAs has been demonstrated in several studies. RRE, U6 spliceosomal snRNA as well as RNAs encoding ribosomal L35a, plasminogen activation inhibitor mRNA-binding protein, NADH dehydrogenase subunit, ADP-ribose pyrophosphatase and several unknown proteins have been identified as potential RNA binding targets for eIF5A (Liu et al., 1997; Xu et al., 2004). In addition, there is a report identifying the specific RNA sequence that eIF5A may bind. This was achieved using the method of systematic evolution of ligands by exponential enrichment (SELEX) (Xu and Chen, 2001). It is estimated that eIF5A may interact with at least 800 different mRNAs, which represent approximately 5% of total mRNAs (Xu et al., 2004).

1.2.6.4 eIF5A interaction with proteins

Motif analysis has indicated that eIF5A contains a stretch of basic amino acids, which is a typical nucleic acid binding domain, clustered at the hypusine site and a leucine-rich stretch, which commonly depicts capability for both protein-protein interaction and RNA-binding, at the C-terminal domain (Liu et al., 1997b). These domains are also present in RNA-binding bimodular proteins such

as Rev and influenza virus NS1 protein (Malim et al., 1989; Wen et al., 1995; Liu et al., 1997C; Li et al., 1998). Therefore, instead of binding only with RNA, eIF5A is thought to use its bimodular structure to interact with both RNA and protein (Xu and Chen, 2000), with several protein targets having been identified (Beninati et al., 1995; Schatz et al., 1998; Singh et al., 1998; Chen and Jao, 1999; Lee et al., 1999; Rosorius et al. 1999; Hofmann et al., 2001; Lipowsky, 2000)

1.2.6.4.1 eIF5A and DHS

Using a yeast two hybrid system to probe a HeLa cell library, Chen and Jao (1999) identified DHS as a binding target of eIF5A. This interaction was confirmed by two other groups using gel mobility-shift assay and tandem affinity purification (Lee et al., 1999; Jao and Chen, 2006). Although DHS exists natively as a tetramer with four potential active sites for eIF5A binding (Joe et al., 1995; Kang et al., 1995; Wolff et al., 1995; Tao and Chen, 1995; Liao et al., 1998), only one eIF5A precursor monomer complexes with each DHS tetramer (Lee et al., 1999). Interestingly, although NAD^+ and spermidine are cofactor and co-substrate, respectively, for the DHS-mediated reaction with eIF5A, it was shown that the formation of the pre-eIF5A-DHS complex is not dependent on either NAD^+ or spermidine, and also that the binding of NAD^+ and spermidine to DHS does not depend on the presence of pre-eIF5A. This was interpreted as indicating that the binding of NAD^+, spermidine and pre-eIF5A to the DHS tetramer occurs in a random order (Lee et al., 1999). In addition, DHS still stably complexes with deoxyhypusine-containing eIF5A after the reaction, and dissociates after the binding of DHH (Lee et al., 1999).

1.2.6.4.2 eIF5A and L5

Ribosomal protein, L5, which binds to 5S rRNA and has been implicated in the intracellular transport of 5S rRNA (Michael and Dreyfuss, 1996), is thought to be a cellular interaction partner for eIF5A (Schatz et al., 1998). Moreover, based on the finding that the nuclear export activity of Rev is enhanced by over-expression of L5 and inhibited by antibodies that recognize eIF5A or L5, it is proposed that Rev is exported in association with an eIF5A-L5 complex (Schatz et al., 1998). In detail, Rev and its binding mRNAs are thought to be exported through the 5S rRNA export pathway by their interaction with L5, and eIF5A may function as an adaptor to enhance the association between viral mRNAs and L5 (Schatz et al., 1998). In addition, eIF5A mutants in which Rev-

mediated nuclear export is inhibited (Bevec et al., 1996) still bind to L5 protein, suggesting that eIF5A and L5 may be involved in intracellular trafficking of substrates other than Rev (Schatz et al., 1998).

1.2.6.4.3 eIF5A and Crm1

In untreated mammalian cells as well as in Xenopus oocytes, eIF5A has been shown to accumulate in the region of the nuclear-pore-complex-associated intranuclear filaments, which is the initial docking site for export substrates on the nuclear-pore-complex (Rosorius et al., 1999). At this site, eIF5A interacts with Crm1, a general nuclear export receptor for substrates containing leucine-rich nuclear export signals (NESs) (Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997). Most importantly, Crm1 is a well-known cellular cofactor for HIV-1 Rev (Bogerd et al., 1998). *In vitro* binding studies have demonstrated that eIF5A is required for efficient interaction of Rev-NES with Crm1 (Hofmann et al., 2001). This has led to the proposal that eIF5A functions as an adaptor to enhance the interaction between Rev and Crm1. However, the interaction between eIF5A and Crm1 has been questioned. First, eIF5A does not have a canonical leucine-rich NES (Valentini et al., 2002), which is required for the interaction of Crm1 with its targets (Kutay and Guttinger, 2005). Second, in a yeast temperature-sensitive Crm1 mutant, there is no nuclear accumulation of eIF5A (Valentini et al., 2002).

There is also evidence that eIF5A may interact with nuclear actin, which is associated with the nucleocytoplasmic filaments of the nuclear-pore-complexes, as well as *Xenopus* oocyte nucleoporins including nup62, nup98, nup153 and CAN/nup214, all of which have been implicated in nuclear export pathways (Hofmann et al., 2001). Consistent with its proposed interaction with these nuclear export-related proteins, eIF5A was shown to be exported from the nucleus to the cytoplasm in a leptomycin B-sensitive manner in African green monkey kidney epithelial cells and in Xenopus oocytes (Rosorius et al., 1999).

1.2.6.4.4 eIF5A and exportin 4

Exportin 4 (Exp4), a novel nuclear export receptor that shares high sequence homology with Crm1, has been suggested to be a much stronger (~1000 fold) binding partner of eIF5A than Crm1 (Lipowsky et al., 2000). It belongs to the importin β superfamily that mediates many of the

nucleocytoplasmic transport events (Lipowsky et al., 2000). As for other members of the importin β superfamily proteins, the cycling of Exp4 between the nucleus and the cytoplasm depends upon GTPase Ran-coordinated, compartment-specific binding and release of cargo (Reviewed by Mattaj and Englmeier, 1998; Gorlich and Kutay, 1999). Ran switches between a GDP- and a GTP-bound form, with RanGTP highly enriched in the nucleus and RanGAP almost exclusively found in the cytoplasm. It has been proposed that Exp4 binds to hypusine-containing eIF5A in the nuclear environment in the presence of RanGTP, forming a trimeric eIF5A-Exp4-RanGTP complex that is subsequently transferred out of the nucleus, and then eIF5A is released from the cargo in the cytoplasm (Lipowsky et al., 2000).

1.2.6.4.5 eIF5A and TGase

eIF5A also appears to be a substrate for some transglutaminases (TGases), including human plasma coagulation Factor XIII (Beninati et al., 1995), guinea pig liver TGases (GPLTG) (Beninati et al., 1995) and tissue transglutaminases (tTGases) (Beninati et al., 1998; Singh et al., 1998). Transglutaminases are enzymes that catalyze covalent cross linking between proteins by forming ε(γ-glutamyl)-lysine isopeptide bonds between peptide-bound glutamine and lysine residues, thereby stabilizing intra- and extra-cellular proteins into macromolecular assemblies (Folk and Finlayson, 1977). TGases are involved in a variety of physiological activities, such as apoptosis, extracellular matrix formation and tumor cell proliferation (Fesus et al., 1987; Fesus et al., 1989; Melino et al., 1994; Baldini et al., 2006). The hypusine residue of eIF5A is thought to function as the acceptor substrate for TGase which, after binding to eIF5A, catalyzes the formation of cross-links between the peptide chains of intracellular proteins and the hypusine residue (Beninati et al., 1995). Furthermore, eIF5A appears to specifically bind to the GDP-bound form, not the GTP-bound form of TGase (Singh et al., 1998). This has led to the proposal that the localization and function of eIF5A are regulated by the GTP-binding/GTPase cycle of TGase. eIF5A is thus retained in the cytoplasm through its association with GDP-bound TGase; upon GTP-GDP exchange, eIF5A is released and returns to the nucleus to participate in trafficking of specific sets of mRNAs or proteins (Singh et al., 1998).

There is high tTGase activity in immune-competent cells of HIV+ persons (Amendola et al., 1994). Based on the observation that induction of tTGase in these cells induces apoptosis (Amendola et al., 1994; Amendola et al., 1996) and that blocking the expression of tTGase in HIV-1 chronically-infected cells significantly induces higher viral production (Amendola et al., 2001), tTGase is thought

to play a role in reducing viral production. Combined with the fact that its binding partner, eIF5A, is the putative cofactor of Rev, it has been suggested that tTGase may act as an effective and natural defence mechanism for the host by binding to eIF5A and retaining it in the cytoplasm, thereby blocking the export of viral mRNA into the cytoplasm and limiting viral production in HIV+ cells (Amendola et al., 2001).

1.2.6.4.6 eIF5A and syntenin

Syntenin, also known as syndecan binding protein, was identified as a binding partner of eIF5A by Li et al. (2004) using yeast two-hybrid screen. Syntenin binds to the cytoplasmic C-termini of various proteins, including syndecans, ephrins, pro-TGF-α, IL-5-specific α-subunit (IL-5Rα) and neurofascin (Grootjans et al., 1997; Torres et al., 1998; Fernandez-Larrea et al., 1999; Cowan et al., 2002; Geijsen et al., 2001; Koroll et al., 2001), and has been reported to play a role in protein trafficking (Fernandez-Larrea et al., 1999), activation of the transcription factor, Sox4 (Geijsen et al., 2001), and in the organization of cell-cell and cell-matrix adhesions (Zimmermann et al., 2001). While syntenin is widely expressed in all human tissues investigated (Zimmermann et al., 2001), it is over-expressed in many types of human cancer (Koo et al., 2002; Boukerche et al., 2005). Recent studies have indicated that syntenin may play a critical role in tumor progression and metastasis (Koo et al., 2002; Helmke et al., 2004; Boukerche et al., 2005).

It has been suggested that the N-terminal domain of eIF5A, but not the hypusine residue, is required for the interaction beween eIF5A and Syntenin (Li et al., 2004). Of interest is the finding that the interaction between eIF5A and syntenin antagonizes the ability of eIF5A to up-regulate p53, suggesting that syntenin may act as a negative regulator of the eIF5A-p53 signaling pathway (Li et al., 2004).

1.2.6.5 mRNA decay

Several studies with yeast and mammalian cells have indicated a possible role for eIF5A in the regulation of mRNA turnover (Zuk and Jacobson, 1998; Veress et al., 2000; Nishimura et al., 2002; Valentini et al., 2002). A yeast temperature-sensitive eIF5A mutant was shown to exhibit decreased decay rates for several mRNAs, as well as a two-fold accumulation of uncapped mRNAs at the restrictive temperature (Zuk and Jacobson, 1998). In another study, mutation of the temperature-

sensitive alleles of the yeast *TIF51A* gene caused defects in the degradation of short-lived mRNAs at the nonpermissive temperature, supporting a role for eIF5A in mRNA decay (Valentini et al., 2002). Since the accumulated RNAs in eIF5A mutants lack the 5'-cap, it has been suggested that eIF5A affects the decay of specific yeast mRNAs by acting downstream of the decapping step and upstream of the degradation by exonuclease (Zuk and Jacobson, 1998; Schrader et al., 2006). Furthermore, the nonsense-mediated decay (NMD) pathway, which targets mRNAs that have acquired premature translation termination codons (Review by Frischmeyer and Dietz, 1999), and Poly(A) binding protein 1, which is essential for mRNA decay (Review by Caponigro and Parker, 1996; Sachs and Varani, 2000), are both thought to be functionally related to the ability of eIF5A to regulate mRNA turnover in yeast (Valentini et al., 2002; Schrader et al., 2006). Inhibition of hypusine formation in mammalian cells using either deoxyspergualin or α-difluoromethylornithine (DFMO) has been shown to induce stabilization and accumulation of several kinds of decapped mRNA, prompting the suggestion that it is the hypusinated form of eIF5A that is involved in mRNA decay (Veress et al., 2000; Nishimura et al., 2002).

1.2.6.6 Cellular organizing activity of eIF5A

Recent studies with yeast temperature-sensitive *TIF51A* mutants have implicated eIF5A in the maintenance of cell wall integrity and actin cytoskeleton organization (Zanelli and Valentini, 2005; Chatterjee et al., 2006), both of which are crucial for progression of the cell cycle from G1 to S phase in yeast (Reviewed by Pruyne et al., 2004). These findings thus correlate with earlier observations that depletion of eIF5A or inhibition of hypusine formation results in cell cycle arrest (Schnier et al., 1991; Park et al., 1993b; Hanauske-Abel et al., 1994; Kang and Hershey, 1994; Chen et al., 1996; Shi et al., 1996b; Tome and Gerner, 1997). In addition, Weir and Yaffe (2004) reported that Mmd1p, which is a DHH homologue in fission yeast, is required for mitochondrial distribution, an event regulated by microtubules (Huffaker et al., 1988; Jacobs et al., 1988). Moreover, disruption of the DHH gene in budding yeast leads to increased sensitivity to the antimicrotubule drug, benomyl, further supporting the contention that hypusine-containing eIF5A is involved in the regulation of microtubule stability in yeast (Weir and Yaffe, 2004).

1.2.6.7 Cell growth and proliferation

1.2.6.7.1 eIF5A and the growth of yeast cells

Hypusinated eIF5A is essential for the growth of the yeast, *Saccharomyces cerevisiae* (Schnier et al., 1991; Sasaki et al., 1996; Park et al., 1998). Strains with both isoforms of eIF5A genes disrupted exhibit slowed or inhibited growth (Schnier et al., 1991) as well as enlarged cells arrested in G1 phase (Kang and Hershey, 1994). Blocking the hypusine modification in yeast either by substitution with a mutant eIF5A gene in which the specific lysine is mutated (Schnier et al., 1991) or by disruption or deletion of the DHS gene (Sasaki et al., 1996; Park et al., 1998) results in loss of cell viability and production of non-viable spores. However, deletion of the DHH gene only slightly inhibits growth (Park et al., 2006), suggesting a critical role for deoxyhypusine-containing eIF5A for the growth of yeast. It should be noted that in higher eukaryotic organisms, i.e., *Caenorhabditis elegans* (Sugimoto, 2004) or *Drosophila melanogaster* (Spradling et al., 1999), disruption of the DHH gene results in recessive lethality, indicating an essential function for hypusine-containing eIF5A.

1.2.6.7.2 eIF5A and the growth of mammalian cells

Mammalian cell growth and proliferation are regulated by the carefully balanced expression of proto-oncogenes to promote cell proliferation and tumour suppressor genes to inhibit proliferation (Reviewed by King and Cidlowski, 1998; Vermeulen et al., 2003). Accumulating evidence suggests that eIF5A and hypusine formation play a role in the regulation of mammalian cell proliferation. First, hypusine modification has been reported to occur in growing cells but not resting cells, and to increase following mitogen stimulation (Cooper et al., 1982). In addition, Chen (1983) found that fetal calf serum, the most widely used growth supplement for cell cultures, stimulates the hypusine modification of eIF5A in mouse neuroblastoma NB-15 cells. As well, inhibition of hypusination by using specific inhibitors of DHS or DHH or by depleting the polyamine, spermidine, inhibits the growth of mammalian cells by inducing reversible arrest at the G1/S boundary of the cell cycle, and the treated cells ultimately die (Park, 1987; Byers et al., 1992 & 1994; Jakus et al., 1993; Park et al., 1993a, b & c; Park et al., 1994; Hanauske-Abel et al., 1994; Chen et al., 1996; Shi et al., 1996b; Tome and Gerner, 1997; Lee et al., 2002; Jasiulionis et al., 2007). These observations have prompted the proposal that the transition from G1 to S phase of the eukaryotic cell cycle is a hypusine-sensitive

event. Indeed, it has been suggested that eIF5A, especially hypusine-containing eIF5A, could perhaps be used as a diagnostic biomarker for *in situ* detection of aberrant cell proliferation (Cracchiolo et al., 2004).

1.2.6.8 eIF5A and apoptosis

Apoptosis, the process by which damaged and unwanted cells are induced to die, is essential for the development and homeostasis of normal tissues (Reviewed by Arends et al., 1990; Dragovich et al., 1998; Hengartner, 2000; Ferri and Kroemer, 2000; Hengartner, 2000). Recent evidence indicates that eIF5A plays a role in apoptosis. For example, suppression of hypusine formation has been shown to induce apoptosis (Tome and Gerner, 1997; Tome et al., 1997; Caraglia et al., 1999; Beninati et al., 1998; Caraglia et al., 2001; Takeuchi et al., 2002). In particular, depletion of spermidine or treatment of cells with specific inhibitors of DHS or DHH, the two enzymes that mediate hypusination, induces cell death (Park et al., 1993b; Hanauske-Abel et al., 1994; Chen et al., 1996; Shi et al., 1996b; Tome and Gerner, 1997; Tome et al., 1997; Jin et al., 2003). It should be noted that these treatments not only inhibit the formation of hypusine-containing eIF5A, but also induce the accumulation of hypusine-absent eIF5A, raising the possibility that it is the unmodified eIF5A that is involved in cell death. Such an interpretation, however, must be tempered by the fact that most of the inhibitors used in these studies are either polyamine analogues or iron chelators (Csonga et al., 1995; Liao et al., 1998; Caraglia et al., 1999; Kim et al., 2006; Park et al., 2006). Thus, in addition to their ability to inhibit hypusination, they also affect a number of other events in cellular metabolism (Chen et al., 1996; Kulp and Vulliet, 1996; Nishimura et al., 2005; Perry et al., 2005), raising the possibility that their ability to induce apoptosis may not reflect inhibition of hypusination.

Additional evidence for the involvement of eIF5A in apoptosis has come from experiments with inhibitors of the ubiquitin-proteasome (UP) pathway. It is well known that inhibition of the UP pathway induces cell cycle arrest as well as apoptosis (Fujita et al., 1996; Drexler, 1997; Kitagawa et al., 1999; Wagenknecht et al., 1999; An et al., 2000; Naujokat et al., 2000; MacLaren et al., 2001; Sassone et al., 2006). Of particular interest is the finding that hypusine-containing eIF5A was shown to be down-regulated coincident with proteasome inhibitor-induced apoptosis, and this was accompanied by an accumulation of hypusine-absent eIF5A (Jin et al., 2003). This finding is consistent with the possibility that unhypusinated eIF5A plays a role in apoptosis. eIF5A has also been shown to be involved in heat-stress induced apoptosis. Incubation of MIA PaCa-2 human

pancreatic carcinoma cells or Jurkat human leukemic T cells at 51 $^{\circ}$C for 30 min suppressed cell growth and led to cell death, and this was associated with almost complete disappearance of hypusine-containing eIF5A (Takeuchi et al., 2002).

1.2.6.8.1 eIF5A and cytokines

Some studies also suggest a regulatory role for eIF5A in cytokine-induced apoptosis. Exposure of human epidermoid carcinoma KB cells to interferon α (IFNα) induced cell growth inhibition and apoptosis, paralleled by a 75% decrease of hypusine synthesis (Caraglia et al., 1997). Treatment with epidermal growth factor (EGF) not only antagonised the anti-proliferative and apoptosis-inducing effects of IFNα in these experiments, but also reconstituted hypusine levels (Caraglia et al., 1999). Furthermore, GC7 and IFNα, which both inhibit hypusine synthesis, have been shown to have a synergistic effect on cell growth inhibition and apoptosis (Caraglia et al., 2003). Thus, these data suggest a pro-apoptotic function of inhibited hypusination, which potentiates the antitumor activity of IFNα.

There is also evidence that eIF5A plays a role in death receptor-mediated apoptosis. Treatment of human lamina cribrosa cells with camptothecin and TNFα induces strong up-regulation of eIF5A, coincident with the induction of apoptosis (Taylor et al., 2004). In addition, blocking the expression of eIF5A using specific siRNA reduces TNFα-induced apoptosis by up to 80% (Taylor et al., 2004).

1.2.6.8.2 eIF5A and tTGase

eIF5A may also play a role in tTGase-induced apoptosis. TGases are involved in a variety of essential physiological activities, including apoptosis (Fesus et al., 1987; Fesus et al., 1989; Melino et al., 1994). tTGase is specifically expressed in cells undergoing apoptosis, and over-expression of tTGase induces apoptosis and inhibits cell proliferation (Beninati et al., 1998; Caraglia et al., 2001). Moreover, stable over-expression of tTGase in mouse embryonic fibroblast BALB/c 3T3 cells induced a 100-fold reduction of hypusine levels, which was attributed to depletion of the free polyamine pool and formation of γ-glutamyl polyamine conjugates (Beninati et al., 1998). These findings led to the proposal that apoptosis induced by tTGase is at least in part attributable to inhibition of hypusine formation (Caraglia et al., 2001).

1.2.6.8.3 eIF5A and p53

There is increasing evidence that eIF5A regulates p53-induced apoptosis. For example, over-expression of eIF5A induces up-regulation of p53 mRNA and protein levels, as well as p53 targets including Bax and p21 (Li et al., 2004). As well, in a recent comparison of protein expression in HCT116 human colon carcinoma cells carrying wild-type p53 and corresponding isogenic p53 null cells, eIF5A was shown to be associated with p53 expression (Rahman-Roblick et al., 2007). DNA damage leading to apoptosis was induced in both cell types, and eIF5A mRNA and protein levels proved to be highly up-regulated in treated p53+/+ cells (Rahman-Roblick et al., 2007). Most interestingly, Rahman-Roblick et al. (2007) also discovered a p53-binding motif in the first intron of eIF5A, thereby verifying the role of eIF5A as a p53 target.

1.2.6.8.4 eIF5A may be involved in the regulation of homeostasis

Several molecules and pathways are partially cross-utilized in opposing processes like cell survival on one hand and cell death on the other. For example, multifunctional transcription factor proteins like p53 (Reviewed by Levine et al., 1997; Sionov and Haupt, 1999; Liebermann et al., 2007), E2F (Loughran and La Thangue, 2000; Muller et al., 2001) and NF-κB (Reviewed by Dutta et al., 2006; Piva et al., 2006) play important roles in the regulation of apoptosis as well as cell survival and cell proliferation. Components of the MAPK signaling cascade including JNK, p38 and ERK also promote survival as well as cell death (Reviewed by Chang et al., 2003; Lin, 2003; Nishina et al., 2003; Nishina et al., 2004; Wada and Penninger, 2004; Lu and Xu, 2006). In response to apoptotic stimuli, these "double-edge" molecules activate both survival and death-promoting pathways, which in turn interplay and compete until one eventually dominates and determines the fate of the cell. It has been suggested that this mechanism assures not only close regulation of these pathways, but also that mutations within one signaling cascade will to some extent affect the opposite process, thus contributing to homeostatic regulatory circuits (Reviewed by Maddika et al., 2007). That eIF5A may regulate both proliferation and apoptosis suggests that it could be involved in the maintenance of homeostasis.

1.2.6.9 eIF5A and senescence

Senescence is a state of terminal arrest in which cells are metabolically active for extended periods, but fail to re-enter the cell cycle even after mitogenic stimulation (Hayflick, 1965). Evidence suggesting that eIF5A may be involved in the regulation of senescence includes the finding that hypusine-forming activity is not only prominently serum-responsive, but also much higher in low passage young primary human embryo lung cells than in late passage senescent cells (Chen and Chen, 1997a). Indeed, the senescing cells have little or no hypusine-forming activity (Chen and Chen, 1997a). Moreover, unhypusinated eIF5A is up-regulated 1.3 fold in human skin epidermis from old individuals (89 years old) compared with that of young individuals (24 years old) (Gromov et al., 2003). The senescence-related function of eIF5A was supported by another study in which eIF5A was shown to decrease during the senescence of human umbilical vein endothelial cells (Eman et al., 2006). It should be noted that the change in hypusine-forming activity with advancing senescence appears to be mainly due to a decrease in DHS activity and in the amount of eIF5A precursor, while the eIF5A mRNA level is unaltered (Chen and Chen, 1997a).

1.2.7 eIF5A and cancer

Several reports have suggested a possible role for eIF5A in the development of cancer. Activation of *myc* oncogene, which is rearranged, amplified or mutated in many human tumor types (Reviewed by Arvanitis Felsher, 2006; Vita and Henriksson, 2006), induces up-regulation of eIF5A mRNA *in vitro* (Coller et al., 2000; Boon et al., 2001). In addition, eIF5A is up-regulated in many human lung adenocarcinomas, and enhanced eIF5A1 protein levels have been found in lung tumors with poor differentiation, K-ras mutations or p53 nuclear accumulation as well as in patients with poor survival prospects (Chen et al., 2003).

1.2.7.1 DHS

DHS, the enzyme mediating the first of two reactions leading to hypusination of eIF5A, has been identified as one of a signature set of amplified genes in cancer metastases (Ramaswamy et al., 2003). In addition, DHS mRNA is over-expressed in several human cancer cell lines, including inflammatory breast cancer cell line Sum149, chronic myeloid leukemia cell line K526, breast adenocarcinoma cell line MCF7, prostate adenocarcinoma cell line PC3, colorectal adenocarcinoma

cell line Colo205, ovarian carcinoma cell line OVCAR4 and epithelial breast cancer cell line HS578T (Golen et al., 1999; Clement et al., 2006). These data suggest that DHS may play a role in cancer development and progression.

1.2.7.2 Oncogenic role of eIF5A2

There are two eIF5A genes in the human genome, *eIF5A1* and *eIF5A2*. *eIF5A2* resides in an unstable region of chromosome 3q26 (Guan et al., 2001), which is an area that often exhibits chromosomal alterations such as translocation, duplication or inversion in human ovarian carcinoma (Brass et al., 1997). Over-expression of eIF5A2 mRNA has been detected in many primary ovarian cancers and ovarian cancer cell lines (Guan et al., 2001; Jenkins et al., 2001) as well as in cells that have been derived from colorectal adenocarcinoma, leukemia and lymphoma (Jenkins et al., 2001; Clement et al., 2003 & 2006). In a study of confirmed human epithelial ovarian cancers, over-expression of eIF5A2 protein was not only detected in 57.8% of the cases, but also was significantly associated with advanced ovarian cancer (Guan et al., 2004). In addition, eIF5A2 has been correlated with cell proliferation. For example, suppression of eIF5A2 expression decreases the growth of UACC 1598 cells, a human ovarian cancer line known to express high levels of eIF5A2 (Guan et al., 2004).

Based on its frequent up-regulation in human overian cancers as well as its apparent role in cell proliferation in such cancers, it has been proposed that eIF5A2 is an oncogene whose expression is related to the development of ovarian cancer (Guan et al., 2001). This contention is supported by the finding that injection of eIF5A2-transfected LO2 normal human liver cancer cells into nude mice induces tumor formation (Guan et al., 2004). Moreover, in a recent study of the gene expression profile of primary tumors from 32 patients who underwent radical surgery for gastric carcinoma, eIF5A2, together with Bik and aurora kinase B, were found to be over-expressed in most of the metastatic lymph node-positive (N+) cases, suggesting that it might be a useful prognostic predictor of metastatic lymph node status (Marchet et al., 2007).

1.2.8 Objectives of the present study

It is clear that eIF5A is an important protein that has been implicated in both cell proliferation and apoptosis. In the present study, the mechanism underlying the pro-apoptotic function of eIF5A

was examined in human cancer cell lines by suppressing its expression using specific siRNA and by over-expressing the protein using adenovirus constructs. Both isoforms of human eIF5A were examined in this study, but greater emphasis was given to eIF5A1. In addition, the roles of the post-translationally modified forms of eIF5A in apoptosis were examined using 2D gel electrophoresis to seperate them and mass spectrometry to confirm their identity.

Chapter 2 Materials and Methods

2.1 Cell Lines

Three cell lines were used in this study. COS-7, an African green monkey kidney fibroblast-like cell line, was purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA). HT-29, a human colon adenocarcinoma cell line, and Hela S3, a human cervical adenocarcinoma cell line, which is a clonal derivative of the human cervical adenocarcinoma cell line, Hela, and is capable of suspension growth, were kind gifts from Anita Antes (University of Medicine and Dentistry of New Jersey, Newark, New Jersey, USA). Hela S3 cells were cultured in Minimum Essential Medium (Eagle) (E-MEM) (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) containing 2 mM L-glutamine, Earle's Balanced Salt Solution adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids (Sigma-Aldrich), 1.0 mM sodium pyruvate (Sigma-Aldrich) and 10% (v/v) double-heat inactivated fetal bovine serum (FBS). COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) with 4mM L-glutamine, Earle's Balanced Salt Solution adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose (Sigma-Aldrich) and 10% double-heat inactivated FBS. HT-29 cells were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 2 mM L-glutamine, Earle's Balanced Salt Solution adjusted to contain 2.2 g/l sodium bicarbonate, 1.0 mM sodium pyruvate, 10 mM HEPES (Sigma-Aldrich) and 10% double-heat inactivated FBS. All media were also supplemented with an antibiotic and antimycotic mixture (Gibco, Invitrogen Canada Inc., Burlington, Ontario, Canada) to contain 100 units/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B. Cultures were maintained in a humidified incubator at 37 °C under conditions of 5% carbon dioxide plus 95% air and were split twice weekly. To split cells that had reached confluence, the medium was removed, and the cells were harvested by incubation in 0.25% trypsin-EDTA (Gibco) until they began to detach. Addition of medium containing 10% FBS stopped the trypsin activity, and one eighth of the cell suspension was transfered to a new T25 flask and diluted with fresh complete media.

2.2 Isolation of protein and cell fractions

2.2.1 Preparation of whole cell lysates

For isolation of whole cell lysates, cells were trypsinized, collected in an Eppendorf tube (Eppendorf Canada, Mississauga, Ontario, Canada), washed twice with phosphate-buffered saline (PBS; 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 and 137 mM NaCl, pH 7.4) and then resuspended in boiling lysis buffer (2% SDS and 50 mM Tris-HCl pH 7.4). For every 5 x 10^5 cells, 200 μl of lysis buffer was used. Lysates were boiled at 95 ^{o}C for 5 min, and then sonicated at 5 volts for 30 seconds using a Sonic Dismembrator Model 100 (Fisher Scientific Ltd., Nepean, Ontario, Canada). All samples were centrifuged at 14,000g for 10 minutes at 4 ^{o}C in a Heraeus Biofuge Pico microcentrifuge (Heraeus, Heraeusstraße, Hanau, Germany) to remove debris. Supernatants were collected and stored at -20 ^{o}C.

2.2.2 Preparation of cytosolic and mitochondrial/membrane extracts

Cytosolic and mitochondrial/membrane extracts were prepared by first trypsinizing cells and washing them in PBS. The cells were resuspended (5 x 10^6 cells / 300 μl) in sucrose buffer (250 mM sucrose, 10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA and 1 mM EDTA) containing 0.025% digitonin (Sigma-Aldrich) to permeabilize the plasma membrane and 1 x protease inhibitor cocktail (Sigma-Aldrich). The suspension was incubated at room temperature for 10 min, and the cell lysates were then cleared by centrifugation at 14,000g for 10 min at 4 ^{o}C. The supernatant was placed in a new Eppendorf tube and designated as the cytosolic fraction.

The resultant pellet of permeabilized cells was subsequently resuspended in 150 μl complete lysis buffer [2 M Thiourea, 7 M Urea, 30 mM Tris, 4% CHAPS (Sigma-Aldrich), 0.5% Triton X-100 (Bioshop Canada Inc.) and 1 x protease inhibitor cocktail] and incubated on ice for 10 minutes with gentle shaking. The sample was then centrifuged at 14,000g for 10 minutes at 4 ^{o}C in a microcentrifuge (Heraeus) to remove debris. The supernatant was transferred to a new Eppendorf tube and designated as the mitochondrial/membrane fraction.

2.2.3 Protein quantitation using bicinchoninic acid

The protein concentration of cell extracts was determined using the bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich) following the manufacturer's instructions. The protein standards were prepared by diluting bovine serum albumin (BSA) protein standard solution (Sigma-Aldrich) with different amounts of cell lysis buffer (2% SDS and 50mM Tris-HCl pH 7.4) to generate a series of BSA solutions of different concentrations, which were 0 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml, 150 μg/ml, 200 μg/ml, 400 μg/ml and 600 μg/ml. Samples were diluted in the cell lysis buffer in a ratio of 1:5. Ten μl of each standard and diluted sample were loaded in duplicate into wells of a 96-well plate. Two hundred μl BCA solution, which was prepared by mixing 50 parts of reagent A with 1 part of reagent B (both provided in the kit), was added to each well. The plate was covered with foil and incubated at 37 ^{o}C for 30 min. Absorbance values were measured at 562 nm using a Versamax Tunable microplate reader (Molecular Devices, Concord, Ontario, Canada). Protein concentrations of the samples were calculated by plotting their absorbances on the standard curve that was constructed from the absorbance values of the standards versus their concentrations.

2.2.4 Protein quantitation using the Bradford method

For samples harvested in lysis buffer containing high concentrations of chemicals that precluded the use of BCA, the Bradford assay was used to measure protein concentration. The standards for the Bradford method were prepared by diluting 1 μg, 2 μg, 2.5 μg, 3 μg, 3.5 μg, 4 μg, 4.5 μg, 5 μg or 6 μg of the BSA protein standard (Sigma-Aldrich) in 600 μl of Bradford solution, which was in turn made by diluting 1 part of the stock Bradford reagent (Bioshop Canada Inc., Burlington, Ontario, Canada) with 3 parts of double-distilled water. Three μl each unknown sample was also diluted in 600 μl of diluted Bradford solution. Absorbance values were measured at 595 nm using a DU-64 spectrophotometer (Beckman, GMI Inc., Ramsey, Minnesota, USA). The protein concentrations of the samples were calculated by plotting their absorbances on the standard curve that was constructed from the absorbances of the standards versus their concentrations.

2.2.5 SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Mini-Protean 3 cell from Bio-Rad (Bio-Rad Laboratories, Mississauga, Ontario, Canada). A 12% or 15% (w/v)

resolving gel was overlaid with a 5% (w/v) stacking gel. Five μg protein sample was diluted 1:3 in 4 x SDS gel loading buffer (320 mM Tris-HCl pH 6.8, 40% (v/v) glycerol and 8% (w/v) SDS) and boiled at 100 $^\circ$C for 10 minutes before being loaded into the bottom of the wells using a special gel-loading pipet tip (Fisher Scientific Ltd.). Five μl of page ruler prestained protein ladder (Fermentas Canada Inc., Burlington, Ontario, Canada) was loaded into a separate well in order to track the running of samples. Proteins were then separated using SDS electrophoresis running buffer (25mM Tris, 250mM glycine and 0.1% (w/v) SDS) at 40 mAmps until the bromophenol blue reached the bottom of the resolving gel. The resolving gel was removed and soaked in chilled transfer buffer [25 mM Tris-HCl pH 8.3, 192mM Glycine and 20% (v/v) methanol] for 20 minutes. Hypond-P PVDF membrane (Amersham Biosciences, Amersham Health Inc., Mississauga, Ontario, Canada) was prepared by soaking in 100% (v/v) methanol for 10 seconds and incubation in chilled transfer buffer for 15 minutes. Two pieces of blotting paper (VWR) were prepared by incubation in chilled transfer buffer for 5 minutes. An electroblotting cassette containing the gel, Hypond-P PVDF membrane and the blotting paper was assembled and placed in the Trans-blot SD semi-dry transfer cell (Bio-Rad Laboratories) according to the manufacturer's instructions. Proteins on the gel were transferred to the PVDF membrane at 22 volts for a period of 36 minutes.

2.2.6 Membrane blocking and incubation with antibodies

Following the transfer, the PVDF membrane was removed from the electroblotting cassette and rinsed briefly in TBST [Tris buffered saline (50 mM Tris-HCl pH 7.4 and 150 mM NaCl) containing 0.1% (v/v) Tween 20]. The non-specific binding sites on the membrane were blocked by incubation in 15 ml of blocking buffer [5% (w/v) skim milk power and 0.05% Tween 20 (v/v) in TBS (50 mM Tris-HCl pH 7.4 and 150 mM NaCl)] for one hour at room temperature with gentle shaking.

The membranes were then incubated in anti-Actin (Calbiochem, VWR CANLAB, Mississauga, Ontario, Canada), anti-Bad (Cell Signaling Technology, New England Biolabs Ltd., Pickering, Ontario), anti-Bax (Cell Signaling Technology), anti-Bcl-2 (Cell Signaling Technology), anti-Bid (Cell Signaling Technology), anti-Bim (Cell Signaling Technology), anti-cytochrome *c* (BD Biosciences, Mississauga, Ontario, Canada), anti-eIF5A1 (BD Biosciences), anti-eIF5A2 (Novus Biologicals Inc., Littleton, Colorado, USA), anti-HA (Roche Applied Science, Roche Diagnostics, Laval, Quebec, Canada), anti-p53 (Cell Signaling Technology) or anti-Puma (Cell Signaling Technology) which were diluted 1:20000, 1:2000, 1:1000, 1:1000, 1:1000, 1:1000, 1:500, 1:20000,

1:1000, 1:1000, 1:1000 or 1:1000, respectively, with primary antibody dilution buffer [0.05% (v/v) Tween 20 and either 5% (w/v) skim powder milk or BSA (Sigma-Aldrich) in TBS] for either 1 hour at room temperature or overnight at 4 ^{o}C with gentle agitation. The blots were then washed with TBST 3 times for 10 minutes each and incubated for 1 hour at room temperature with gentle agitation in HRP-conjugated secondary antibody, which was anti-mouse IgG (Sigma-Aldrich), anti-mouse IgM (Calbiochem) or anti-Rabbit IgG (Calbiochem) diluted according to the manufacturers' instructions in secondary antibody dilution buffer [0.05% (v/v) Tween 20 and 1% (w/v) skim powder milk in TBS]. The membranes were then washed with TBST 3 times for 10 minutes each at room temperature before detection.

2.2.7 Detection of protein

ECL Plus Western blotting detection reagents (Amersham Health Inc.) were used following the manufacturer's instructions. Basically, detection solution A and B were mixed in a ratio of 40:1, and 2 ml of the mixture was used for each 8 cm x 10 cm membrane. Excess wash buffer was drained off the membrane, and the membrane was placed in a clean, dry Petri dish with the protein side facing up. The membrane was incubated in the detection mixture at room temperature for 5 minutes and then removed from the Petri dish and placed between two layers of transparent plastic wrap. Air bubbles were smoothed out with a glass rod. The wrapped blot was then placed in an X-ray film cassette with the protein side up. In a dark room with only the duplex super safelight (Thomas Instrument Company, Inc., Palmyra, Virginia, USA) on, a piece of clear blue X-ray film (Fuji Photo Film Canada Inc., Mississauga, Ontario, Canada) was exposed to the membrane for 30 seconds in the cassette. The film was then developed and dried in the AGFA CP 1000 X-ray films processor (AGFA Inc., Toronto, Ontario, Canada). The exposure time was adjusted as necessary.

2.2.8 Stripping and reprobing the membrane

In order to probe the same membrane with different antibodies, the membrane was stripped of bound antibody and re-probed several times. In detail, the membrane was submerged in 50 ml of stripping buffer (100 mM 2-mercaptoethanol, 2% (w/v) SDS and 62.5 mM Tris-HCl pH 6.7) and incubated at 50 ^{o}C for 30 minutes with occasional agitation. The membrane was then briefly rinsed with distilled water and washed in large volumes of TBST twice for 15 minutes each with gentle

shaking. The membrane was then incubated in blocking buffer for 1 hour and re-probed with another antibody.

2.3 Plasmid construction and transfection

2.3.1 Cloning and construction of pHM*eIF5A1* and pHM*eIF5A2*

The plasmids, pHM*eIF5A1* and pHM*eIF5A2*, were constructed by Catherine Taylor in Dr. Thompson's laboratory. For this purpose, human eIF5A1 (access number: NM_001970) and human eIF5A2 (access number: NM_020390) were cloned by RT-PCR from total RNA isolated from RKO human colorectal carcinoma cells using the GenElute mammalian RNA miniprep kit (Sigma-Aldrich) according to the manufacturer's protocol for adherent cells. The primers used for eIF5A1 were: forward, 5'-CGAGTTGGAATCGAAGCCTC-3'; and reverse, 5'-GGTTCAGAGGATCACTGCTG-3'. The primers used for eIF5A2 were: forward, 5'-AAACTACCATCTCCCCTGCC-3'; and reverse, 5'-TGCCCTACACAGGCTGAAAG-3'. The resulting products were subcloned into pGEM-T Easy vector and sequenced. The resulting plasmid containing eIF5A1 was used as a template for PCR using the following primers: forward, 5'-GCCAAGCTTAATGGCAGATGATTTGG-3'; and reverse 5'-CCTGAATTCCAGTTATTTTGCCATGG-3'. The resulting plasmid containing eIF5A2 was used as a template for PCR using the following primers: forward, 5'-ATCAAGCTTGCCCACCATGGCAGACG-3'; and reverse 5'-AACGAATTCCATGCCTGATGTTTCCG-3'. The PCR products were subcloned into the *Hind* III and *EcoR* I sites of pHM6 (restriction enzymes as well as the vectors were all purchased from Roche Applied Science), which is a mammalian epitope tag expression vector, thereby generating pHM*eIF5A1* and pHM*eIF5A2*, respectively. eIF5A in this plasmid has HA, a nonapeptide epitope tag from influenza hemagglutinin, attached to its N-terminus. pHM*eIF5A1* and pHM*eIF5A2* were validated by sequencing. pHM*lazZ*6, which was supplied by the manufacturer (Roche Applied Science), was used as the control vector. It features a 3 kb fragment containing the β-galactosidase gene cloned in frame with the N-terminal HA tag and C-terminal His6 (six histidine residue epitope). Protein expressions of pHM*eIF5A1*, pHM*eIF5A2* and pHM*lacZ6* are enhanced by the efficient immediate-early human cytomegalovirus (CMV) promoter sequence. In addition, both vectors contain antibiotic resistance genes that permit selection in both bacteria and mammalian cells.

2.3.2 Preparation of eIF5A1 with a point-mutation

A site-specific mutation of eIF5A1 at a specific lysine residue was obtained by PCR using pHM*eIF5A1* DNA as the template. Using the forward and reverse primers, 5'-GCCAAGCTTAATGGCAGATGATTTGG-3' and 5'-GTG$\boxed{\text{CGC}}$GCCAGTC$\underline{\text{TTCGAA}}$GTAG-3', respectively, a 158 bp portion of the coding region of eIF5A1containing a point-mutation converting lysine$_{50}$ to alanine (indicated by a rectangle) as well as the *Sfu* I restriction enzyme site (underlined) was obtained. Using the forward and reverse primers, 5'-CTAC$\underline{\text{TTCGAA}}$GACTGGC$\boxed{\text{GCG}}$CAC-3' and 5'-CCTGAATTCCAGTTATTTTGCCATGG-3', respectively, the remaining 347 bp segment of the coding region of eIF5A1 also bearing a point mutation converting lysine$_{50}$ to alanine (indicated by a rectangle) and the restriction enzyme site for *Sfu* I (underlined) was synthesized. The two PCR products were isolated on a 2 % (w/v) agarose gel in 1 x TAE buffer (40 mM Tris, 2 mM Na$_2$EDTA, glacial acetic acid to pH 8.3) at 80 volts. DNA fragments were cut from the gel and purified using Perfectprep gel clean-up kit (Eppendorf Canada) according to the manufacturer's instructions. Extracted 158 bp and 347 bp fragments were both digested with *Sfu* I (Roche Applied Science) at 37 ^{o}C for 3 hours, and then incubated together with T4 ligase (Fermentas Canada Inc.) overnight at 16 ^{o}C. The ligation product was isolated on a 2 % (w/v) agarose gel in 1 x TAE buffer at 80 volts and purified using the Perfectprep gel clean-up kit. The purified ligation product was subsequently sub-cloned into the *Hind* III and *EcoR* I sites of the pHM6 vector. This construct of mutated eIF5A1 was validated by sequencing and referred to as pHM*eIF5A1(K50A).*

2.3.3 *Escherichia coli* transformation

pHM*eIF5A1*, pHM*eIF5A2*, pHM*eIF5A1(K50A)* and pHM*lacZ6* plasmids were separately transformed into competent *Escherichia coli* DH5α cells. Briefly, 10 μl of plasmid was added to 200 μl of DH5α cell suspension, and the mixture was incubated on ice for 30 minutes. The mixture was then heat-shocked at 42 ^{o}C for 90 seconds. The cells were chilled on ice for 1 to 2 minutes before being mixed with 800 μl 2 x YT medium (16 g/l Tryptone, 10 g/l Yeast extract and 5 g/l NaCl, pH 7.0) and incubated at 37 ^{o}C for 45 minutes with shaking. The cells were then quickly spun down and resuspended in 200 μl of 2 x YT medium. The transformation culture was transferred onto a Luria-Bertani (LB) agar plate (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl and 15g/l agar, pH 7.0) supplemented with 50 μg/ml ampicillin. The plate was incubated overnight at 37 ^{o}C. Only the transformants were able to grow on the antibiotic-containing plate because of the ampicillin resistance

that is provided by the pHM6 vector. A single colony was inoculated into 5 ml of LB broth (10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl, pH 7.0) supplemented with 50 μg/ml ampicillin and incubated overnight at 37 $^{\circ}$C with shaking. Plasmid DNA was isolated using the Wizard plus miniprep DNA purification system (Promega, Fisher Scientific Ltd., Nepean, Ontario, Canada), and then digested with *EcoR* I and *Hind* III for one hour at 37 $^{\circ}$C. The digestion product was fractionated on a 1% (w/v) agarose gel and visualized under a UV lamp to verify the presence of the *eIF5A1* or *eIF5A1(K50A)* insert. Plasmid DNA was sequenced by the sequencing facility of York University (Toronto, Ontario, Canada).

2.3.4 Purification of plasmid DNA

Escherichia coli cells transformed with pHM*eIF5A1*, pHM*eIF5A2*, pHM*eIF5A1(K50A)* or pHM*lacZ*6 were aseptically streaked on LB agar plates containing 50 μg/ml ampicillin. The plates were incubated overnight at 37 $^{\circ}$C and stored at 4 $^{\circ}$C for up to a month. Single fresh colonies were aseptically inoculated into 50 ml 2 x YT medium supplemented with 50 μg/ml ampicillin and incubated overnight at 37 $^{\circ}$C with gentle shaking. Plasmid DNA was then harvested using the GenElute high performance plasmid miniprep kit (Sigma-Aldrich) following the manufacturer's instructions. DNA product was purified by mixing with 100 mM sodium acetate (pH 5.2) and 64.5% (v/v) ethanol and incubation at -20 $^{\circ}$C for at least one hour. The mixture was centrifuged at 14,000g for 30 minutes at 4 $^{\circ}$C. The supernatant was carefully removed without disturbing the pellet, which was then washed twice by resuspension in 70% (v/v) ethanol and centrifugation at 14,000g for 30 minutes at 4 $^{\circ}$C. The final supernatant was aspirated off carefully, and the pellet was air-dried in a fume hood to allow the ethanol to evaporate thoroughly. The dry DNA pellet was resuspended in tissue-culture-grade water (Sigma-Aldrich). DNA concentration and purity were determined spectrophotometrically by measuring the absorbance at 260 nm, 280 nm and 320 nm using a DU-64 spectrophotometer (Beckman).

2.3.5 Transfection of mammalian cells using electroporation

COS-7 cells were trypsinized and resuspended in DMEM containing 10% FBS at a concentration of 1.5 x 10^6 cells/ml. 15 μg of pHM*eIF5A1*, pHM*eIF5A1(K50A)* or pHM*lacZ*6 DNA was diluted in 50 μl Opti-MEM I reduced medium (Gibco), and then mixed with 650 μl of diluted

COS-7 cells. The mixture was transferred to a 2 mm gap electroporation cuvette (BTX Technologies Inc., Hawthorne, New york, USA), which was then inserted in the slide of the pulser chamber of a T820 ElectroSquarePorator (BTX Technologies Inc.). DNA was electroporated into the cells at 1.5 kilovolts and 90 microseconds per pulse for 1 to 2 pulses. The cells were then transferred into one well of a 6-well plate, which was pre-loaded with complete medium, and the concentration of FBS was brought up to 10%. The medium was changed 24 hours after the transfection. Cell lysates were harvested 48 hour after electroporation and subjected to analysis.

2.3.6 LacZ staining

Forty-eight hours after electroporation, COS-7 cells transfected with pHM*lacZ*6 were stained in order to determine the transfection efficiency. The medium was removed, and the cells were washed with PBS twice, fixed with 0.5% (w/v) glutaraldehyde in PBS at room temperature for 10 minutes, and then washed twice with PBS containing 1 mM $MgCl_2$. They were then incubated in lacZ staining solution [5 mM $K_4Fe(CN)_6$$\cdot$$3H_2O$, 5 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$ and 0.1% (w/v) X-gal in PBS] at 37 ^{o}C until the desired level of staining was achieved. The staining solution was then removed, and the cells were washed with PBS. Numbers of stained cells and unstained cells were counted separately to determine the transfection efficiency.

2.3.7 Labeling eIF5A with [^{3}H]-spermidine

COS-7 cells were transfected with pHM*eIF5A1*, pHM*eIF5A1(K50A)* or pHM*lacZ*6 by electroporation as described above. Six hours after transfection, 40 μCi/ml [terminal methylene ^{3}H (N)]-spermidine (PerkinElmer Life Science, Boston, Massachusetts, USA) with or without 1 mM aminoguanidine (AM) was added to each well. Forty-two hours later, the cells were washed three times with PBS to remove free [^{3}H]-spermidine. Cell lysates were then harvested as described in section 2.2.1. Proteins as well as protein markers were fractionated on an SDS-PAGE gel containing a 15% resolving gel and a 5% stacking gel, and subsequently transferred to a piece of Hypond-P PVDF membrane as described in section 2.2.6. The membrane was incubated in Amplify fluorographic reagent (Amersham Health Inc.) for 30 minutes to enhance the radioactivity signal. A piece of Hyperfilm (Amersham Health Inc.) was incubated with the membrane in a cassette for 10 days at -80 ^{o}C, and then developed to visualize the [^{3}H]-labeled protein. The membrane was then wetted with

methanol, washed twice for 10 minutes with PBS, and subjected to Western blot analysis using anti-eIF5A1 and anti-HA as described in section 2.2.7.

2.3.8 Immunoprecipitation of eIF5A

COS-7 cells transfected with pHM*eIF5A1* were incubated in complete medium supplemented with 40 μCi/ml [terminal methylene ^{3}H (N)]-spermidine as well as 1 mM AM. At 48 and 72 hours after transfection, the cells were washed twice with ice-cold PBS and lysed by incubation in ice-cold IP lysis buffer [150 mM NaCl, 1% (v/v) NP40, 50 mM Tris-HCl pH 8.0 and 1 x protease inhibitor cocktail] for 30 minutes on ice with gentle shaking. The cells were scraped from the bottom of the plate and, together with the lysis buffer, transferred to an Eppendorf tube and centrifuged at 14,000g for 10 minutes at 4 ^{o}C. The supernatant was transferred to a fresh Eppendorf tube. Cellular proteins were pre-cleared by incubation with Protein G plus-agarose suspension (Calbiochem) as well as anti-mouse IgG (Sigma), and the supernatant was then incubated in anti-eIF5A1 (BD Biosciences) at a ratio of 100:1 (v/v) at 4 ^{o}C overnight with gentle rotation. Protein G-plus agarose was added to the mixture the next morning, and the combined mixture was incubated at 4 ^{o}C for 2 hours with gentle rotation. The mixture was spun down at 14,000g for 10 seconds, and the beads were washed five times with ice-cold PBS. The protein associated with the agarose beads was then eluted by boiling in 1 x SDS PAGE gel loading buffer at 100 ^{o}C for 10 minutes. Immunoprecipitation (IP) product was then subjected to SDS-PAGE, autoradiography and immunoblot analysis as described in section 2.3.7.

2.4 Induction of apoptosis

HT-29 cells or Hela S3 cells were treated with Actinomycin D, sodium nitroprusside or MG-132, a proteasome inhibitor, in order to induce apoptosis. Specifically, cells were incubated in 0.5 μg/ml Actinomycin D (Calbiochem), 3 mM sodium nitroprusside (Sigma-Aldrich) or 5 μM MG-132 (Sigma-Aldrich) in complete medium for periods of up to 24 hours. Serum starvation was used as a means of inducing cell stress leading to apoptosis. For this purpose, cells were cultured under normal growth conditions until they reached about 30 to 40% confluence. The medium was then removed, and the cells were incubated in medium containing 0% FBS for 72 hours.

2.5 siRNA studies

2.5.1 Designing siRNA

siRNA specifically targeted to human eIF5A1 as well as a control siRNA were designed by Catherine Taylor and obtained from Dharmacon (Chicago, Illinois, USA). The eIF5A1 siRNA, which targets the 3′UTR of eIF5A1 mRNA (GenBank Accession No BC085015), has the following sequence: sense strand, 5'-GCUGGACUCCUCCUACACAdTdT-3'; and antisense strand, 3'-dTdTCGACCUGAGGAGGAUGUGU-5'. The control siRNA has the reverse sequence of the eIF5A1 siRNA and is not identical to any known human gene product. The control siRNA has the following sequence: sense strand, 5'-ACACAUCCUCCUCAGGUCGdTdT-3'; and antisense strand, 3'-dTdTUGUGUAGGAGGAGUCCAGC-5'.

2.5.2 siRNA transfection using Lipofectamine 2000

HT-29 or Hela S3 cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen Canada Inc.) following the protocol provided by the manufacturer. On the day before the transfection, cells were seeded in either a 96-well plate for cell proliferation and viability studies or a 24-well plate for Western blot analysis so that they would reach 30-50% confluence at the time of transfection. For the reaction in each well of the 96-well plate, 4.08 pmol siRNA and 0.16 μl Lipofectamine 2000, which were separately diluted in 8 μl Opti-MEM I reduced serum medium (Gibco), were mixed together and incubated at room temperature for 20 minutes to allow the formation of siRNA-Lipofectamine 2000 complex. For the reaction in each well of the 24-well plate, 25.5 pmol siRNA and 1 μl Lipofectamine 2000, which were separately diluted in 50 μl Opti-MEM I reduced serum medium (Gibco), were used. The medium was removed from the cells, and the cells were incubated in the siRNA-Lipofectamine 2000 complexes at 37 $^{\circ}$C in a CO_2 incubator to initiate transfection. Four to six hours later, the medium was diluted 1:2 with RPMI 1640 containing 30% FBS, so that the concentration of FBS was brought up to 10%. The medium was replaced with RPMI 1640 containing 10% FBS at 24 hours after the initiation of transfection, and the cells were then subjected to specified treatments.

2.5.3 XTT cell viability assay

XTT [2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)] allows measurement of cell viability. This is made possible by the fact that XTT, a yellow-colored tetrazolium salt, is cleaved to a soluble orange-colored formazan dye by the mitochondrial succinatetetrazolium reductase system of metabolically active cells, a reaction that can be quantified by measuring the absorbance at 490 nm using a microplate reader.

For measurements of viability, HT-29 cells were seeded on a 96-well plate, transfected with either eIF5A1 siRNA or control siRNA, and then subjected to either no further treatment or to apoptotic stimuli (see section 2.4). The XTT reagent solution and the activation solution (both provided in the kit from Roche Applied Science) were thawed in a 37 $^{\circ}$C water bath and mixed at a ratio of 50:1. 50 μl of the mixture was loaded into each well and incubated with the cells in a 37 $^{\circ}$C incubator until color had formed. Absorbance values were measured at 490 nm and 630 nm (the latter is used as reference absorbance) using a Versamax Tunable microplate reader (Molecular Devices).

2.5.4 BrdU cell proliferation assay

BrdU (5-bromo-2'-deoxyuridine) was used to measure cell proliferation. In proliferating cells, BrdU is incorporated into DNA, and the incorporation can be detected by immunoassay. Thus, whereas XTT is a cell proliferation assay that measures cell metabolic activity, BrdU measures cell proliferation by detecting newly synthesized DNA in the cells.

The BrdU cell proliferation ELISA colorimetric assay (Roche Applied Science) was performed following the manufacturer's instructions. HT-29 cells seeded in a 96-well plate were treated with eIF5A1 siRNA or control siRNA and then incubated with diluted BrdU labeling solution at 37 $^{\circ}$C for 2 hours to allow incorporation of BrdU into the DNA of proliferating cells. After removing the labeling medium, the cells were fixed, and the DNA was partially denatured by incubation in FixDenat for 30 minutes at room temperature. The cells were then incubated in anti-BrdU-POD for 90 minutes at room temperature to allow binding of the antibody to BrdU incorporated into newly synthesized DNA. Then, excess antibody was removed, and the 96-well plate was rinsed three times for five minutes with gentle shaking using washing solution provided in the kit. Finally, substrate solution was added to the cells. In the presence of hydrogen peroxide, peroxidase (POD) catalyzed the oxidation of diacylhydrazides-like luminal, emitting light. The signal was quantified by

measuring the absorbance at 370 nm and 492 nm (the latter is used as reference wavelength) using a Versamax Tunable microplate reader (Molecular Devices).

2.5.5 ELISA detection of cell death

During apoptosis, mono- and oligo-nucleosomes consisting of cores of histones and DNA chains coiled around histones are released into the cytoplasm coincident with the degradation of chromosomal DNA. The cell death detection ELISA kit purchased from Roche Applied Science was used to measure apoptotic cell death by detection of these cytoplasmic histone-associated-DNA-fragments.

HT-29 cells seeded on a 96-well plate were treated with eIF5A1 siRNA or control siRNA and subjected to apoptotic stimuli (see section 2.4). To measure apoptosis, the microplate was centrifuged at $200g$ using an IEC 21000R centrifuge (IEC, Needham Heights, Maryland, USA) for 10 minutes. The supernatant was carefully removed, and the cell pellet was resuspended in the lysis buffer provided in the kit and incubated for 30 minutes at room temperature to release the cytoplasmic fraction. The plate was then centrifuged at $200g$ for 10 minutes, and 20 μl of supernatant, which was the cytoplasmic fraction, was carefully transferred into a streptavidin-coated microplate for analysis. Eighty μl of mixture containing anti-histone-biotin and anti-DNA-POD was also loaded into each well. The plate was tightly sealed with adhesive cover foil and incubated at room temperature for 2 hours with gentle shaking. During the incubation, the antibody mixture bound to the histone and DNA components of the nucleosomes, and the immunocomplex was thus bound to the streptavidin-coated microplate by biotin. Unbound antibodies were removed, and the plates were rinsed 3 times with incubation buffer provided in the kit. ATBS (2,2-Azino bis-3-ethylbenzothiazoline-6-sulforic acid diammonium salt) solution was added to each well. The reaction between ATBS and POD-linked complex allowed color formation; therefore, the amount of apoptosis-associated nucleosomes was detected and quantified by measuring the absorbance value at 405 nm using a Versamax Tunable microplate reader (Molecular Devices).

2.5.6 Cell cycle analysis

Hela S3 cells were transfected with eIF5A1 siRNA or control siRNA as described above. At 24, 48 and 72 hours after transfection, the medium was collected, and the cells were treated with

0.25% Trypsin-EDTA for one minute or until they began to detach. Detached cells were combined with the media collected previously, and the mixture was centrifuged at 300g for 3 minutes using an IEC Centra CL2 centrifuge (IEC). The cells were washed once with PBS, and then resuspended in 0.5 ml PBS. The cell suspension was transferred into a 14 ml Corning tube (Lowell, Massachusetts. USA), and cells were fixed by adding with 4.5 ml ice-cold 70% (v/v) ethanol. The fixed cells were stored at -20 ^{o}C for up to four weeks. On the day of analysis, cells were centrifuged for 5 minutes at 300g. Ethanol was decanted thoroughly, and the cells were resuspended in 1.5 ml PBS and allowed to recover by incubation at room temperature for 10 minutes. The recovered cells were spun down at 300g for 5 minutes, resuspended in PI staining solution [40 μg/ml propidium iodide (Sigma-Aldrich) and 200 μg/ml RNase A ((Bioshop Canada Inc.) in PBS] at 1 x 10^6 cells/ml and incubated at room temperature in darkness for 45 minutes. The final cell suspension was pipetted through a nylon 6/6 mesh screen cloth with 50 micron openings (Small Parts Inc., Miami lakes, Florida, USA) to remove large multicellular aggregates. Subsequently, the treated cells were analyzed using a BD FACSVantage SE system (BD Bioscience) with an argon laser source. 15,000 cells per sample were acquired in list mode and analyzed with WinMDI 2.8 software (Flow cytometry core facility, Purdue University, West Lafayette, Indiana, USA).

2.5.6.1 Cell cycle arrest using GC7

GC7, which is an inhibitor of hypusination, was used as a control for the cell cycle analysis. Hela S3 cells were incubated in 50 μM GC7 (Biosearch Technologies Inc., Novato, California, USA) in complete medium for 24, 48 or 72 hours, and then subjected to cell cycle analysis.

2.6 Adenovirus studies

2.6.1 Generation of adenovirus expressing eIF5A

Adenovirus (Adenovirus 5 serotype, E1, E3-deleted) expressing human eIF5A1, eIF5A2 or eIF5A1 bearing a single point mutation (K50→A50) [eIF5A(K50A)] that inhibits hypusination were constructed using the AdMax™ Hi-IQ system (Microbix Biosystems Inc., Toronto, Canada) by Dr. Songmu Jin in Dr. Thompson's laboratory.

The eIF5A cDNAs were amplified by PCR using pHM*eIF5A1,* pHM*eIF5A2* and pHM*eIF5A1(K50A)* plasmid DNAs as template and ligated into the *Sma* I site of the adenovirus

shuttle vector pDC516(μio). The sequences of the PCR primers were: forward, 5′-GCCAAGCTTAATGGCAGATGATTTGG-3′; and reverse, 5′-CCTGAATTCCAGTTATTTTGCCATGG-3′. The adenovirus genomic plasmid vector pBHGfrt(del)E1,3FLP and the shuttle vectors were propagated in *Escherichia coli* DH5α and purified using Qiagen EndoFree plasmid mega kit (Qiagen Inc., Mississauga, Ontario, Canada). 293-IQ cells (Microbix Biosystems Inc.) in 60 mm culture plates that were 60 to 80% confluent were transfected with 5 μg each of the adenovirus genomic plasmid pBHGfrt(del)E1,3FLP and shuttle vector, pDC516(io)-eIF5A1, pDC516(io)-eIF5A2 or pDC516(io)-eIF5A1(K50A) using the CaCl$_2$ method recommended by Microbix Biosystems Inc.. Plaques appeared after 7 to 10 days of incubation at 37 ^{o}C, and the resulting adenoviral particles [Ad-eIF5A1, Ad-eIF5A2 and Ad-eIF5A1(K50A)] were amplified in 293-IQ cells. Pure adenovirus stocks were prepared by CsCl gradient ultracentrifugation according to the protocol provided by Microbix Biosystems Inc. An adenovirus vector expressing LacZ (Ad-LacZ; serotype 5; E1,E3-deleted) was purchased from Qbiogene Inc. (Irvine, California, USA) and used as a control and reporter in these experiments. The Ad-LacZ adenovirus was amplified and purified in the same manner as the Ad-eIF5A1, Ad-eIF5A2 and Ad-eIF5A1(K50A) virus constructs.

2.6.2 Induction of apoptosis by infection with adenovirus eIF5A

In order to induce apopotosis, HT-29 or Hela S3 cells were seeded at 1×10^5 cells per well on a 6-well tissue culture plate and infected with adenovirus constructs the next day with 4000 plaque forming units per cell (pfu/cell) for HT-29 cells or 500 pfu/cell for Hela S3 cells in medium containing 2% FBS. Additional medium was added to the cells at four hours after infection, and the concentration of FBS was brought to 10%.

2.6.3 DeadEnd fluorometric TUNEL assay of apoptosis

TdT-mediated dUTP Nick-End Labeling (TUNEL) enables specific detection and quantitation of apoptosis within a cell population. The enzyme terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals and then catalyzes the addition of fluorescein-12-dUTP which allows the direct visualization of cells containing fragmented DNA by fluorescence microscopy.

The TUNEL assay was carried out using a Promega kit and following the manufacturer's instructions. HT-29 cells were seeded on an eight-chamber culture slide (Falcon, BD Biosciences, Mississauga, Ontario, Canada). After treatment with adenovirus eIF5A constructs to induce apoptosis, the medium was removed, and the cells were washed twice with PBS, fixed by incubation in freshly diluted 3.7% (v/v) methanol-free formaldehyde solution (Polysciences Inc., Warrington, Pennsylvania, USA) in PBS at 4 $^{\circ}$C for 20 minutes, and then permeabilized in 0.2% (v/v) Triton X-100 for 5 minutes. The cells were then equilibrated in equilibration buffer provided in the kit for 5 minutes and then incubated in the TdT incubation buffer, which is a mixture of the equilibration buffer, nucleotide mixture and TdT enzyme, in a humidified environment at 37 $^{\circ}$C for 60 minutes. The cells were immersed in 2 x SSC (300 mM NaCl, 30 mM sodium citrate pH 7.0) for 15 minutes at room temperature to terminate the reaction, and then washed three times with PBS to remove unincorporated fluorescein-12-dUTP. The cells were subsequently stained with 5 μg/ml Hoechst 33358 (Sigma-Aldrich) for 10 minutes at room temperature. The slide was covered with Crystoseal 60 (Richard-Allan Scientific, Kalamazoo, Michigan, USA) as the mounting medium, and the edge was sealed with clear nail polish (Revlon Canada Inc., Mississauga, Ontario, Canada). The cells were visualized directly by fluorescence microscopy (Carl Zeiss Canada Ltd., Toronto, Ontario, Canada) using a UV filter (UV-G 365, filter set 487902) to observe the nucleus and a fluorescein filter (Green H546, filter set 48915) to observe the apoptotic cells. Pictures were taken with a camera connected to the microscope, and total cells and apoptotic cells, respectively, were counted. The percentage of apoptotic cells was calculated by dividing the number of apoptotic cells by the total number of cells.

2.6.4 Annexin V/PI assay of apoptosis

Apoptosis was also measured using Annexin V/PI. This assay detects the translocation of the phospholipid, phosphatidylserine (PS), from the inner leaflet of the plasma membrane to the outer leaflet during the early stage of apoptosis.

The assay was performed using the Annexin V-FITC apoptosis detection kit I (BD Bioscience) according to manufacturer's instructions. In detail, after the induction of apoptosis by treatment with adenovirus eIF5A constructs, Hela S3 cells were detached by trypsinization, washed twice with cold PBS and resuspended in 1 x binding buffer provided in the kit at a concentration of 1 x 10^6 cells/ml. One hundred μl of diluted cells (1 x 10^5 cells) were transferred to a FACs tube (Falcon), and mixed with 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI). The mixture

was incubated at room temperature for 15 minutes in the dark, and then combined with 400 μl of 1 x binding buffer to terminate the reaction. The cells were sorted using a BD FACSVantage SE system (BD Bioscience) with an argon laser source. 15,000 cells were counted for each sample. The data were analyzed using WinMDI 2.8 software. Unstained cells, cells stained with Annexin V-FITC alone (no PI), cells stained with PI alone (no Annexin V-FITC) and Actinomycin D-treated cells were used to set the quadrants.

2.6.5 Trypan blue measurement of cell death

For measurement of cell death using trypan blue, Hela S3 cells treated with adenovirus eIF5A constructs were trypsinized and resuspended in PBS at 1 - 2 x 10^5 cells/ml. Twenty μl of 0.5% (w/v) Trypan blue stain was added to each 100 μl cell suspension, and mixed thoroughly. After 3 to 5 minutes, the cells were loaded into a hemocytometer, and stained and unstained cells, respectively, were counted. Cells that excluded the dye were viable, and the non-viable cells absorbed the dye and appeared blue. The percentage of viable cells was calculated by dividing the number of unstained cells by the total number of cells.

2.6.6 Measurement of mitochondrial transmembrane potential following induction of apoptosis

The induction of apoptosis can also be detected by measuring changes in mitochondrial transmembrane potential ($\Delta\Psi_m$). In the present study, DiOC6(3), a dye that permeates through the plasma membrane, was used to detect loss of $\Delta\Psi_m$ following the induction of apoptosis by treatment with adenovirus eIF5A constructs. At low concentration (50 to 100 nM), the dye will accumulate in the mitochondrial matrix under the influence of the $\Delta\Psi_m$. A decrease in $\Delta\Psi_m$ will result in less dye binding to the cells, and can be detected by the measurement of fluorescence intensity using flow cytometry.

Hela S3 cells treated with adenovirus eIF5A constructs were trypsinized and diluted in MEM containing 10% (v/v) FBS to 2 x 10^6 cells/ml. One μl of 1.75 x 10^4 nM DIOC6(3) (Sigma-Aldrich) was added to each 350 μl cell suspension so that its working concentration was 50 nM. The cell-dye mixture was protected from light and incubated at 37 ^{o}C for 20 minutes. After incubation, cells were washed twice with PBS, and resuspended in 500 μl of PBS. Samples were kept on ice and analyzed

within 1 hour using a BD FACSVantage SE system with an argon laser source. For each sample, 15,000 cells were acquired in list mode and analyzed with WinMDI 2.8 software.

2.6.7 Caspase assay

The activation of caspases reflecting the induction of apoptosis by treatment with adenovirus eIF5A constructs was measured using the caspase detection kits from Calbiochem and following the manufacturer's instructions. Kits that specifically target caspase 3, 8 and 9 were used in separate experiments. The assay is based on the fluorescenct markers, FITC-DEVD-FMK, FITC-IETD-FMK and FITC-LEHD, which are cell-permeable, non-toxic inhibitors that bind irreversibly to activated caspase 3, 8 and 9 in living cells, respectively. Cells with active caspases are easily detected by measuring fluorescence intensity using flow cytometry.

Hela S3 cells, which had been treated with adenovirus eIF5A constructs to induce apoptosis, were trypsinized and resuspended in complete medium containing 10% (v/v) FBS at 1×10^6 cells/ml. Three hundred μl of cell suspension was mixed with 1 μl of FITC-DEVD-FMK, FITC-IETD-FMK or FITC-LEHD and incubated for 1 hour in a 37 $^{\circ}$C incubator with 5% CO_2. The cells were then centrifuged at 300g for 5 min, and the supernatant was removed. The cells were washed twice with the wash buffer provided in the kit, and finally resuspended in 300 μl of wash buffer. Samples were kept on ice and protected from light. The cells were sorted using a Coulter Epics XL-MCL flow cytometry system (Beckman Coulter Canada Inc., Mississauga, Ontario, Canada) with an argon laser source. For each sample, 25,000 cells were acquired in list mode and analyzed with WinMDI 2.8 software.

2.7 2-Dimensional gel electrophoresis and mass spectrometry

2.7.1 2-Dimensional gel electrophoresis

The post-translationally modified forms of eIF5A1 were fractionated by 2-dimensional (2D) gel electrophoresis and identified by Western blotting. HT-29 or Hela S3 cells were used for these experiments. The growth medium was removed, and the cells were washed twice with ice-cold PBS. Lysates were obtained by incubating the cells in cold lysis buffer [7 M Urea, 2 M Thiourea, 30 mM Tris, 4% (w/v) CHAPS and 1 x protease inhibitor cocktail] for 30 minutes with gentle shaking followed by sonication at 5 volts for 30 seconds using a Sonic Dismembrator Model 100. Debris was

57

removed by centrifugation at 14,000g for 10 minutes. Protein concentration was measured using the Bradford method.

The first dimensional isoelectric focusing (IEF) was performed with an Ettan IPGphor Isoelectric Focusing System (Amersham Health Inc.) according to the manufacturer's instructions. Immobiline DryStrips (7 cm, pH 4-7; Amersham Health Inc.) were rehydrated in rehydration buffer [8 M Urea, 2% (w/v) CHAPS, 0.2% (w/v) DTT, 0.5% (v/v) pH 4-7 IPG buffer and 0.002% (v/v) Bromophenol blue] containing 5 μg or 0.3 μg of cell lysate at room temperature for 12 hours. The isoelectric focusing was then performed at 500 volts for 30 minutes, 1000 volts for 10 minutes, and 2500 volts for 100 minutes. Proteins on the IPG strip gel were reduced by incubation in SDS equilibration buffer [50mM Tris-HCl pH 8.8, 6M Urea, 30% (v/v) Glycerol, 2% (w/v) SDS and 0.002% (w/v) bromophenol blue] containing 10 mg/ml dithiothreitol (DTT) (Fermentas Canada Inc.) at room temperature for 15 minutes with gentle shaking, and then alkylated by incubation in 25 ml SDS equilibration buffer containing 25 mg/ml iodoacetamide (IAA) (Sigma-Aldrich) for 15 minutes at room temperature in the dark with gentle shaking. The IPG strip was then carefully put on top of a 15% SDS-PAGE resolving gel and sealed with agarose sealing solution [0.5% (w/v) agarose and 0.002% (w/v) bromophenol blue in SDS electrophoresis buffer (25mM Tris, 250mM glycine and 0.1% (w/v) SDS)]. Proteins on the IPG strips were then separated by SDS-PAGE and transferred to a Hypond-P PVDF membrane (Amersham Health Inc.). The membrane was then subjected to Western blot analysis using specific antibody for eIF5A1 (BD Bioscience).

2.7.2 Mass spectrometry

The separated post-translationally modified forms of eIF5A1 were sequenced by Zhenyu Cheng from Dr. McConkey's laboratory using mass spectrometry (MS). For this purpose, the first dimensional isoelectric focusing was conducted using 24 cm Immobiline DryStrips (24 cm, pH 4.5-5.5; Amersham Health Inc.). The strips were rehydrated in rehydration buffer [8 M urea, 2% (w/v) CHAPS, 0.2% (w/v) DTT, 0.5% (v/v) pH 4.5-5.5 IPG buffer and 0.002% (w/v) Bromophenol blue] containing 400 μg of cell lysate at room temperature for 12 hours. The isoelectric focusing was performed at 500 volts for 1 hour, 1000 volts for 3 hours, 5000 volts for 5 hours, and 8000 volts for 10 hours. Proteins on the IPG strip gel were then separated by SDS-PAGE.

The 2D gels were then stained with Bio-Safe Coomassie (Bio-Rad Laboratories) for 2 hours and destained with water overnight with gentle shaking. Spots of interest were excised from the gel

and cut into 1 mm cubes. Gel pieces were washed three times for five minutes each with HPLC-grade water and then destained twice for 10 minutes each with 50 mM NH_4HCO_3/ 50% (v/v) acetonitrile (ACN). Gel particles were dehydrated by vortexing in 100 µl 100% (v/v) ACN for 10 minutes. Proteins were reduced by incubation of the gel particles in 10 mM DTT in 100 mM NH_4HCO_3 at 50°C for 30 minutes, and alkylated by incubation in 55 mM IAA in 100 mM NH_4HCO_3 for 30 minutes in the dark. The gel particles were then washed for 10 minutes with 100 mM NH_4HCO_3 and dehydrated again with 100 µl 100% (v/v) ACN. The gel particles were then air-dried and rehydrated for ten minutes in 10 µl 20 ng/µl endoproteinase solution [either trypsin or *Staphylococcus aureus* Protease V8 (GluC)] (both from Promega). Then 50 µl of 50 mM NH_4HCO_3 was added to the gel pieces, and the proteins were digested at 37 °C for 16 hours.

The peptides produced by trypsin digestion were extracted by adding 50 µl HPLC-grade water and vortexing for 10 minutes. The supernatant was transferred to a collecting tube that contained 5 µl of 5% (v/v) formic acid (FA) in 50% (v/v) ACN. The gel particles were further extracted twice with 75 µl of 5% (v/v) FA in 50% (v/v) ACN and vortexing for 5 minutes. The supernatants from these extractions were combined with the original extraction. The volume in the collecting tube was reduced to 10 µl using a Speedvac. Samples were cleaned using the C-18 ZipTip system (Millipore, Billerica, Massachusetts, USA) followed by addition of 2 µl 1% (v/v) FA to protonate the peptides for mass spectrometry.

Mass spectrometry of the peptides was performed on a Waters-Micromass Q-TOF Ultima Mass Spectrometer (Waters Ltd., Mississauga, Ontario, Canada) using nano-spray injection as the sample delivery method. Both peptide mass fingerprinting analysis and MS/MS profiling using PEAKS software (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) were used to obtain amino acid sequence data.

2.8 Confocal microscopy

Confocal microscopy was used to ascertain the subcellular localization of eIF5A1 protein after labeling cells with eIF5A1-specific primary antibody and FITC-labeled secondary antibody. For this purpose, Hela S3 cells were seeded in a glass bottom culture dish (MatTek Cooperation, Ashland Massachusetts) and incubated in 400 nM MitoTracker Red CMXRos (Invitrogen Canada Inc.) in complete medium for 45 minutes under normal growth conditions. After staining, the medium was removed, and the cells were washed twice with pre-warmed PBS and fixed at 37 °C for 15 minutes

with 3.7% (v/v) formaldehyde (Polysciences Inc.) which was freshly diluted in PBS. After the solvents were removed, the cells were rinsed twice with PBS and incubated for 5 minutes in PBS solution containing 100 mM glycine. The cell membranes were then permeabilized by incubation in 0.2% (v/v) Triton X-100 at room temperature for 5 minutes, and the cells were washed twice with PBS and incubated for 1 hour at room temperature or overnight at 4 $^{\circ}$C in anti-eIF5A1 (BD Bioscience), which was diluted either 1:100 or 1:250 in PBS containing 1% (w/v) BSA. After the primary antibody was removed, cells were washed with PBS twice and then incubated for 1 hour at room temperature in FITC-conjugated anti-mouse IgG (Sigma-Aldrich) diluted 1:200 in PBS. The cells were then washed twice with PBS, incubated in 1 μM Sytox Blue (Invitrogen Canada Inc.) at room temperature for 15 minutes, washed with PBS twice and covered with PBS. Samples were viewed using a LSM 510 META laser scanning confocal microscope (Carl Zeiss Canada Ltd.) equipped with Argon, Diode 405, Hene1 and Hene2 laser lines. Images were analyzed using the LSM5 Image Examiner software (Carl Zeiss Canada Ltd.).

Chapter 3 Results

3.1 Role of eIF5A1 in cell proliferation and maintenance of cell viability

3.1.1 Blocking the expression of eIF5A1 has no effect on cell proliferation or cell viability

There are several reports indicating that eIF5A and hypusine formation are essential for the growth of yeast cells (Schnier et al., 1991; Kang and Hershey, 1994; Sasaki et al., 1996; Park et al., 1998). It has also been reported for mammalian cells that inhibitors of DHS induce cell cycle arrest at G1 phase (Park et al., 1993b; Hanauske-Abel et al., 1994; Chen et al., 1996; Shi et al., 1996b; Tome and Gerner, 1997). However, the inhibitors of DHS used in these experiments are not specific for the DHS reaction and may affect a number of other events in cellular metabolism (Chen et al., 1996; Nishimura et al., 2005), and thus there is no direct evidence linking eIF5A to mammalian cell growth.

In order to determine more definitively whether eIF5A is required for growth of mammalian cells, the effects on cell proliferation of siRNA specifically targeting human eIF5A1 were determined. HT-29 human colorectal adenocarcinoma cells were transfected with either eIF5A1 siRNA or control siRNA. Figure 1A illustrates that eIF5A1 expression is almost completely suppressed by 72 hours after transfection. At intervals of 0, 24, 48, 72, 96, and 120 hours after transfection, cell viability was measured using the XTT assay which depicts mitochondrial activity, and cell proliferation was measured using the BrdU assay which portrays DNA synthesis accompanying cell division. The growth curves constructed from both assays revealed that suppressing the expression of eIF5A1 using specific siRNA has no effect on cell proliferation or cell viability, suggesting that eIF5A1 is not essential for the growth of mammalian cells (Figures 1B and C).

3.1.2 Blocking the expression of eIF5A1 has no effect on cell cycle progression

To further assess the possibility that eIF5A1 is involved in cell division, the effect of eIF5A siRNA on cell cycle progression was examined. Hela S3 cells were transfected with eIF5A1 siRNA or control siRNA. Figure 2A demonstrates that eIF5A1 expression was strongly suppressed within 72 hours. Cell cycle progression was analyzed at 24, 48 and 72 hours after transfection using PI staining. The data indicate that suppressing the expression of eIF5A1 has no effect on cell cycle progression

Figure 1: Suppressing the expression of eIF5A1 with specific siRNA has no effect on cell proliferation.

HT-29 cells were transfected with either eIF5A1 siRNA or control siRNA. eIF5A1 expression levels were determined at 72 hour after transfection. Cell proliferation and cell viability were measured at 0, 24, 48, 72, 96, and 120 hours after transfection. **A)** Western blot probed with anti-eIF5A1 and anti-actin. Each lane contained 5 μg of protein. Actin served as a loading control. A1: eIF5A1 siRNA-transfected cells; con: control siRNA-transfected cells; un: untreated. Results shown are representative of those obtained in 3 independent experiments. **B)** Determination of cell viability using the XTT assay. Data are expressed as percentage of the viability of cells before the transfection. **C)** Determination of cell proliferation using the BrdU assay. Data are expressed as percentage of the proliferation of cells before transfection. For **B** and **C,** each experiment was repeated four times, and the values shown are means +/- standard error. Asterisks (**) denote values determined not to be significantly different from the corresponding control values by independent t-test ($p > 0.5$).

A)

A1   con   un

Anti-eIF5A1

Anti-actin

B)

C)

← siRNA eIF5A1   ← siRNA control
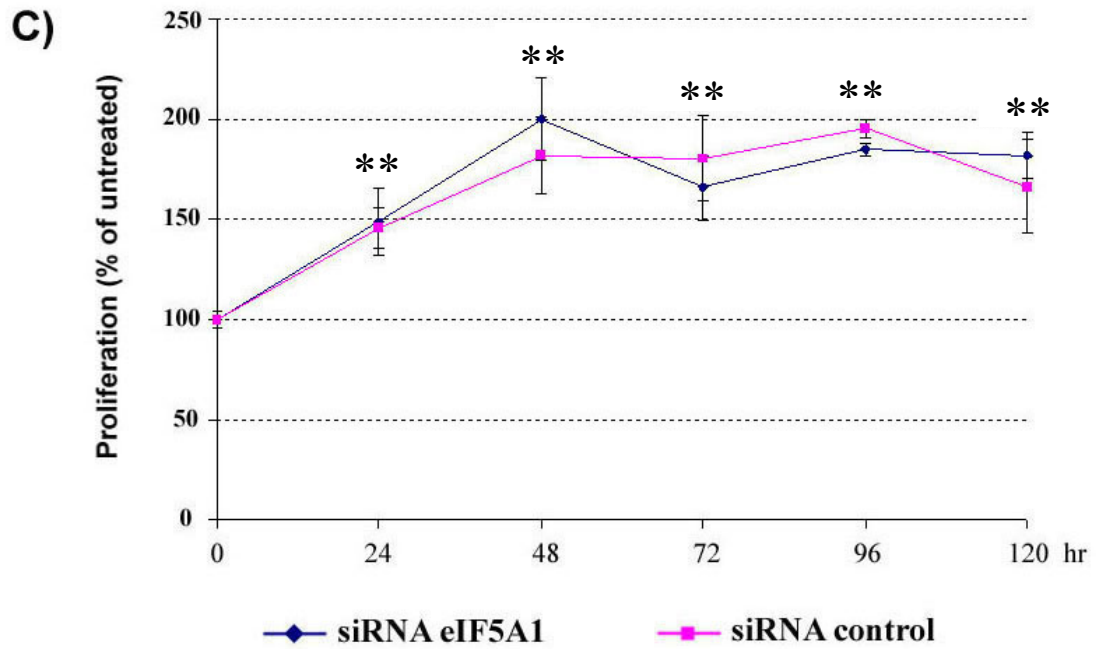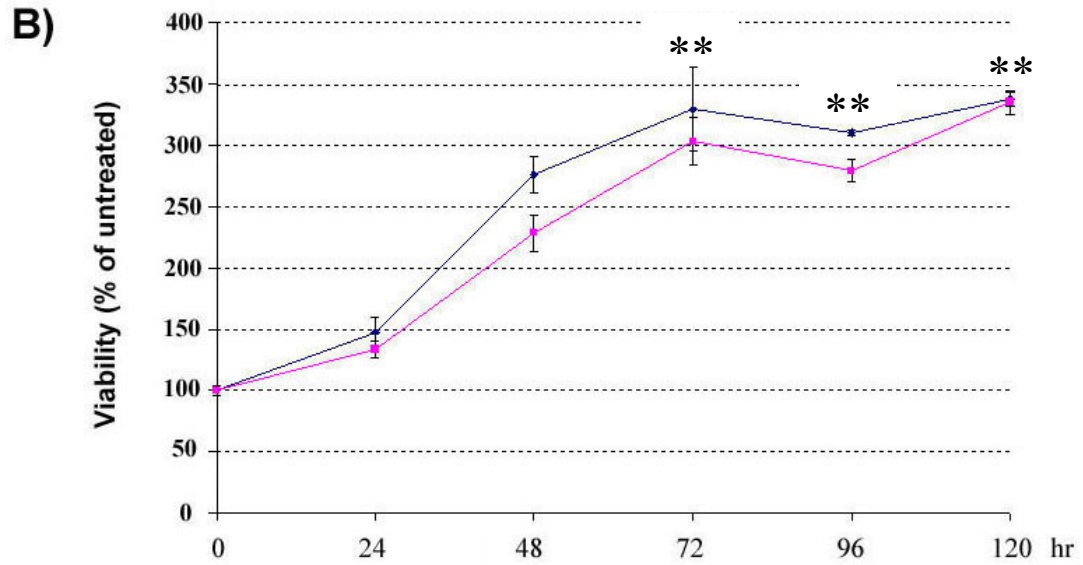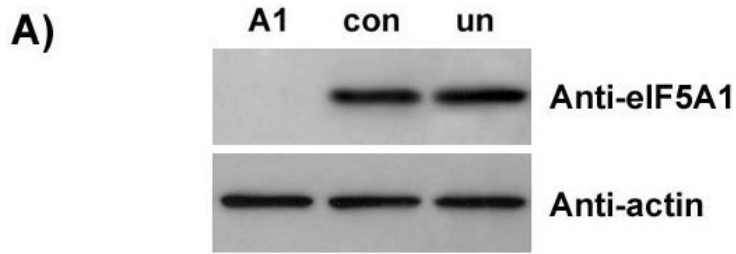
Figure 2: Suppressing the expression of eIF5A1 with specific siRNA has no effect on cell cycle progression.

Hela S3 cells were transfected with either eIF5A1 siRNA or control siRNA. eIF5A1 expression levels were examined 72 hours after transfection. Cell cycle analysis was conducted at 24, 48 and 72 hours after transfection. **A)** Western blot probed with anti-eIF5A1 and anti-actin. Each lane contained 5 μg of protein. Actin served as a loading control. A1: eIF5A1 siRNA-transfected cells; con: control siRNA-transfected cells; un: untreated. Results shown are representative of those obtained in 3 independent experiments. **B)** Flow cytometer analysis of cell-propidium iodide (PI) conjugates illustrating cell cycle progression. Cells were harvested at 24, 48 and 72 hours after transfection, stained with 40 μg/ml PI and sorted by flow cytometetry. X axis: fluorescence intensity of cell-PI conjugates; Y axis: relative cell number. Cells treated with 50 μM GC7 for 24, 48 and 72 hours were used as control. M1: apoptotic cells; M2: cells in G1/G0 phase; M3: cells in S phase; M4: cells in G2/M phase. Results shown are representative of those obtained in 3 independent experiments.

A)

un   A1   con

Anti-eIF5A1

Anti-actin

B)

0 hr

24 hr

48 hr

72 hr

GC7   siRNA eIF5A1   siRNA control

65
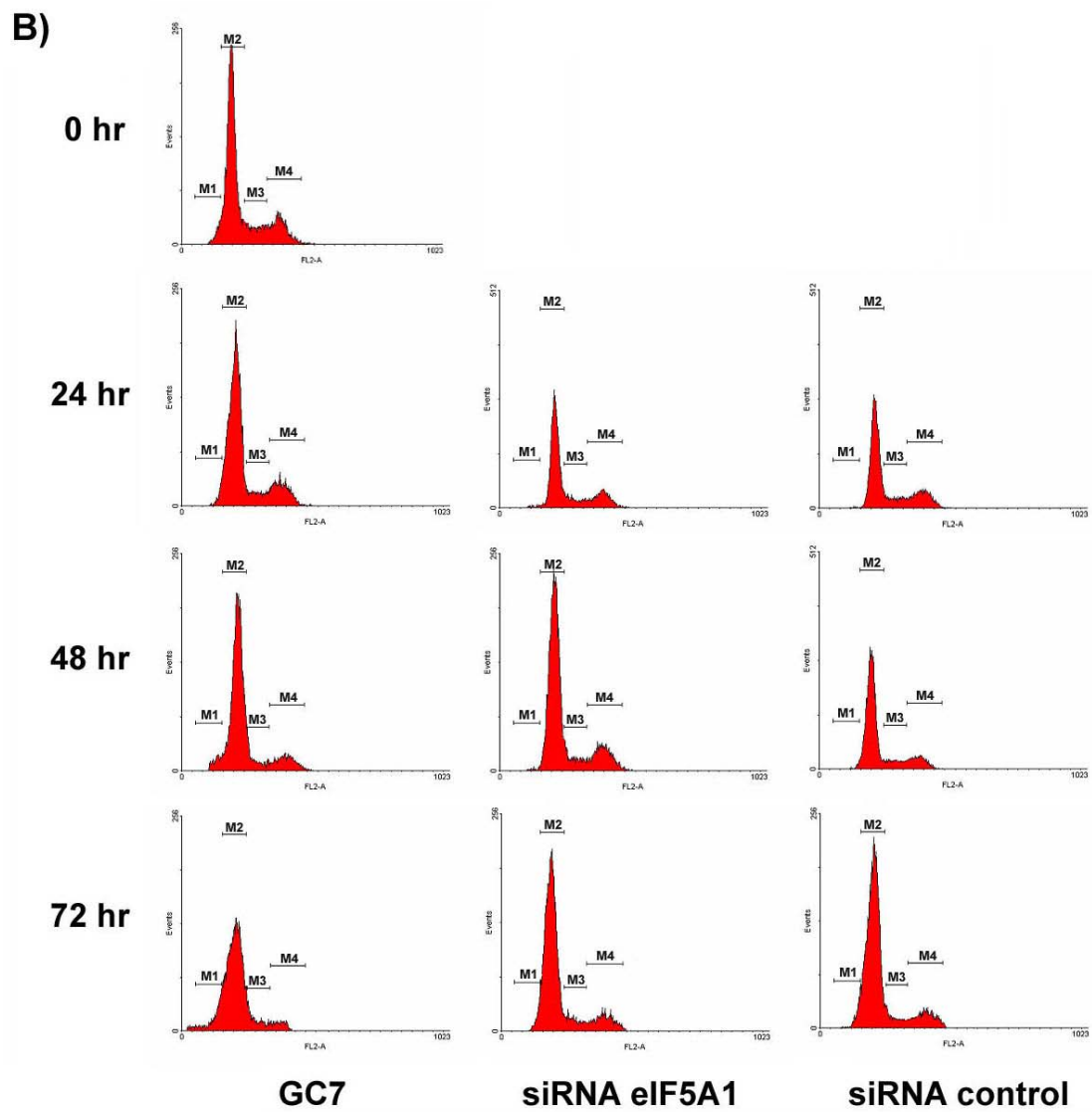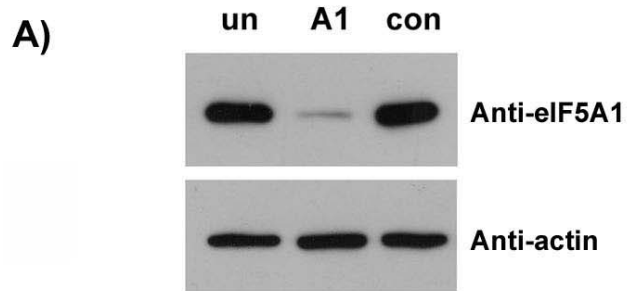
(Figure 2B). In contrast, cells that were treated with GC7, an inhibitor of DHS, for 72 hours were arrested at the G1 phase (Figure 2B). This finding is consistent with previous reports that CG7 induces cell cycle arrest at the G1 phase (Park et al., 1993b; Hanauske-Abel et al., 1994; Chen et al., 1996; Shi et al., 1996b; Tome and Gerner, 1997). However, that suppression of eIF5A1 expression does not disrupt cell cycle progression suggests that the arrest at G1 is due either to an effect of GC7 other than its ability to inhibit DHS or to the accumulation of unhypusinated eIF5A.

3.2 Protection against programmed cell death by eIF5A1 siRNA

There is accumulating evidence suggesting a regulatory role for eIF5A in apoptosis (Tome and Gerner, 1997; Tome et al., 1997; Caraglia et al., 1997, 1999 & 2003; Beninati et al., 1998; Caraglia et al., 2001; Takeuchi et al., 2002; Jin et al., 2003, Taylor et al., 2004 & 2007). In light of the fact that eIF5A appears to have no effect on cell proliferation (Figures 1 and 2), the possibility that suppression of eIF5A1 with specific siRNA could protect cells from death induced by pro-apoptotic stimuli was examined.

3.2.1 eIF5A1 siRNA reduces SNP-induced cytotoxicity

In one set of experiments, sodium nitroprusside (SNP), a well known nitric oxide (NO) donor, was used to induce apoptosis. SNP generates reactive oxygen species (ROS) during the redox cycling of nitroprusside, as well as NO, iron and cyanide (Ramakrishna Rao and Cederbaum, 1996). These products induce genotoxicity. At mM concentrations, SNP has been shown to induce apoptosis in various cell lines (Cui et al., 1994; Sumitani et al., 1997; Tsi et al., 2001; Olivier et al., 2005; Kwak et al., 2006; Gui et al., 2007; Wang et al., 2007).

HT-29 cells were transfected with eIF5A1 siRNA or control siRNA. After 48 hours, cells were treated with 3 mM SNP for another 24 hours under normal conditions of growth. The XTT assay was then used to measure cell viability. The data indicate that, whereas SNP has cytotoxic effects on cells transfected with eIF5A1 siRNA or control siRNA, there is a 220% enhancement in viability for eIF5A1 siRNA-transfected cells compared to those transfected with control siRNA (Figure 3A). This corresponded with a strong reduction in eIF5A1 protein levels in eIF5A1 siRNA-transfected cells in comparison with levels in the control cells (Figure 3B).

Figure 3: Suppressing the expression of eIF5A1 using specific siRNA reduces SNP- and Actinomycin D-induced cell death.

A) HT-29 cells were transfected with either eIF5A1 siRNA or control siRNA. Forty-eight hours after transfection, the cells were treated with 3 mM SNP or 0.5 μg/ml Actinomycin D for an additional 24 hours. Cell viability was measured using the XTT assay. The data are normalized to the value for untransfected cells which was set at 100%. The experiment was repeated three times, and the values shown here are means for the three experiments +/- standard error. Asterisks (*) denote values determined to be significantly different from the corresponding control values by independent t-test ($p < 0.001$). **B)** HT-29 cells were transfected with eIF5A1 siRNA or control siRNA. After 48 hours, the cells were treated with 3 mM SNP or 0.5 μg/ml of Actinomycin D for 24 hours. Cell lysates were collected and subjected to immunoblotting analysis using eIF5A1-specific antibody. Equal loading of the samples was evaluated by probing with anti-actin. A1: eIF5A1 siRNA; con: control siRNA; un: untranfected. The results shown are representative of those obtained in 3 independent experiments.
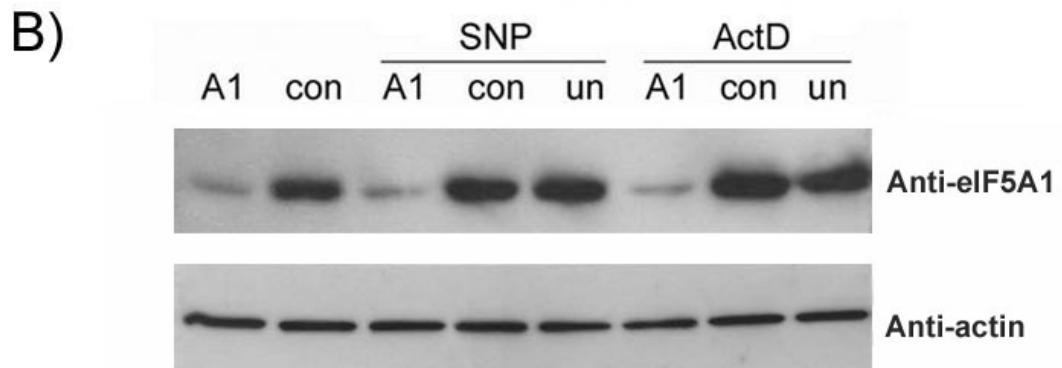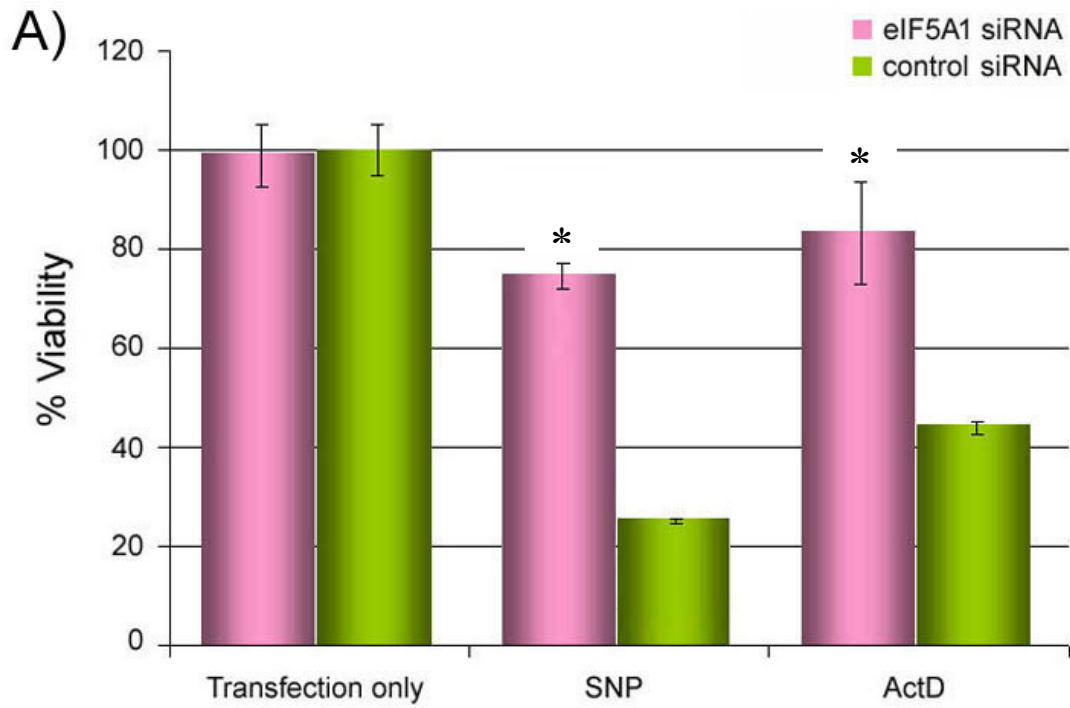
3.2.2 eIF5A1 siRNA reduces Actinomycin D-induced cytotoxicity

Actinomycin D is known to induce apoptosis by binding to DNA duplexes and repressing DNA replication and transcription (Clark and Ellem, 1966; Guy and Taylor, 1978; Cherry and Hsu, 1982; Mori et al., 1984; Sobell, 1985). Accordingly, the effects of eIF5A1 on Actinomycin D-induced cytotoxiciy were also examined. HT-29 cells were again transfected with eIF5A1 siRNA or control siRNA, and 48 hours later were treated with 0.5 μg/ml Actinomycin D for 24 hours. Cell viability was measured using the XTT assay, and although suppression of eIF5A1 expression using specific siRNA did not totally block Actinomycin D-induced cytotoxicity, it enhanced cell viability by ~100% in comparison with cells transfected with control siRNA (Figure 3A). In addition, Western blot analysis showed that the protein level of eIF5A1 in eIF5A1 siRNA-transfected cells was very low in comparison with control cells (Figure 3B).

3.2.3 eIF5A1 siRNA reduces MG-132-induced apoptosis

MG-132 is a potent and irreversible proteasome inhibitor, and has been shown to induce apoptosis in several cell lines (Fujita et al., 1996; Drexler, 1997; Kitagawa et al., 1999; Wagenknecht et al., 1999; An et al., 2000; Naujokat et al., 2000; MacLaren et al., 2001; Sassone et al., 2006). In these experiments, HT-29 cells transfected with eIF5A1 siRNA or control siRNA for either 24 or 48 hours were treated with 5 μM MG-132 for another 24 hours. Western blot analysis indicated that the protein level of eIF5A1 was strongly suppressed in the eIF5A1 siRNA-transfected cells (Figure 4B). For cells transfected with siRNA for 24 hours and then exposed to MG-132 for another 24 hours, there was no difference in levels of apoptosis between the eIF5A1 siRNA-transfected cells and those transfected with control siRNA (Figure 4A). In contrast, for cells which were transfected with siRNA constructs for 48 hours and then exposed to MG-132 for another 24 hours, there was about a 60% decrease in the percentage of apoptotic cells in samples transfected with eIF5A1 siRNA compared with those transfected with control siRNA (Figure 4A). Moreover, this protection correlated with stronger suppression of eIF5A1 protein at 72 hours than at 48 hours. These observations suggest that eIF5A is involved in MG-132-induced apoptosis.

Figure 4: Suppressing the expression of eIF5A1 using specific siRNA reduces MG-132-induced apoptosis.

A) Cells were transfected with either eIF5A1 siRNA or control siRNA. After 24 or 48 hours, the cells were treated with 5 μM MG-132 for another 24 hours. Apoptosis was measured by using the Cell Death Detection ELISA kit. Data are expressed as percentage of the value for cells transfected with control siRNA for 48 hours and treated with 5 μM MG-132 for an additional 24 hours. Each experiment was repeated four times, and the values shown are means +/- standard error for these four experiments. Asterisk (*) denotes value determined to be significantly different from the corresponding control value by independent t-test ($p < 0.001$). **B)** Immunoblotting analysis of lysates of the cells which were treated as in **A** using specific anti-eIF5A1 and anti-actin. A1: eIF5A1 siRNA; con: control siRNA; -: without; +: with. The results are representatives of those obtained in 3 independent experiments.

A)

B)
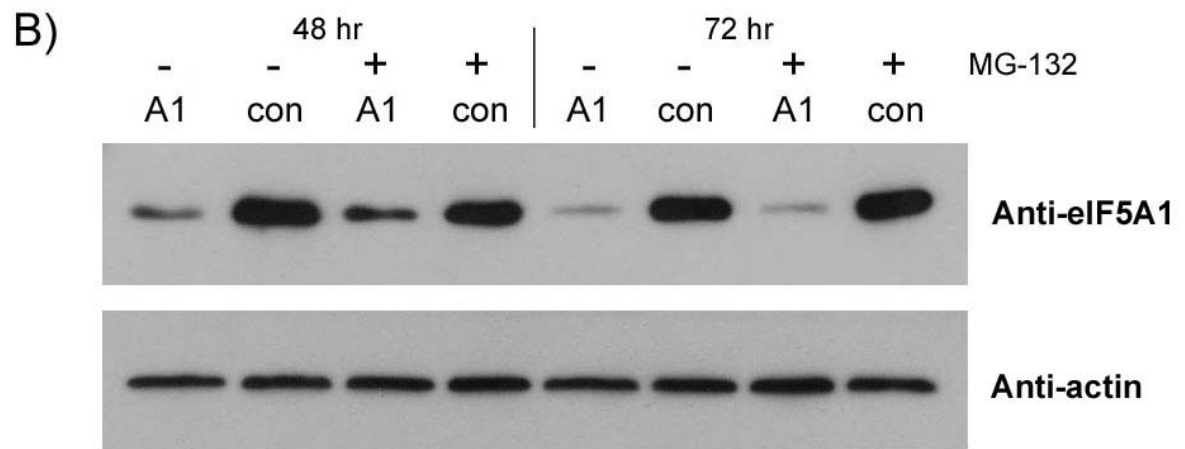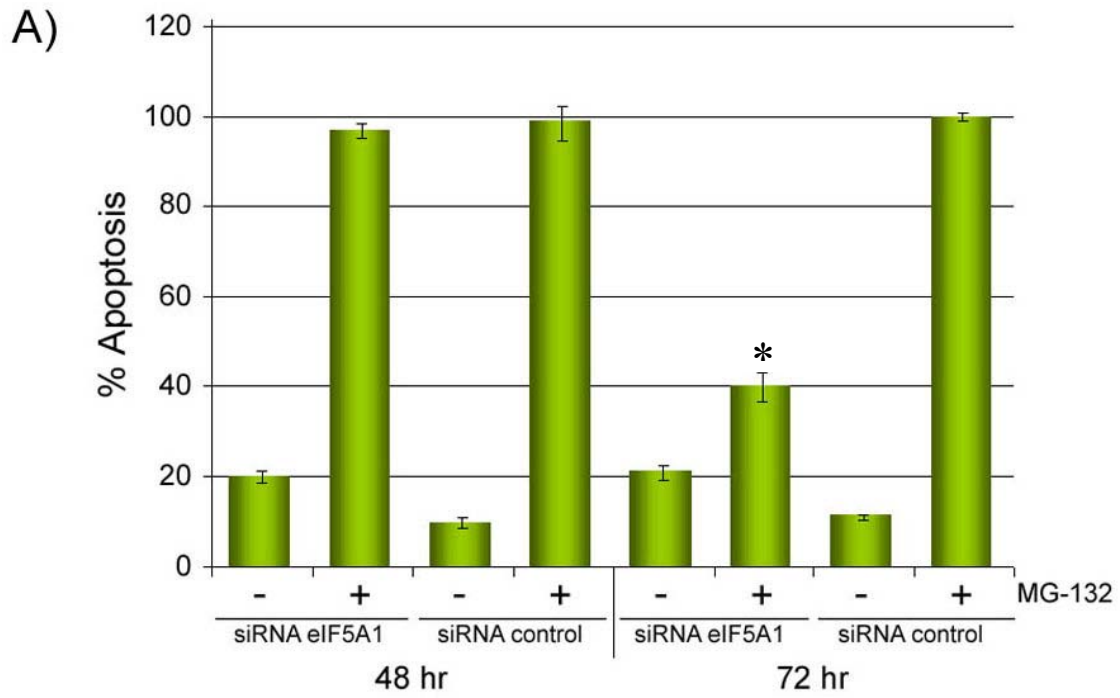
3.2.4 eIF5A1 siRNA reduces serum starvation-induced stress

The effects of serum starvation on cell cycle arrest and apoptosis have been well described (Cherney et al., 1994; Dahm-Daphi et al., 2000; Zolzer, 2000; Oya et al., 2003; Li et al., 2006). Since serum-starvation is one of the major stimulants of apoptosis, this treatment was also used to assess the efficacy of eIF5A1 siRNA as an agent of protection against cell death.

HT-29 cells were transfected with eIF5A1 siRNA or control siRNA in serum-reduced Opti-MEM without FBS. At 6 hours after transfection, the medium was removed and replaced with RPMI with or without FBS. After another 66 hours, the cells were observed under a microscope. Untransfected cells as well as those transfected with eIF5A1 siRNA or control siRNA were cultured in medium containing 10% FBS and reached 100% confluence within 72 hours after transfection reflecting normal growth (Figure 5). In contrast, both untransfected and control siRNA-transfected cells cultured in medium without FBS for 72 hours did not reach confluence and were rounded and enlarged (Figure 5). However, eIF5A1 siRNA-transfected cells cultured in medium without FBS for 72 hours grew normally and reached confluence (Figure 5). Indeed, there was no morphological difference between eIF5A1 siRNA-transfected cells cultured under conditions of serum-depletion and those cultured in the presence of a normal serum supplement (Figure 5).

3.3 Induction of programmed cell death by up-regulation of eIF5A1 expression

The finding in the present study that suppressing the expression of eIF5A1 with specific siRNA provides protection against cell death induced by a variety of pro-apoptotic stimuli supports the contention that eIF5A plays a role in apoptosis. In order to investigate this possibility further, the possibility that up-regulation of eIF5A might induce apoptosis was examined.

3.3.1 Over-expression of eIF5A using adenovirus

Adenovirus constructs of wild-type eIF5A1 (Ad-eIF5A1), mutated eIF5A1 with $lysine_{50}$ changed to alanine [Ad-eIF5A1(K50A)] and wild-type eIF5A2 (Ad-eIF5A2) were used as gene delivery vectors in these experiments. An adenovirus LacZ construct was used as a negative control.

The optimization of adenovirus infection for HT-29 cells is illustrated in Figure 6. The cells were infected with different amounts of adenovirus lacZ construct and stained for β-galactosidase

Figure 5: Suppressing the expression of eIF5A1 using specific siRNA reduces serum starvation-induced cell death.

HT-29 cells transfected with eIF5A1 siRNA or control siRNA were cultured in growth medium with or without 10% FBS for 72 hours. Microscopic images of the cells at the end of 72 hours culturing periods are illustrated. The micrographs are representative of those obtained in 3 independent experiments. Magnification: 100 X.

	with 10% FBS	without FBS
Untransfected		
siRNA eIF5A1		
siRNA control		
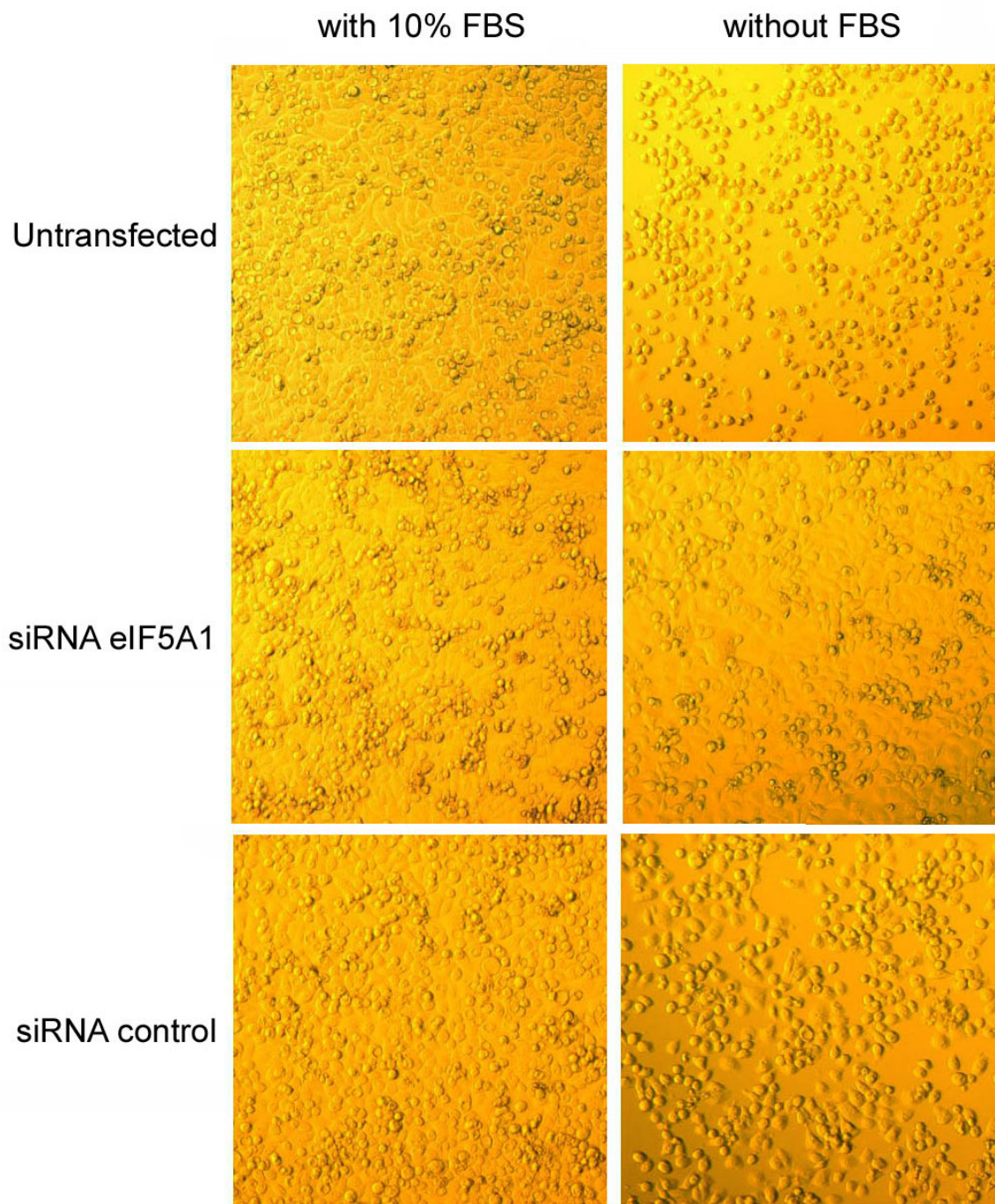
activity at 48 hours after infection (Figure 6C). The infection efficiency was more than 90% when the cells were infected with 4000 pfu/cell of adenovirus construct, and there were no apparent adverse effects on cell morphology at this level of infection (Figure 6C). In HT-29 cells infected with 4000 pfu/cell Ad-eIF5A1, Ad-eIF5A1(K50A)) or Ad-eIF5A2, the protein levels of eIF5A1 or eIF5A2 were highly up-regulated at 48 and 72 hours after0 infection (Figure 6A). Two eIF5A antibodies were used in the Western blot analysis. While they were both able to recognize eIF5A1 as well as eIF5A2, anti-eIF5A1 (BD Biosciences) appeared to interact with eIF5A1 better, and anti-eIF5A2 (Novus Biologicals Inc.) appeared to preferentially bind to eIF5A2 (Figure 6A).

Another cell line, Hela S3, was also infected with different adenovirus eIF5A constructs. However, lower amounts of adenovirus construct were required for this cell line. Indeed, the transfection efficiency in Hela S3 cells reached more than 70% when the cells were infected with 500 pfu/cell of adenovirus constructs (data not shown). The protein levels of eIF5A1 and eIF5A2 in HT-29 cells and Hela S3 cells infected with 4000 pfu/cell and 500 pfu/cell, respectively, of adenovirus constructs are illustrated in Figure 6B, and it is apparent that the adenovirus infection induces strong up-regulation of eIF5A in both cell lines.

3.3.2 Over-expression of eIF5A induces apoptosis as well as cytotoxicity in HT-29 cells

HT-29 cells were infected with different types of adenovirus construct at 4000 pfu/cell, and apoptosis was measured 48 hours after infection using the TUNEL assay. Cells infected with Ad-lacZ served as control. Ad-eIF5A1, Ad-eIF5A1(K50A) and Ad-eIF5A2 all proved to be strong inducers of apoptosis. Within 48 hours of infection, 23%, 22% and 28% of the cells were undergoing apoptosis in samples infected with Ad-eIF5A1, Ad-eIF5A1(K50A) and Ad-eIF5A2, respectively (Figures 7 and 8). Virtually no apoptosis was observed in cells infected with Ad-lacZ (Figures 7 and 8).

Infection of HT-29 cells with eIF5A adenovirus constructs also induced strong cytotoxic effects. In these experiments, HT-29 cells were infected with different adenovirus constructs at 4000 pfu/cell. After 4 days, cell viability was measured using the XTT assay. The viability of cells infected with Ad-eIF5A1, Ad-eIF5A1(K50A) and Ad-eIF5A2 was reduced by 77%, 77% and 80%, respectively, in comparison with the viability of cells infected with Ad-lacZ (Figure 9A).

Figure 6: Infection with Ad-eIF5A1, Ad-eIF5A1(K50A) or Ad-eIF5A2 induces up-regulation of eIF5A expression.

A) HT-29 cells were infected with 4000 pfu/cell of adenovirus constructs. At indicated times after infection, cell lysates were collected and subjected to immunoblot analysis using specific antibodies against eIF5A, eIF5A2 and actin. A1: Ad-eIF5A1; A1M: Ad-eIF5A1(K50A); A2: Ad-eIF5A2; Z: Ad-lacZ; 48: 48 hours after infection; 72: 72 hours after infection. The results shown are representative of those obtained in 3 independent experiments. **B)** Hela S3 cells and HT-29 cells were infected with 500 pfu/cell and 4000 pfu/cell adenovirus constructs, respectively. At 48 hours after infection, cell lysates were collected and subjected to immunoblot analysis using specific antibodies against eIF5A and actin. A1: Ad-eIF5A1; A1M: Ad-eIF5A1(K50A); A2: Ad-eIF5A2; Z: Ad-lacZ. The results shown are representative of those obtained in 3 independent experiments. **C)** HT-29 cells were infected with Ad-lacZ at 500, 1000, 2000 or 4000 pfu/cell. At 48 hours after infection, the cells were stained for β-galactosidase activity. The results shown are representative of those obtained in 3 independent experiments. Magnification: 100 X.

A)

	A1	M	A2	Z
	48 72	48 72	48 72	48 72	hr

eIF5A2 —
eIF5A1 — **Anti-eIF5A1**

eIF5A2 —
eIF5A1 — **Anti-eIF5A2**

Anti-actin

B)

Hela | HT-29

A1 M A2 Z | A1 M A2 Z

eIF5A2 —
eIF5A1 — **Anti-eIF5A1**

Anti-actin

C)

500 pfu/cell   1000 pfu/cell

2000 pfu/cell   4000 pfu/cell

77
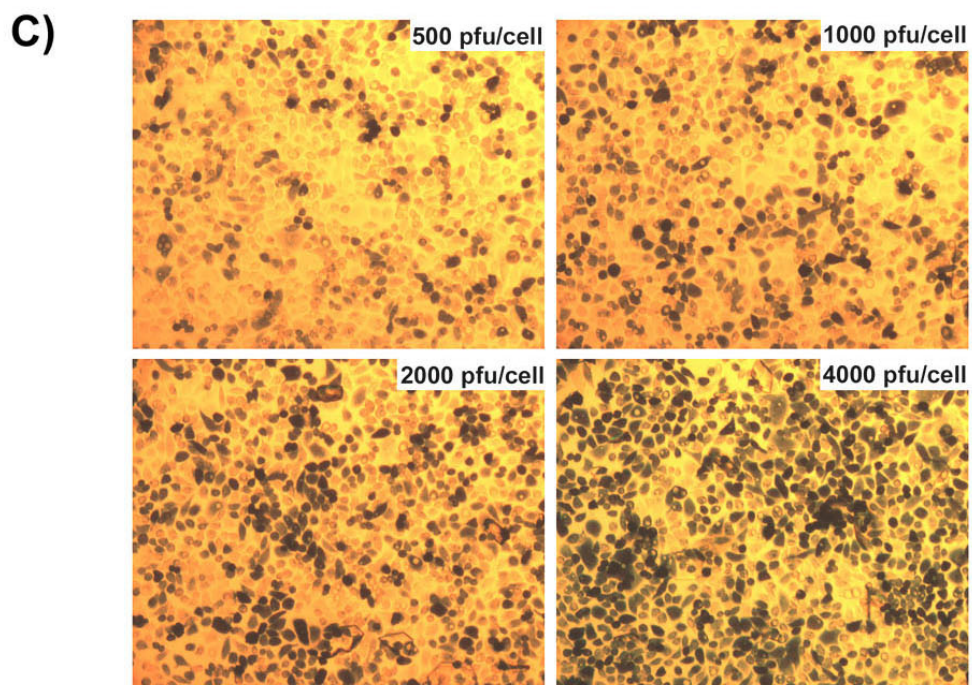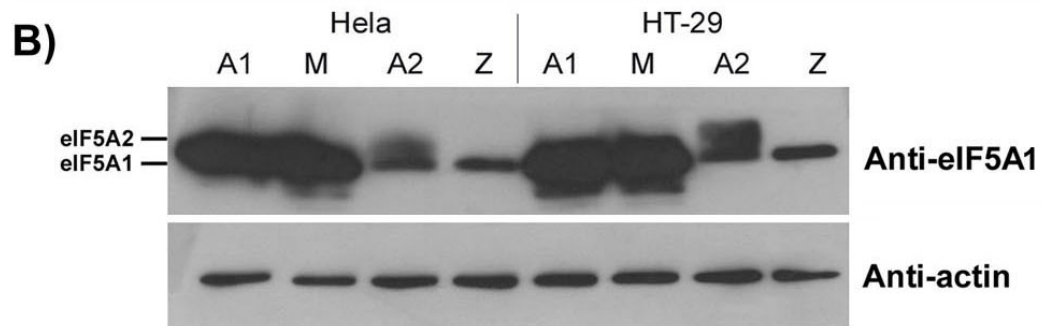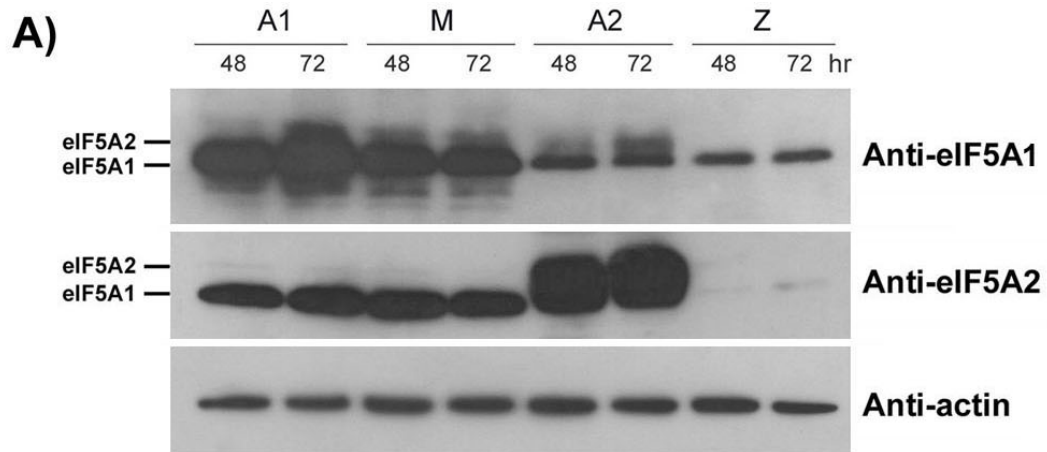
Figure 7: TUNEL labeling of HT-29 cells infected with adenovirus-eIF5A.

HT-29 cells were infected with different types of adenovirus construct. After 48 hours, the cells were fixed and stained with TUNEL and Hoechst 33258. For each sample, the same field was observed by fluorescence microscopy using two different filters. A fluorescein filter was used to visualize TUNEL stained apoptotic cells (green-colored, left column); a UV filter was used to visualize nuclei (blue-colored, right column). The results shown are representatives of those obtained in 3 independent experiments. Magnification: 100 X.

TUNEL   Hoechst

Ad-eIF5A1

Ad-eIF5A1(K50A)

Ad-eIF5A2

Ad-lacZ
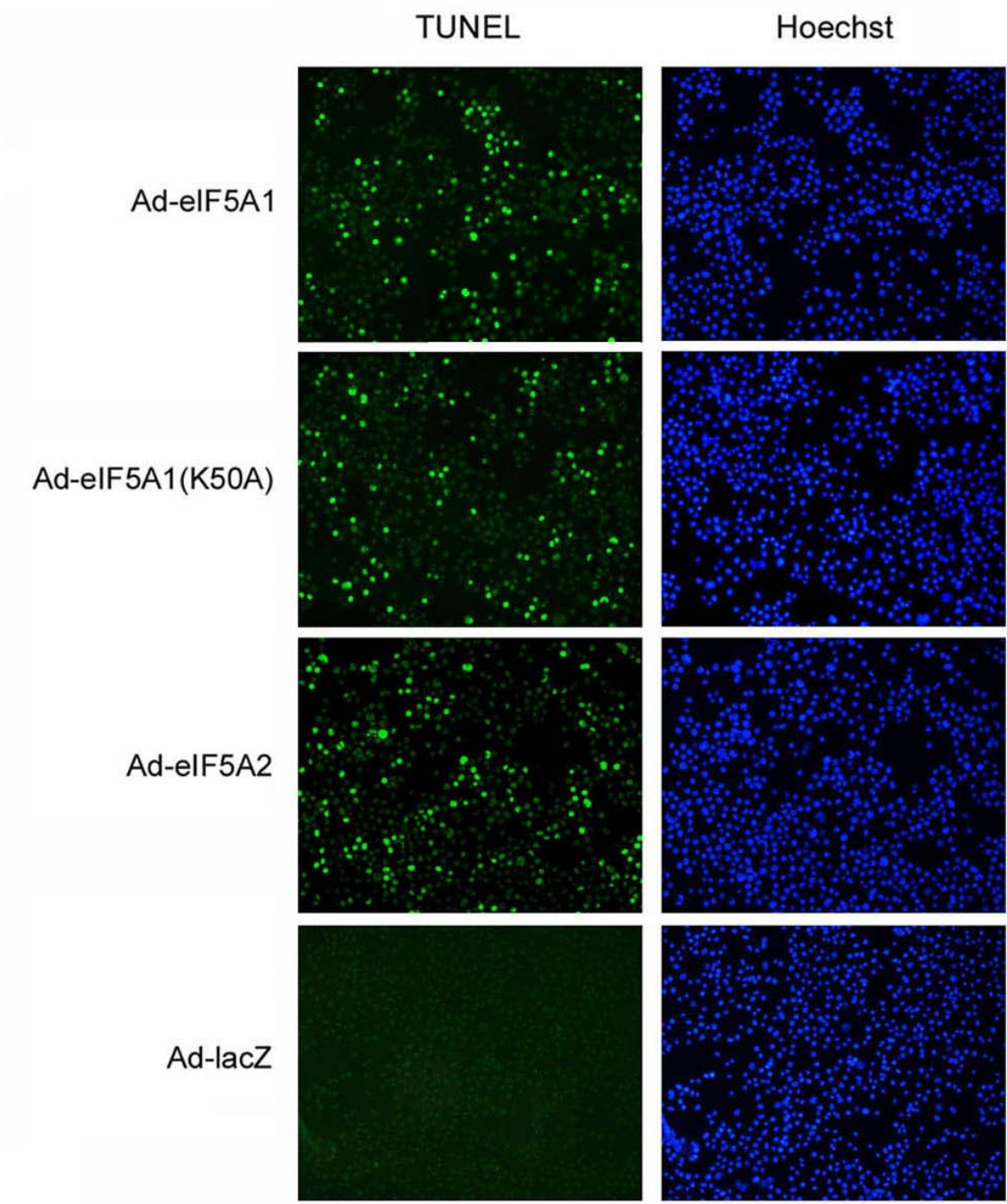
Figure 8: Quantitation of apoptosis induced in HT-29 cells by infection with adenovirus-eIF5A.
HT-29 cells were infected with different types of adenovirus construct. After 48 hours, the cells were fixed and stained with TUNEL and Hoechst 33258. For each sample, the same field was observed by fluorescence microscopy using two different filters. The percentage of cells undergoing apoptosis was calculated by dividing the number of apoptotic cells by the number of Hoechst-stained cells. Four different fields were randomly chosen for each experiment, and the whole experiment was repeated three times. Values are means +/- standard error. Asterisks (*) denote values determined to be significantly different from the corresponding control value (Ad-lacZ) by one-way ANOVA followed by post hoc Scheffe's test ($p < 0.001$).
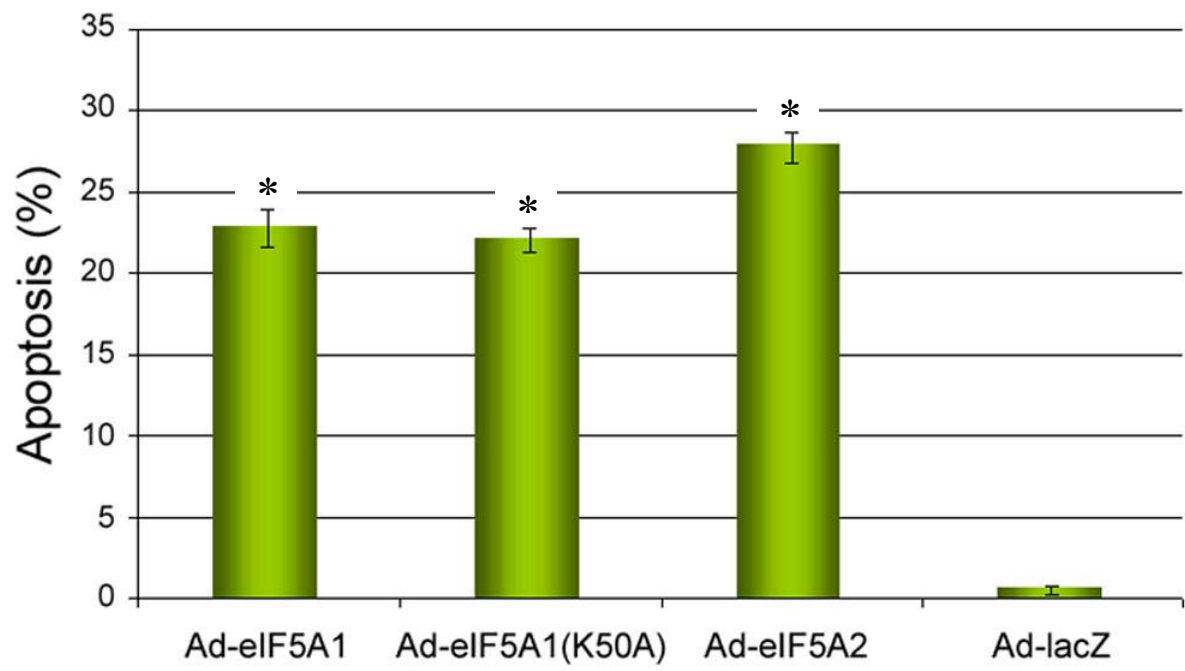
Figure 9: Effect of over-expression of eIF5A on cell growth and viability.

A) HT-29 cells were infected with different types of adenovirus construct. After 4 days, cell viability was measured using the XTT assay. The values for Ad-eIF5A infected cells are normalized to that for Ad-lacZ infected cells, which was set at 100%. Values are means +/- standard error for 3 independent experiments. Asterisks (*) denote values determined to be significantly different from the corresponding control value (Ad-lacZ) by one-way ANOVA followed by post hoc Scheffe's test ($p < 0.001$). **B)** Hela S3 cells were infected with different types of adenovirus constructs. Cells were harvested 24, 48 and 72 hours after infection, and stained with trypan blue. The percentage of viable cells was calculated by dividing the number of unstained (viable) cells by total number of cells (stained plus unstained). The values are means +/- standard error for 3 independent experiments.

A)

B)
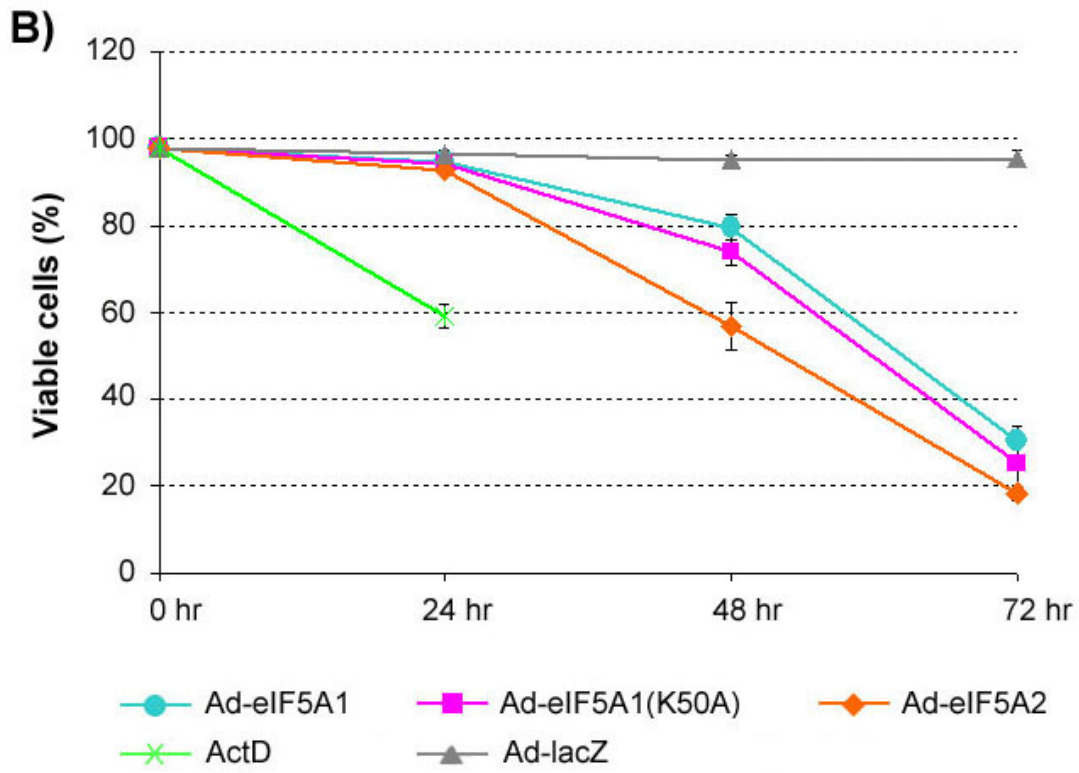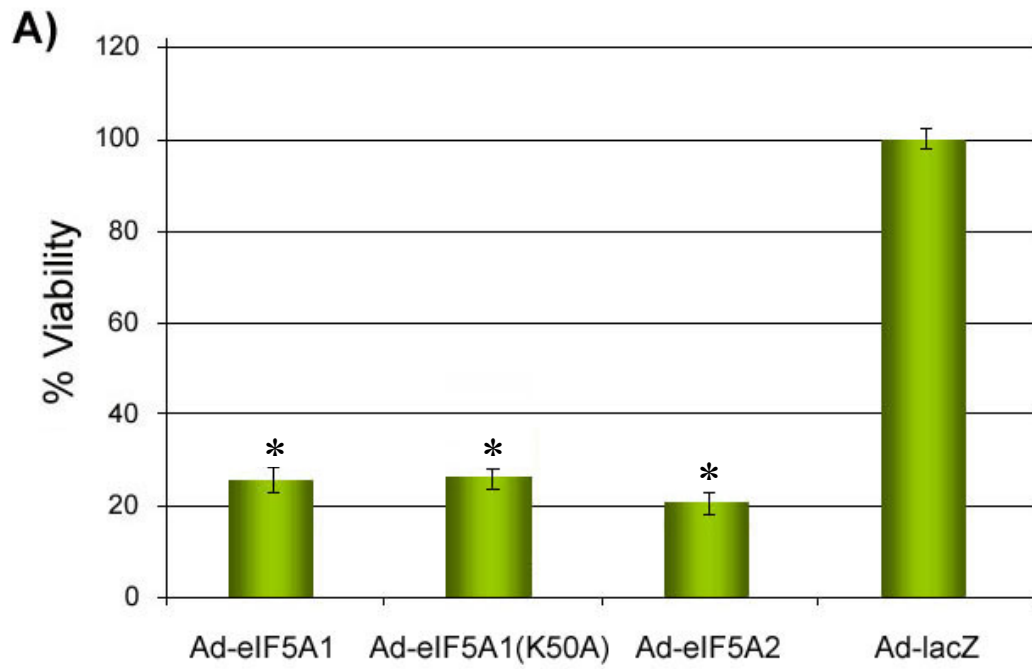
3.3.3 Over-expression of eIF5A induces apoptosis as well as cytotoxicity in Hela S3 cells

Ad-eIF5A1, Ad-eIF5A1(K50A) and Ad-eIF5A2 also had strong pro-apoptotic effects in Hela S3 cells. Cells were infected with different types of adenovirus construct at 500 pfu/cell. At intervals of 24, 48 and 72 hours after infection, they were harvested, stained with Annexin V-FITC/PI and analyzed by flow cytometry. This analysis enables quantitation of viable cells, cells at an early stage of apoptosis, cells at a late stage of apoptosis as well as dead cells. Representative data presented as dot plots are illustrated in Figure 10. The fluorescence intensity of cell-Annexin V-FITC conjugates is plotted on the X axis and fluorescence intensity of cell-PI conjugates on the Y axis. Quadrants were set using the data for unstained cells, cells stained with Annexin V-FITC alone, cells stained with PI alone and Actinomycin D-treated cells.

Analysis of the dotplot data indicated that there was no induction of apoptosis in cells treated with Ad-eIF5A within 24 hours of infection. (Figures 10 and 11). The treatment with Actinomycin D served as a positive control. However by 48 hours after infection there was clear evidence of apoptosis for cells infected with Ad-eIF5A1, Ad-eIF5A1(K50A) or Ad-eIF5A2. Specifically, the proportion of cells at early and late stages of apoptosis was ~20% for Ad-eIF5A1 infected cells and ~40% for Ad-eIF5A1(K50A)- and Ad-eIF5A2-infected cells (Figure 11). At 72 hours after infection, the percentage of viable cells had decreased to less than 30% for all of the Adenovirus-eIF5A-infected cells, the percentage of cells at the early stage of apoptosis was ~12% and the percentage of cells at the late stage of apoptosis was about 50 to 60% (Figure 11). Interestingly, although Ad-eIF5A1, Ad-eIF5A1(K50A) and Ad-eIF5A2 all proved to be potent inducers of apoptosis, the pro-apoptotic activity of eIF5A2 appeared to be stronger than those for eIF5A1 and eIF5A1(K50A), and eIF5A1(K50A) appeared to be a slightly stronger inducer of apoptosis than eIF5A1 (Figure 11). It should be noted, however, that although the number of plaque forming units (pfu) of adenovirus used for infection was the same for each of the adenovirus constructs, their infection forming units (ifu) may be different, and this could explain the observed differences in pro-apoptotic activities of Ad-eIF5A1, Ad-eIF5A1(K50A) and Ad-eIF5A2. In addition, Ad-lacZ was used as the control vector, and it is clear that it did not induce apoptosis in Hela S3 cells (Figures 10 and 11).

The viability of Hela S3 cells infected with different adenovirus construct was measured by trypan blue-staining. Cells were infected with different types of adenovirus construct at 500 pfu/cell. At 24, 48, and 72 hours after infection, cells were collected and stained with trypan blue. The majority of cells infected with Ad-eIF5A1, Ad-eIF5A1(K50A) or Ad-eIF5A2 were still viable at 24

Figure 10: Annexin V/PI staining of adenovirus-infected cells.

Hela S3 cells were infected with adenovirus constructs as indicated. The cells were harvested at 24, 48 and 72 hours after infection, stained with Annexin V-FITC and PI, and analyzed by flow cytometry. Cells treated with 0.5 μg/ml Actinomycin D served as a positive control. X axis: fluorescence intensity of cell-Annexin V-FITC conjugates; Y axis: fluorescence intensity of cell-PI conjugates. Quadrants designate the following: (1) lower left: viable cells; (2) lower right: early-stage apoptotic cells; (3) upper right: late-stage apoptotic cells; (4) upper left: dead cells. Results shown are representative of those obtained in 4 independent experiments.

Untreated

24 hr ActD

24 hr

48 hr

PI

72 hr

Ad-eIF5A1   Ad-eIF5A1(K50A)   Ad-eIF5A2   Ad-lacZ

Annexin V

86
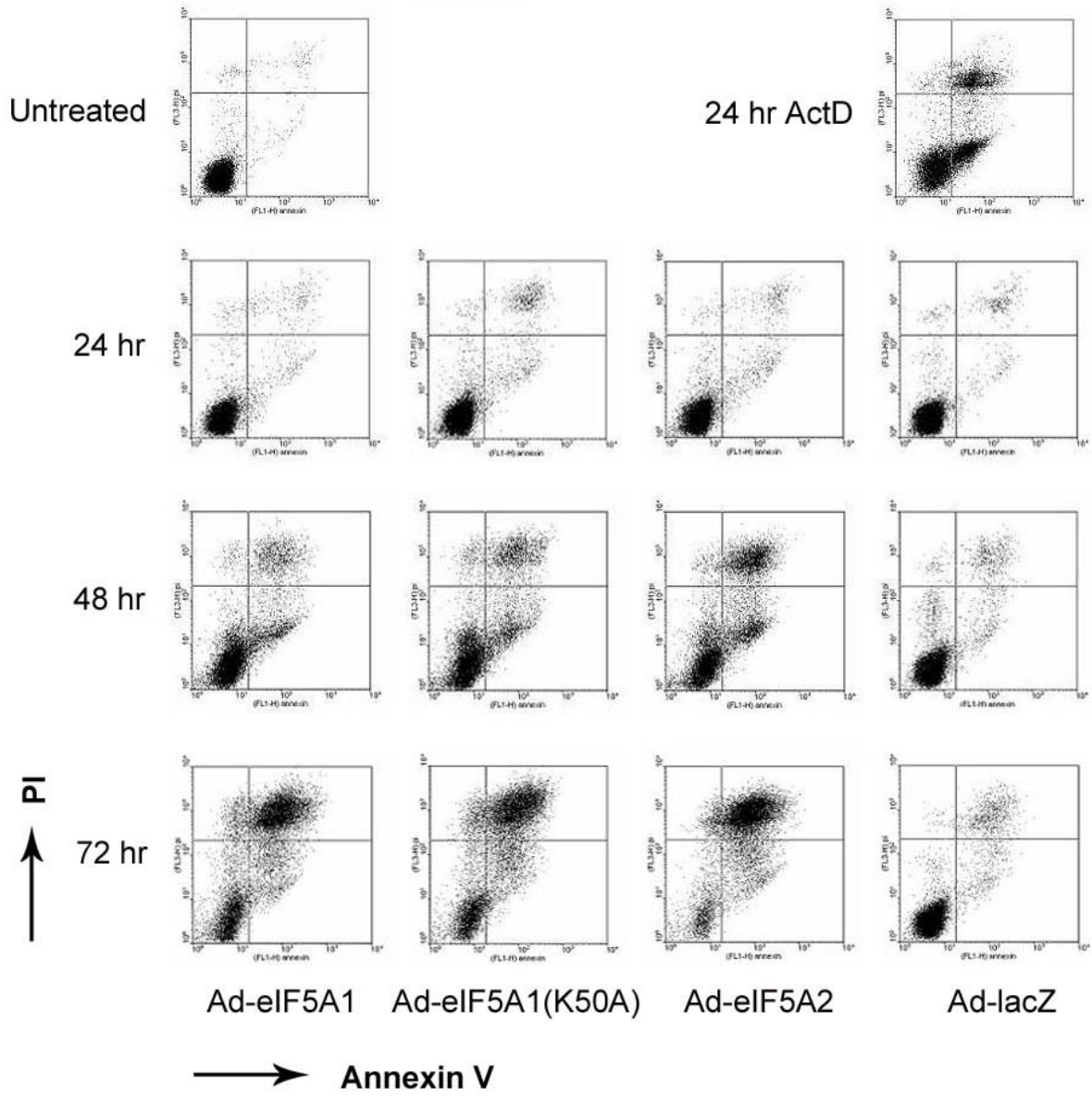
Figure 11: Percentages of cells sorted into quadrants by flow cytometry.

Hela S3 cells were infected with different types of adenovirus construct. Cells were harvested at 24, 48 and 72 hours after infection, stained with Annexin V-FITC and PI, and analyzed by flow cytometry. Cells treated with 0.5 μg/ml Actinomycin D served as a positive control. Quantitation of flow cytometric cell counts in 4 different quadrants is illustrated. UL: upper left, dead cells. UR: upper right, late-stage apoptotic cells. LL: lower left, viable cells. LR: lower right, early stage apoptotic cells. Each experiment was repeated four times, and the values shown are means +/- standard error.
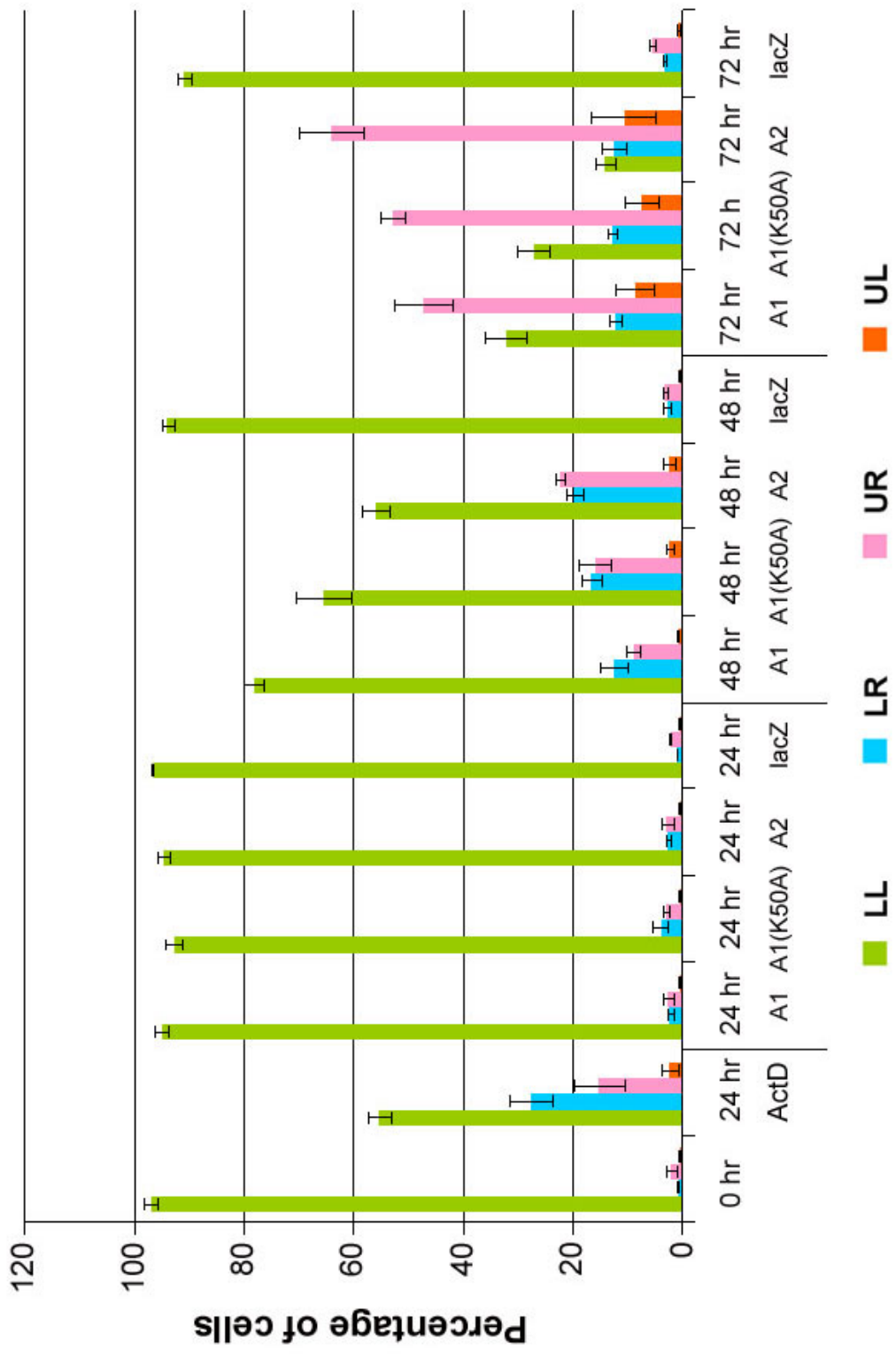
hours after infection (Figure 9B). However, the viability decreased dramatically between 24 hours and 72 hours after infection in a time-dependent manner. At 72 hours, up to 80% of the cells were non-viable, whereas virtually 100% of the control cells infected with Ad-lacZ were viable (Figure 9B). Cells treated with Actinomycin D served as a positive control (Figure 9B). .

3.4 Pro-apoptotic molecular targets of eIF5A

It is clear that eIF5A1, eIF5A1(K50A) and eIF5A2 are all capable of inducing apoptosis in cancer cell lines. In light of this, additional studies were undertaken to identify molecular targets in the various pathways leading to apoptosis that are regulated by eIF5A.

3.4.1 Over-expression of eIF5A induces activation of caspases 3, 8 and 9

Activation of caspases is a well established index of apoptosis (Reviewed by Cohen, 1997; Nicholson, 1999). In order to determine whether Ad-eIF5A1-, Ad-eIF5A1(K50A)- or Ad-eIF5A2-induced apoptosis is caspase-dependent, the effects of infection with these adenovirus constructs on the activities of executioner caspase 3 as well as initiator caspases 8 and 9 were examined. Hela S3 cells were infected with different types of adenovirus construct at a concentration of 500 pfu/cell. At 48 and 72 hours after infection, cells were collected and stained with fluorescence-conjugated peptides specifically targeting caspase 3, caspase 8 or caspase 9.

At 48 hours after infection, all three caspases were activated in Ad-eIF5A1-, Ad-eIF5A1(K50A)- and Ad-eIF5A2- infected cells (Figures 12, 13 and 14). In Ad-eIF5A1 and Ad-eIF5A1(K50A)- infected cells, up to 25% of the cells exhibited activated caspase 3, up to 30% exhibited activated caspase 8 and up to 25% exhibited activated caspase 9 (Figure 15). For Ad-eIF5A2- infected cells, the incidence of activation was higher (~47%) for each of caspase 3, 8 and 9 (Figure 15). By 72 hours after infection, 65 to 70% of cells infected with Ad-eIF5A1 or Ad-eIF5A1(K50A) exhibited activated caspases 3, 8 and 9, and ~90% of cells infected with Ad-eIF5A2 exhibited activated caspases 3, 8 and 9 (Figure 15). Cells treated with Actinomycin D and cells infected with Ad-lacZ served as positive and negative controls, respectively (Figures 12, 13, 14 and 15).

Figure 12: Over-expression of eIF5A activates caspase 3.

Hela S3 cells were infected with different types of adenovirus construct for 48 or 72 hours, or treated with 0.5 μg/ml Actinomycin D for 24 hours. Cells were subsequently collected, incubated with FITC-DEVD-FMK, which is a fluorescent dye-conjugated inhibitor against caspase 3, and then analyzed by flow cytometry. The data are presented as histograms of FITC-DEVD-FMK-labeled cells. X axis: fluorescence intensity of cell-FITC-DEVD-FMK conjugates; Y axis: relative cell number. Cells with inactivated caspase 3 are sorted within the second log decade of the X axis, and cells with activated caspase 3 are sorted within the third and forth log decades. Results shown are representative of those obtained in 4 independent experiments.

Untreated

24 hr ActD

Ad-eIF5A1

Ad-eIF5A1(K50A)

Ad-eIF5A2

Ad-lacZ
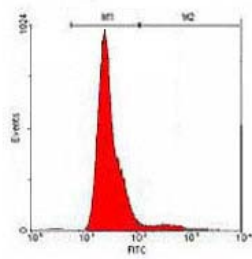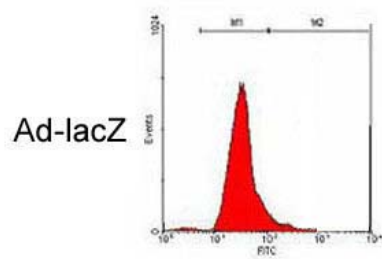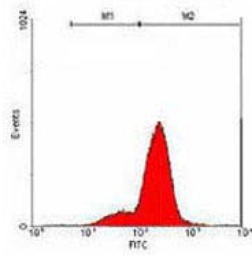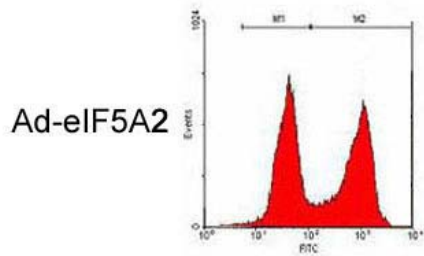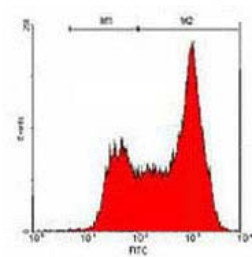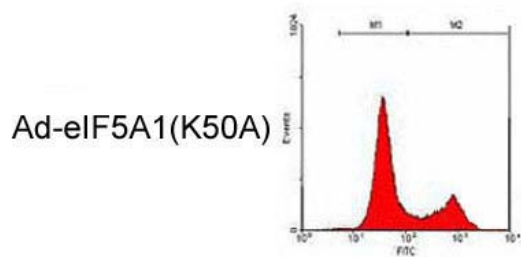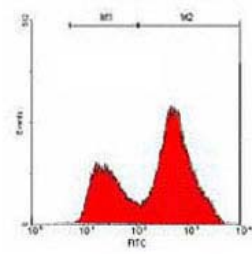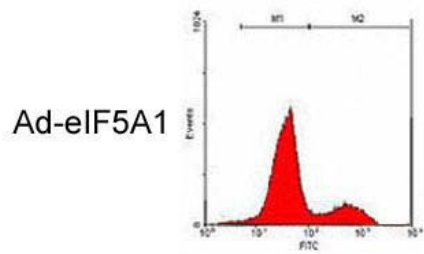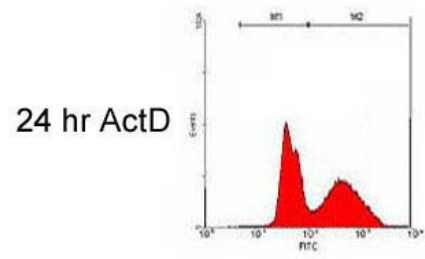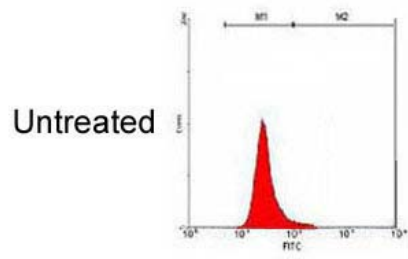
48 hr

72 hr

Figure 13: Over-expression of eIF5A activates caspase 8.

Hela S3 cells were infected with different types of adenovirus construct for 48 or 72 hours, or treated with 0.5 μg/ml Actinomycin D for 24 hours. Cells were subsequently collected, incubated with FITC-IETD-FMK, which is a fluorescent dye-conjugated inhibitor against caspase 8, and then analyzed by flow cytometry. The data are presented as histograms of FITC-IETD-FMK-labeled cells. X axis: fluorescence intensity of cell-FITC-IETD-FMK conjugates; Y axis: relative cell number. Cells with inactivated caspase 8 cells are sorted within the second log decade of the X axis, and cells with activated caspase 8 are sorted within the third and forth log decades. Results shown are representative of those obtained in 4 independent experiments.

Untreated

24 hr ActD

Ad-eIF5A1

Ad-eIF5A1(K50A)

Ad-eIF5A2

Ad-lacZ
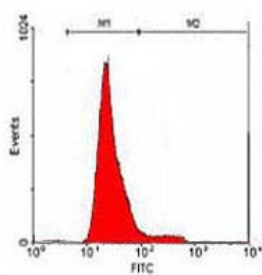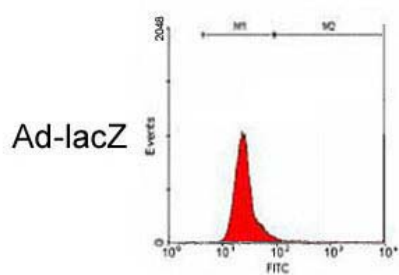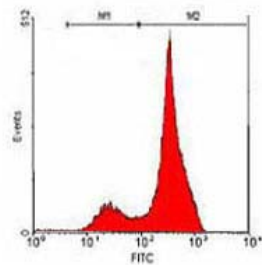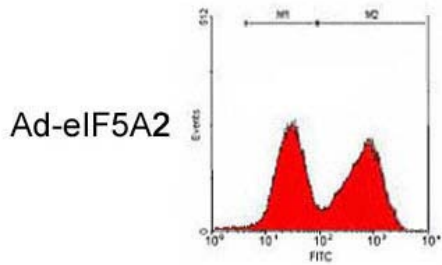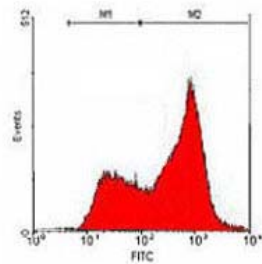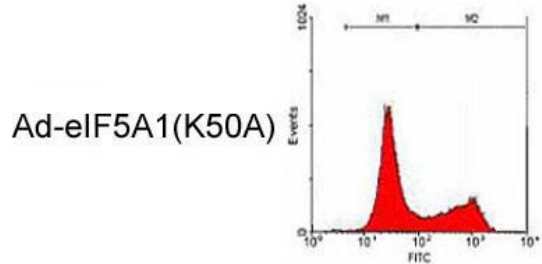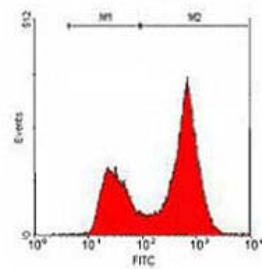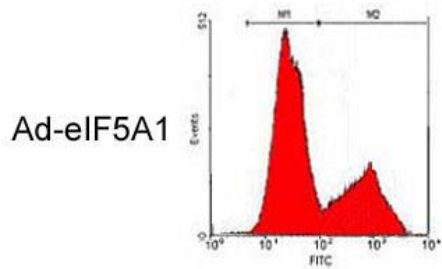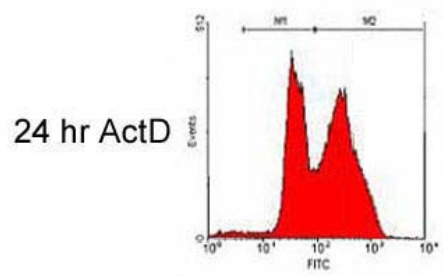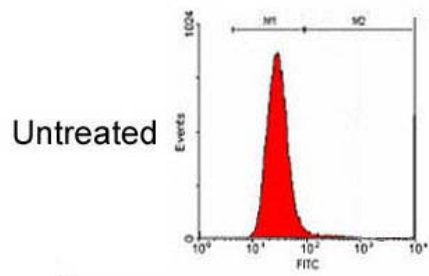
48 hr

72 hr

Figure 14: Over-expression of eIF5A activates caspase 9.

Hela S3 cells were infected with different types of adenovirus construct for 48 or 72 hours, or treated with 0.5 μg/ml Actinomycin D for 24 hours. Cells were subsequently collected, incubated with FITC-LEHD-FMK, which is a fluorescent dye-conjugated inhibitor against caspase 9, and then analyzed by flow cytometry. The data are presented as histograms of FITC-LEHD-FMK-labeled cells. X axis: fluorescence intensity of cell-FITC-LEHD-FMK conjugates; Y axis: relative cell number. Cells with inactivated caspase 9 cells are sorted within the second log decade of the X axis, and cells with activated caspase 9 are sorted within the third and forth log decades. Results shown are representative of those obtained in 4 independent experiments.

Untreated

24 hr ActD

Ad-eIF5A1

Ad-eIF5A1(K50A)

Ad-eIF5A2

Ad-lacZ

48 hr

72 hr

95
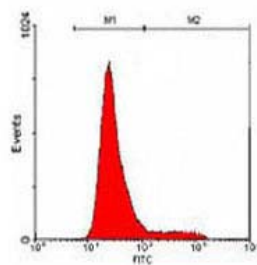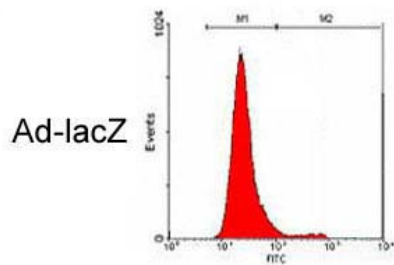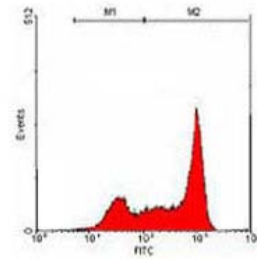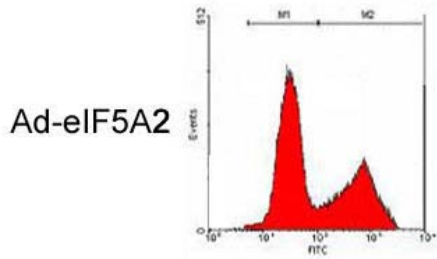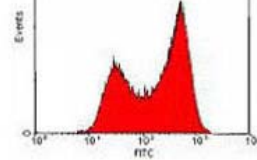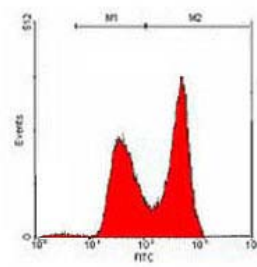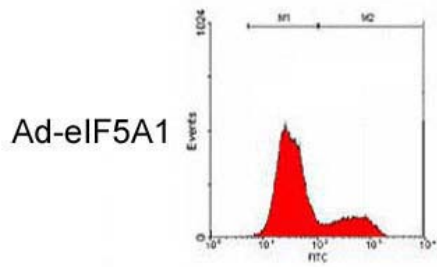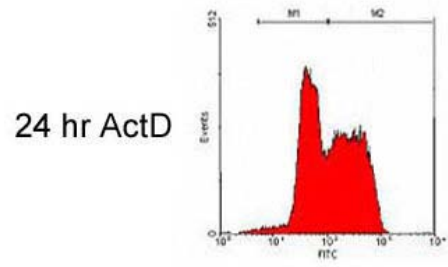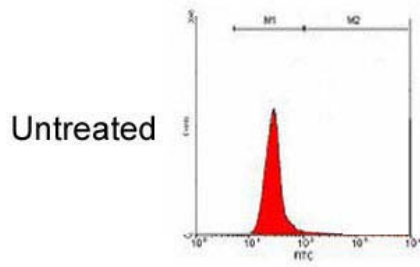
Figure 15: Quantitation of flow cytometric data portraying activation of caspase 3, caspase 8 and caspase 9.

Hela S3 cells were infected with adenovirus constructs as indicated for 48 or 72 hours, or treated with Actinomycin D for 24 hours. Cells were collected, labeled with FITC-DEVD-FMK, FITC-IETD-FMK or FITC-LEHD-FMK, and analyzed by flow cytometry. The data were analyzed using WinMDI 2.8. Percentages of cells with activated caspase 3, 8 or 9 were calculated. Each experiment was repeated 4 times, and the results shown are means +/- standard error.
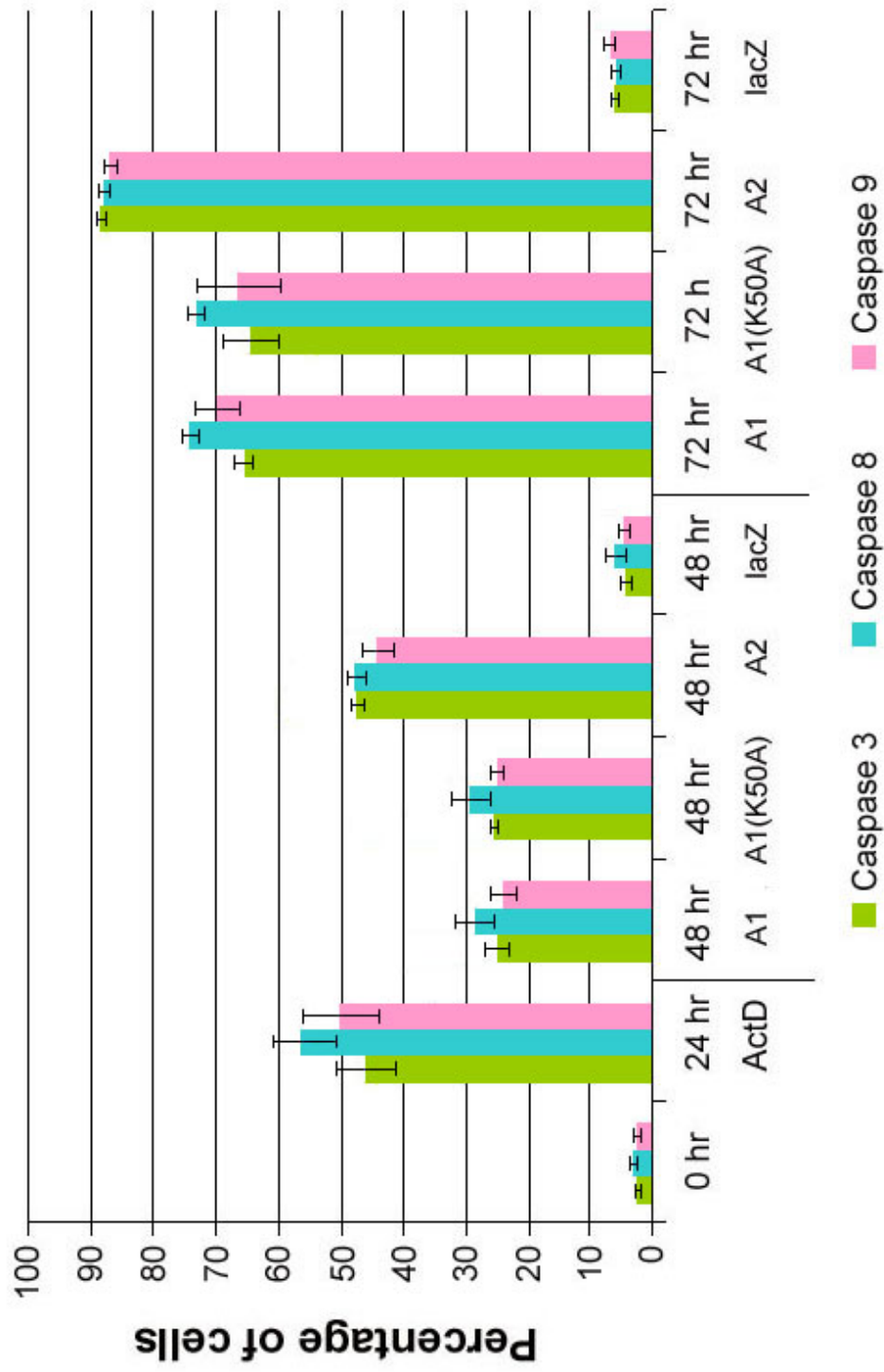
3.4.2 Over-expression of eIF5A induces loss of mitochondrial transmembrane potential ($\Delta\Psi_m$)

It is clear from the data in Figures 14 and 15 that caspase 9, which is the initiator caspase for the mitochondria-based apoptotic pathway, is activated in Ad-eIF5A1-, Ad-eIF5A1(K50A)- and Ad-eIF5A2-infected cells. In an effort to further understand the involvement of eIF5A in the changes in mitochondria that accompany the onset of apoptosis, the possibility that up-regulation of eIF5A results in loss of mitochondrial transmembrane potential ($\Delta\Psi_m$), a well established apoptotic trait (Vayssiere et al., 1994; Petit et al., 1994; Zamzami et al., 1995; Marchetti et al., 1996), was examined. $\Delta\Psi_m$ was measured using the membrane potential-sensitive dye, DiOC6(3). Hela S3 cells were infected with different types of adenovirus construct at a concentration of 500 pfu/cell. At 24, 48 and 72 hours after infection, the cells were stained with DiOC6(3) and analyzed by flow cytometry.

Over the entire 72 hours experimental period, the dye was retained within mitochondria in ~98% of untreated cells and cells infected with Ad-lacZ reflecting structural integrity of the mitochondrial membrane system and retention of $\Delta\Psi_m$ (Figure 16). By 24 hours, Ad-eIF5A1-, Ad-eIF5A1(K50A)- and Ad-eIF5A2-infected cells still exhibited normal $\Delta\Psi_m$ values (Figure 16A). However, after 48 hours of infection reductions in $\Delta\Psi_m$ were evident for all of the Ad-eIF5A-infected cells. The percentage of cells with normal $\Delta\Psi_m$ decreased by ~25% for Ad-eIF5A1-infected cells and by ~40% for Ad-eIF5A1(K50A)- and Ad-eIF5A2-infected cells (Figure 16B). By 72 hours, only about 50% of cells infected with Ad-eIF5A1, Ad-eIF5A1(K50A) or Ad-eIF5A2 exhibited normal $\Delta\Psi_m$ (Figure 16B). Cells treated with Actinomycin D and cells infected with Ad-lacZ served as positive and negative controls, respectively (Figure 16).

3.4.3 Over-expression of eIF5A induces release of cytochrome *c*

The release of cytochrome *c* from mitochondria into the cytosol is an early event in the mitochondrial pathway of apoptosis. Once in the cytosol, cytochrome *c* binds to Apaf-1 to form the cytochrome *c*: Apaf-1 complex which directly induces activation of caspase 9 (Li et al., 1997; Srinivasula et al., 1998; Zou et al., 1999). To determine whether the effects of up-regulated eIF5A on mitochondria included induction of cytochrome *c* release, Hela S3 cells were infected with 500 pfu/cell Ad-eIF5A1. At 7, 24, 31, 48, 55 and 72 hours after infection, cytosolic and membrane fractions were isolated and subjected to immunoblot analysis using specific antibody against cytochrome *c*. In untreated cells as well as in cells infected with Ad-eIF5A1 for up to 31 hours, cytochrome *c* localized only in the membrane fraction indicating that it was still entrapped in the

Figure 16: Effect of over-expression of eIF5A on mitochondrial transmembrane potential.

A) Hela S3 cells were infected with different types of adenovirus construct. At intervals of 24, 48 and 72 hour after infection, cells were collected and incubated in growth medium containing 50 nM DiOC6(3) for 45 minutes at 37 ^{o}C in a humidified chamber. The cells were then analyzed by flow cytometry. X axis: fluorescence intensity of cell-DiOC6(3) conjugates; Y axis: relative cell number. Cells with normal mitochondrial transmembrane potential register within the third and forth log decade of the X axis, whereas cells with decreased mitochondrial transmembrane potential register within the first and second log decade. Cells treated with Actinomycin D or infected with Ad-lacZ served as positive and negative controls, respectively. Results shown are representative of those obtained in 4 independent experiments. **B)** Quantitation of flow cytometric data illustrating changes in mitochondrial transmembrane potential following infection with different types of adenovirus construct. Cells treated with Actinomycin D or infected with Ad-lacZ served as positive and negative controls, respectively. Each experiment was repeated 4 times, and the results shown are means +/- standard error.

A)

Untreated

24 hr ActD

24 hr

48 hr

72 hr

Ad-eIF5A1   Ad-eIF5A1(K50A)   Ad-eIF5A2   Ad-lacZ

B)

ActD   Ad-eIF5A1   Ad-eIF5A1(K50A)   Ad-eIF5A2   Ad-lacZ
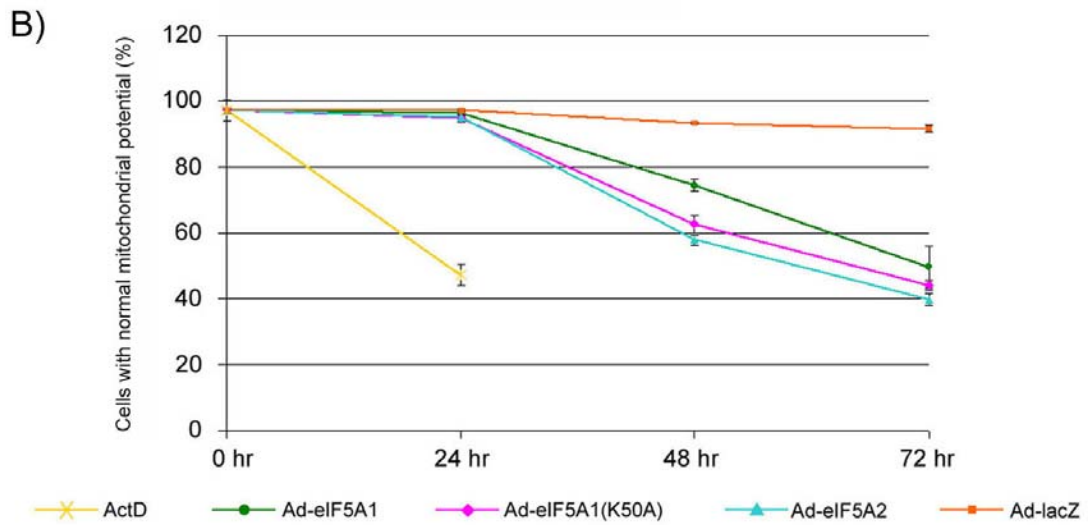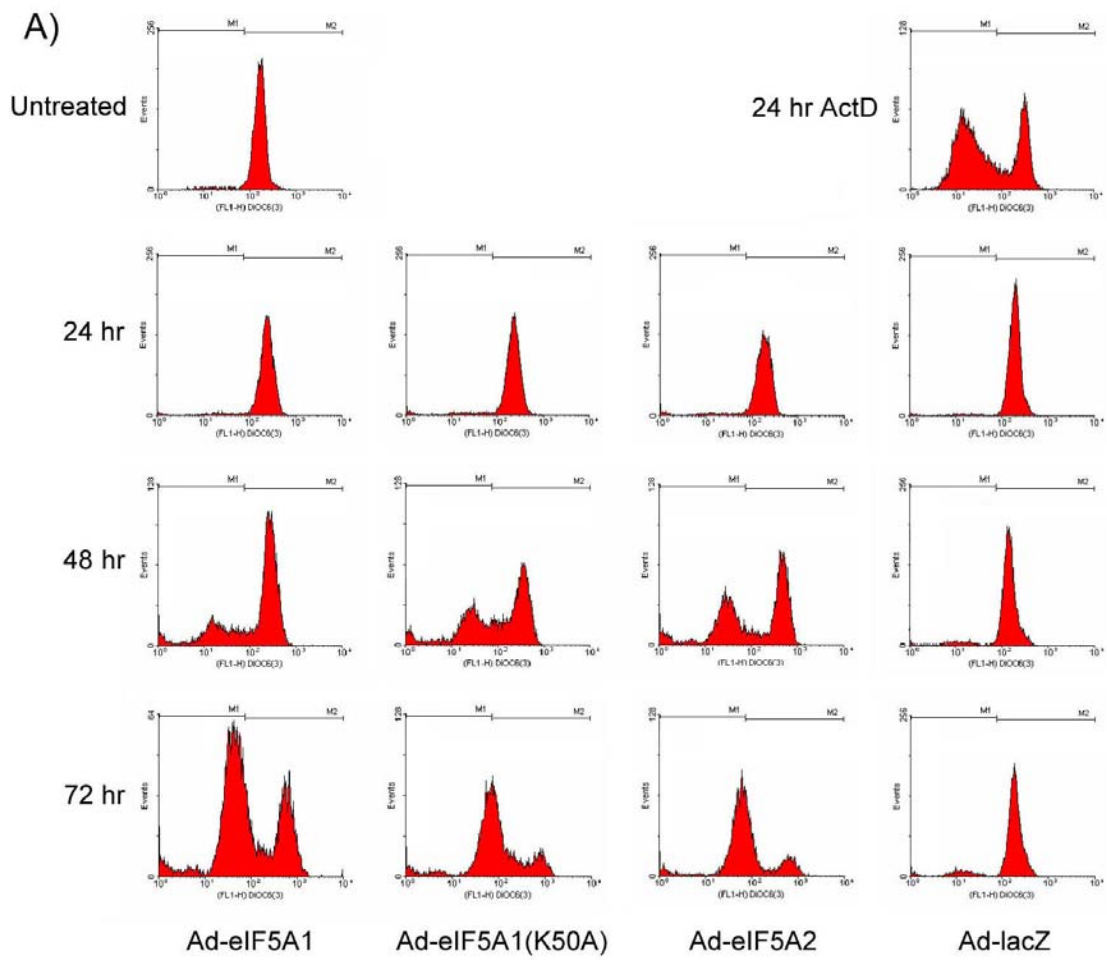
mitochondrial intermembrane space (IMS) (Figure 17). However, by 48 hours after infection translocation of cytochrome *c* from the mitochondria to the cytosol was evident, and after 55 hours there were almost equal proportions of cytochrome *c* in the membrane fraction and in the cytosol (Figure 17).

3.4.4 Over-expression of eIF5A induces translocation of Bax

Bax, which is a pro-apoptotic Bcl-2 family protein, usually resides as a monomer in the cytosol and, to a lesser extent, loosely associates with the mitochondrial outer membrane (Suzuki et al., 2000; Schinzel et al., 2004). In response to some apoptotic stimuli, Bax forms oligomers and translocates to the mitochondrial outer membrane (Schinzel et al., 2004). To determine whether eIF5A influences Bax migration, Hela S3 cells were infected with Ad-eIF5A1 at 500 pfu/cell. Cytosolic and membrane fractions were isolated at 7, 24, 31, 48, 55 and 72 hours after infection and subjected to Western blot analysis using specific antibody against Bax. In keeping with the established cytosolic localization of Bax in cells that are not undergoing apoptosis (Suzuki et al., 2000; Schinzel et al., 2004), in untreated cells most of the Bax protein was localized in the cytosol, with only a small amount located in the membrane fraction (Figure 17). However, within 24 hours of infection with Ad-eIF5A1 there was clear evidence for migration of Bax from the cytosol to the membrane fraction, and by 31 hours after infection, there were almost equal proportions of Bax in the cytosol and in the fraction containing mitochondria (Figure 17). By 48 hours after infection, almost all of the Bax protein was associated with the membrane fraction (Figure 17).

3.4.5 Effects of eIF5A over-expression on other proteins of the Bcl-2 family

Other members of the Bcl-2 family of proteins are also known to be involved in apoptosis (Reviewed by Adams and Cory, 2001; Newmeyer and Ferguson-Miller, 2003). Accordingly, the effects of eIF5A up-regulation on levels of the Bcl-2 proteins, Bid, Puma, Bim, Bad as well as Bax were examined. Specifically, Hela S3 cells were infected with Ad-eIF5A1, Ad-eIF5A1(K50A), Ad-eIF5A2 and Ad-lacZ, which served as a negative control. Cell lysates were harvested at 24, 48 and 72 hours after infection and subjected to Western blot analysis using antibodies for Bid, Puma, Bim, Bad and Bax. Bim, a pro-apoptotic BH3-only Bcl-2 family protein, was up-regulated within 48 hours in Ad-eIF5A1- and Ad-eIF5A2-infected cells (Figure 18). Indeed, not only was Bim_{EL} up-regulated,

Figure 17: Over-expression of eIF5A1 induces translocation of cytochrome *c* and Bax.
Hela S3 cells were infected with 500 pfu/cell of Ad-eIF5A1. At the indicated times after infection, cytosolic and membrane fractions were isolated, and the proteins were fractionated by SDS-PAGE and analyzed by Western blotting using anti-cytochrome *c* and anti-Bax. Each lane was loaded with 5 μg of protein. Equal loading of the samples was evaluated by blotting the membrane with antibody for Actin. C: cytosolic fraction; M: membrane fraction containing mitochondria. Results shown are representative of those obtained in 3 independent experiments.
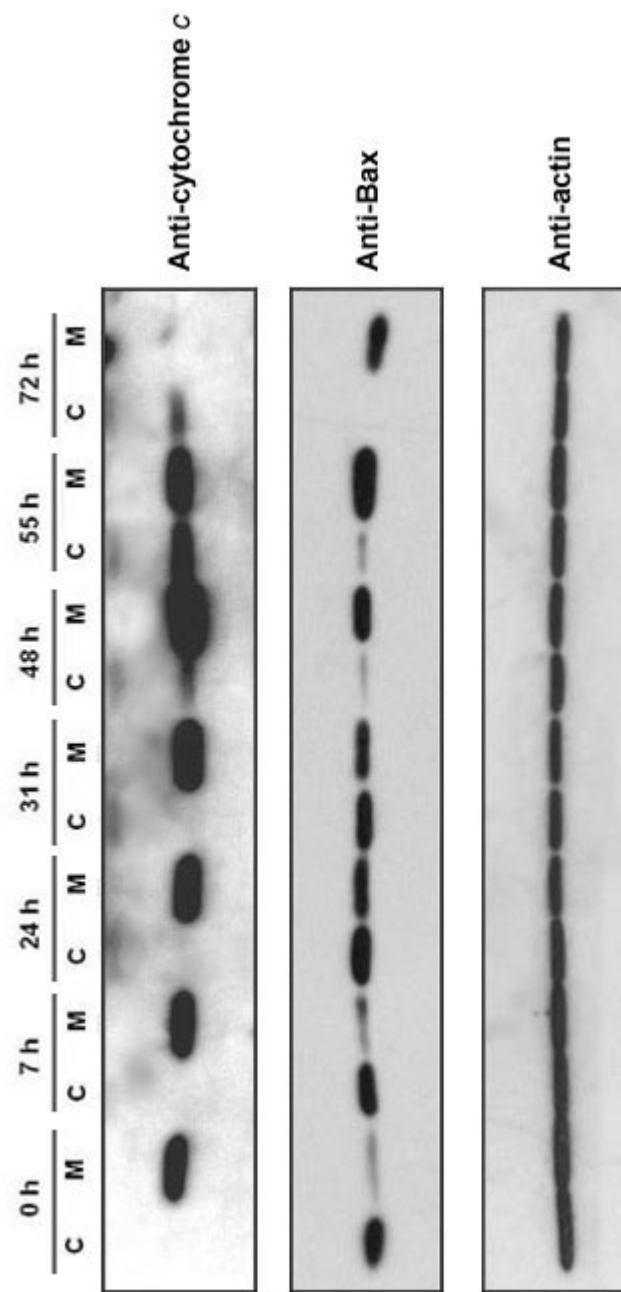
there was also induction of the alternative splicing forms of Bim (Figure 18). However, Bid and Puma, which are also pro-apoptotic BH3-only proteins, were down-regulated in Ad-eIF5A1-, Ad-eIF5A1(K50A)- and Ad-eIF5A2-infected cells (Figure 18). Moreover, cleaved Bid was not observed (Figure 18) indicating that eIF5A-induced activation of caspase 8 does not directly lead to activation of the intrinsic apoptotic pathway. No significant changes in the levels of Bad were apparent following infection (Figure 18). Nor was there a change in the level of Bax (Figure 18) notwithstanding the fact that up-regulation of eIF5A induced migration of Bax from the cytosol to mitochondria (Figure 17). However, it has been suggested previously that the localization of Bax on the mitochondrial outer membrane is in itself sufficient to induce mitochondrial outer membrane permeabilization (Jurgensmeier et al., 1998; Marzo et al., 1998; Narita et al., 1998; Shimizu et al., 1999; Tikhomirov and Carpenter, 2005).

3.4.6 Over-expression of eIF5A2 induces up-regulation of cleaved Bcl-2

The effects of eIF5A2 on levels of Bcl-2, which is a pro-survival member of the Bcl-2 family, were also examined in Hela S3 cells. In light of the apparent pro-apoptotic effect of eIF5A, one might have expected a down-regulation of Bcl-2 following infection with Ad-eIF5A. However, the basal level of Bcl-2 proved to be very low even in untreated Hela S3, and this precluded any assessment of down-regulation (Figure 19). This notwithstanding, there was a strong up-regulation/induction of a cleaved form of Bcl-2 in Ad-eIF5A2-infected cells. This cleaved Bcl-2 was weakly expressed at 24 hours after infection, and strongly upregulated in a time-dependent manner thereafter (Figure 19). Furthermore, there was a slight change in its size between 31 and 48 hours after infection. In addition, this cleaved Bcl-2 was only induced in Ad-eIF5A2-infected cells, not in Ad-eIF5A1-, Ad-eIF5A1(K50A)- or Ad-lacZ-infected cells. Nor was it apparent in cells treated with Actinomycin D (Figure 19).

3.4.7 Over-expression of eIF5A induces up-regulation of p73, a homolog of p53

The tumor suppressor gene, p53, is one of the most important oncogenes discovered to date. It is activated in the event of DNA damage-induced apoptosis, and it also protects normal cells by induction of cell cycle arrest and DNA repair (Reviewed by Fuster et al., 1997; Sionov and Haupt, 1999; Haupt et al., 2003; Helton and Chen, 2007). Although Hela cells contain both of the wild-type

Figure 18: Effect of the over-expression of eIF5A on p53 and pro-apoptotic Bcl-2 family proteins.

Hela S3 cells were infected with different types of adenovirus construct. At the indicated times after infection, cell lysates were collected and subjected to Western blot analysis for p53, Bid, Puma, Bim, Bax, and Bad. Each lane contained 7 μg of protein. Equal loading was evaluated by blotting the membrane with antibody for Actin. A1: Ad-eIF5A1; M: Ad-eIF5A1(K50A); A2: Ad-eIF5A2; Z: Ad-lacZ. Results shown are representative of those obtained in 3 independent experiments.
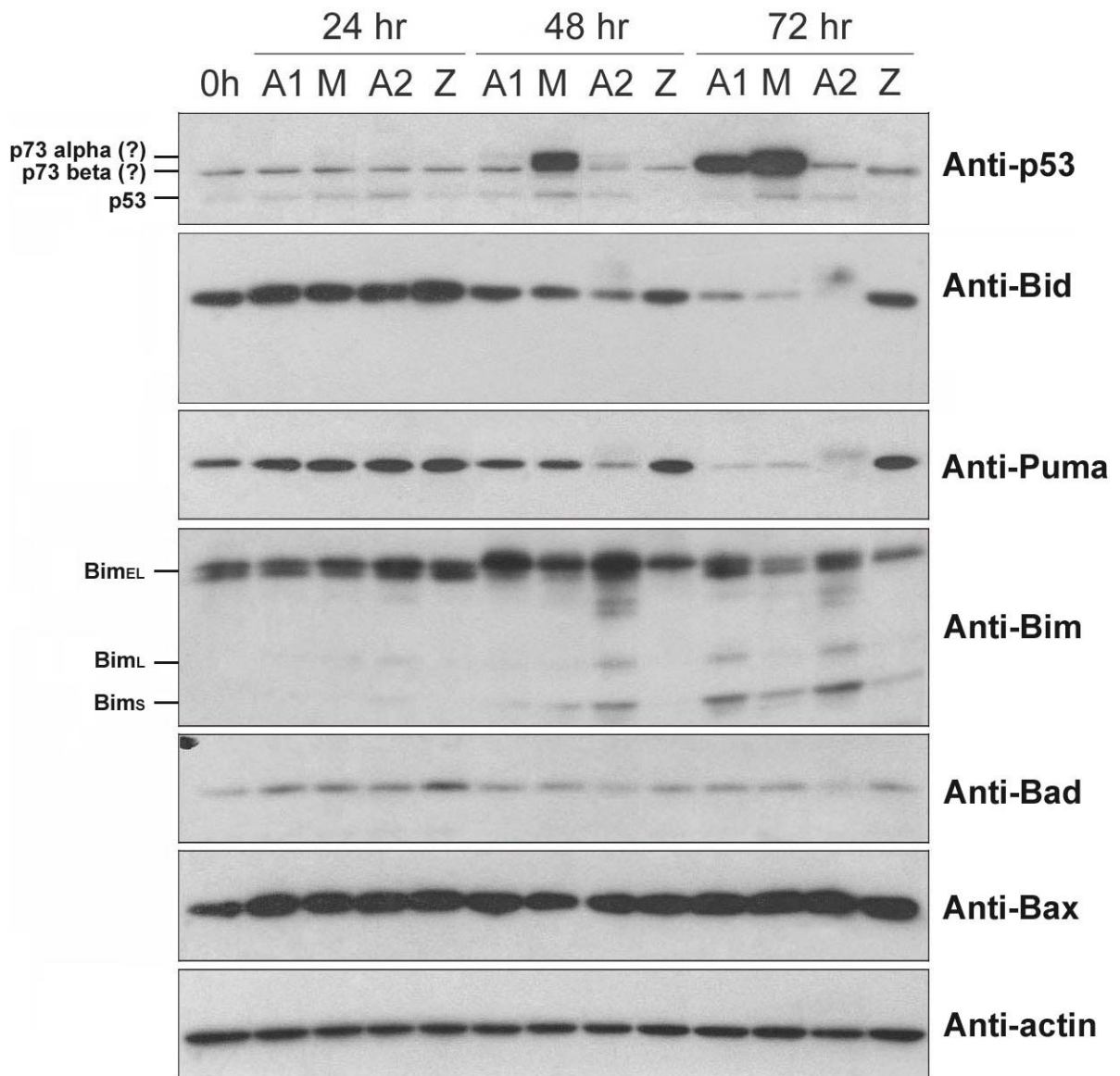
Figure 19: Over-expression of eIF5A2 induces cleaved Bcl-2 protein.

A) Hela S3 cells were infected with different types of adenovirus construct. Cell lysates were harvested at indicated times and subjected to Western blot analysis for Bcl-2. Each lane contained 7 μg of total cellular protein. Results shown are representative of those obtained in 3 independent experiments. **B)** Hela S3 cells were either treated with Actinomycin D for 24 hours or infected with 500 pfu/cell of Ad-eIFA2. Cell lysates were harvested at indicated times and subjected to immunoblot analysis using antibodies for Bcl-2. Equal loading of the samples was evaluated by blotting the membrane with antibody for Actin. Un: untreated; ActD: treated with Actinomycin D for 24 hours. Results shown are representative of those obtained in 3 independent experiments.
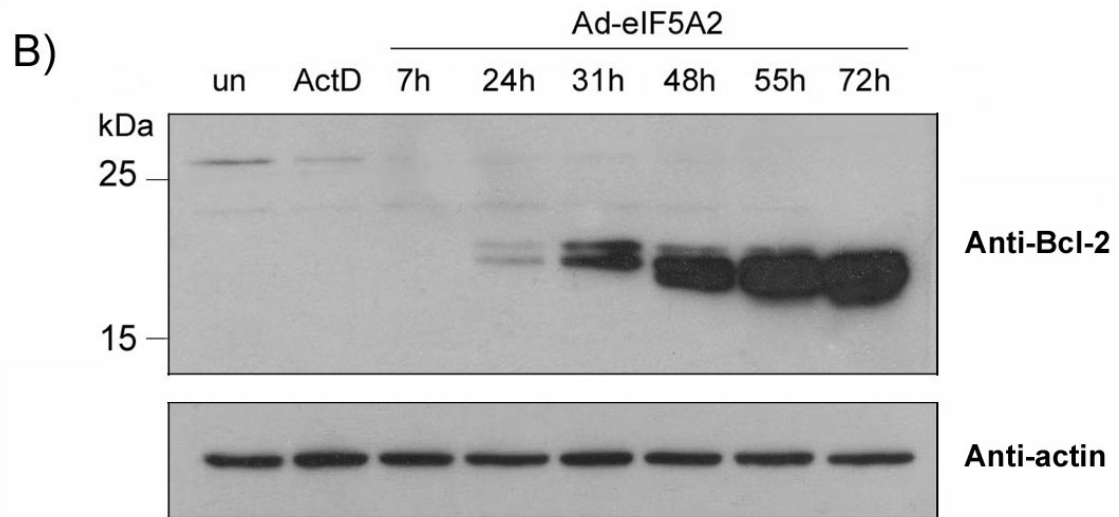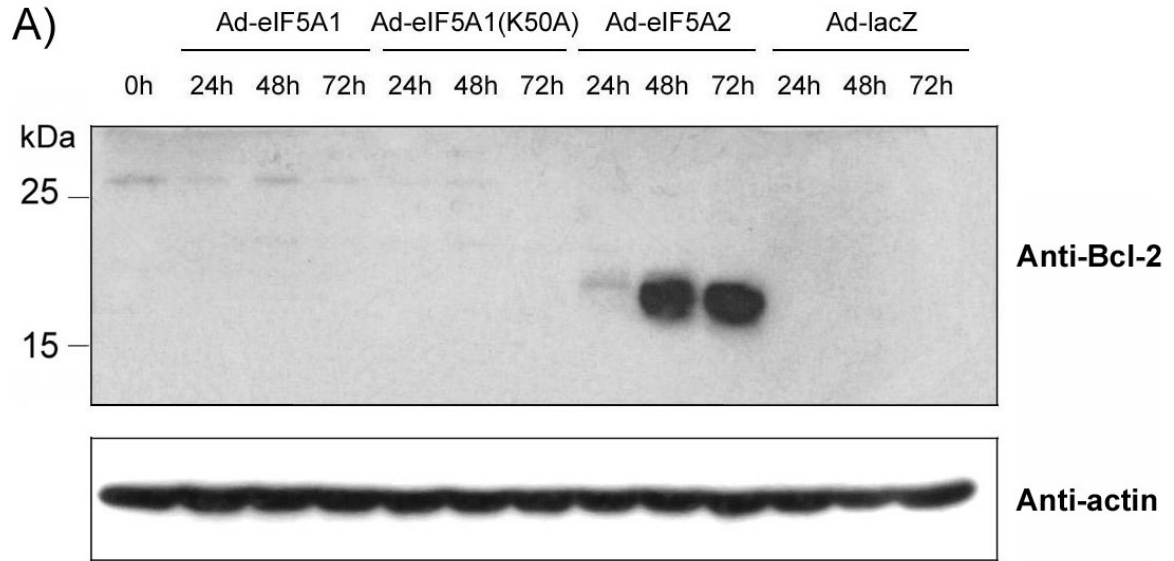
p53 alleles and p53 mRNA is translationally active in Hela cells, their basal level of p53 is very low, and thus p53 is effectively inactive (Scheffner et al., 1991). This reflects the fact that Hela cells are transformed with human papillomavirus (HPV) whose E6 gene product interacts with p53, resulting in its rapid degradation through the ubiquitin-proteasome pathway (Scheffner et al., 1990; Liang et al., 1993 & 1995; Thomas et al., 1996; Song et al., 1998; Talis et al., 1998). As a clonal derivative of Hela cells, Hela S3 cells also contain functionally inactive p53 (Bai et al., 2004).

To evaluate the effect of eIF5A up-regulation on p53 and its homologs, Hela S3 cells were infected with different types of adenovirus constructs and subjected to Western blot analysis using polyclonal antibody against p53 at 24, 48 and 72 hours after infection. The level of p53 was low and remained essentially unchanged during apoptosis induced by the over-expression of eIF5A1, eIF5A1(K50A) or eIF5A2 (Figure 18). However, there was strong up-regulation of a protein with a MW of about 70 to 80 kDa in Ad-eIF5A1- and Ad-eIF5A1(K50A)-infected cells (Figure 18). This protein is deemed to be p73, which has high homology with p53, inasmuch as the p53 antibody used in this analysis is polyclonal and very likely to cross-react with p73. There are two alternatively spliced p73 mRNA isoforms, giving rise to p73α (full length form; MW ~80 kDa) and p73β (shorter from; MW ~70 kDa) (Kaghad et al., 1997; Marin et al., 1998). Assuming this tentative identification of the protein is correct, the data in Figure 18 indicate that p73α and β were highly up-regulated in Ad-eIF5A1-infected cells at 72 hours after infection and in Ad-eIF5A1(K50A)-infected cells at 48 and 72 hours after infection. (Figure 18).

3.5 Post-translationally modified forms of eIF5A

eIF5A is post-translationally modified by two enzymes, deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DHH), which mediate the formation of deoxyhypusinated eIF5A and hypusinated eIF5A, respectively. Specifically, lysine$_{50}$ is converted to deoxyhypusine by DHS, which in turn is converted to hypusine by DHH (Maoka and Nakajima, 1973; Park et al., 1981, 1982 & 1988; Abbruzzese et al., 1986 & 1988; Wolff et al., 1990). In light of this, a series of experiments were conducted to determine whether the pro-apoptotic activity of eIF5A is attributable to a specific post-translationally modified form of the protein.

3.5.1 Over-expression of eIF5A1(K50A) induces an accumulation of lysine$_{50}$ eIF5A

eIF5A is the only protein known to contain the unique amino acid, hypusine (Cooper et al., 1983). Hypusine-containing eIF5A is essential for the growth of yeast (Schnier et al., 1991; Sasaki et al., 1996; Park et al., 1998). For mammalian cells, there are reports that inhibition of hypusine formation induces cell cycle arrest as well as apoptosis (Park et al., 1993b; Hanauske-Abel et al., 1994; Chen et al., 1996; Shi et al., 1996b; Tome and Gerner, 1997). In order to determine whether apoptosis induced by the over-expression of eIF5A1 is associated with hypusine modification, 2-dimensional (2D) electrophoresis combined with Western blot analysis was used to resolve the different post-translationally modified forms of eIF5A1 protein.

Initially, cell lysates of untreated cells, cells treated with GC7, an inhibitor of DHS which mediates the first step in the hypusine modification, and cells treated with DFO, an inhibitor of the enzyme DHH which mediates the second step in the hypusine modification, were analyzed in order to identify tentatively the post-translationally modified forms of eIF5A in the 2D Western blot. Three spots of eIF5A1 with different *pI* values were observed (Figure 20). Based on the effects of the inhibitors on the relative levels of the eIF5A spots, the one on the right (spot C) was tentatively designated as hypusine-containing eIF5A1; the one on the left (spot A) was tentatively designated as eIF5A1 with unmodified lysine$_{50}$; and the one in the middle (spot B) was tentatively designated as deoxyhypusine-containing eIF5A. In cells treated with DHS or DHH inhibitor, although there was the expected accumulation of unmodified and deoxyhypusine-containing eIF5A, respectively, the amount of hypusine-containing eIF5A was almost the same as in untreated cells (Figure 20). This presumably reflects the long half-life of hypusinated eIF5A protein, which has been shown to be more than seven days in mouse mammary carcinoma FM3A cells (Nishimura et al., 2005) and to be very long as well in CHO and JURKAT T-cells (Torrelio et al., 1987; Bergeron et al., 1998). In Ad-eIF5A1(K50A)-infected cells, there was an accumulation of lysine$_{50}$-unmodified eIF5A1 (spot A) (Figure 20), which is consistent with the fact that this mutated form of eIF5A1 is incapable of interacting with DHS. However, in Ad-eIF5A1-infected cells there was a large accumulation of both lysine$_{50}$-unmodified eIF5A1 (spot A) and deoxyhypusine-containing eIF5A1 (spot B) (Figure 20). At face value and assuming the tentative identities of these spots are correct, this suggests that the high levels of eIF5A expression inhibit DHH.

110

Figure 20: Ad-eIF5A1 and Ad-eIF5A1(K50A) induce over-expression of unhypusinated eIF5A1. HT-29 cells were infected with Ad-eIF5A1 or Ad-eIF5A1(K50A), and cell lysates were harvested 48 hours after infection. Lysates of untreated cells, cells treated for 72 hours with 50 μM GC7, an inhibitor of DHS, and cells treated for 72 hours with 500 μM DFO, an inhibitor of DHH, were also collected. The lysates were fractionated by 2D electrophoresis and subjected to immunoblot analysis using specific antibody against eIF5A1. For both Ad-eIF5A1- and Ad-eIF5A1(K50A)-infected cells, 0.3 μg of proteins was loaded onto pH 3-10 7 cm Immobiline DryStrips for the first dimension separation by isoelectric focussing; for the other cell lysates, 5 μg of protein was loaded onto the DryStrips. A: acidic form (tentatively identified as lysine$_{50}$-containing eIF5A); B: intermediate form (tentatively identified as deoxyhypusine-containing eIF5A); C: basic form (tentatively identified as hypusine-containing eIF5A). Results shown are representative of those obtained in 3 independent experiments.
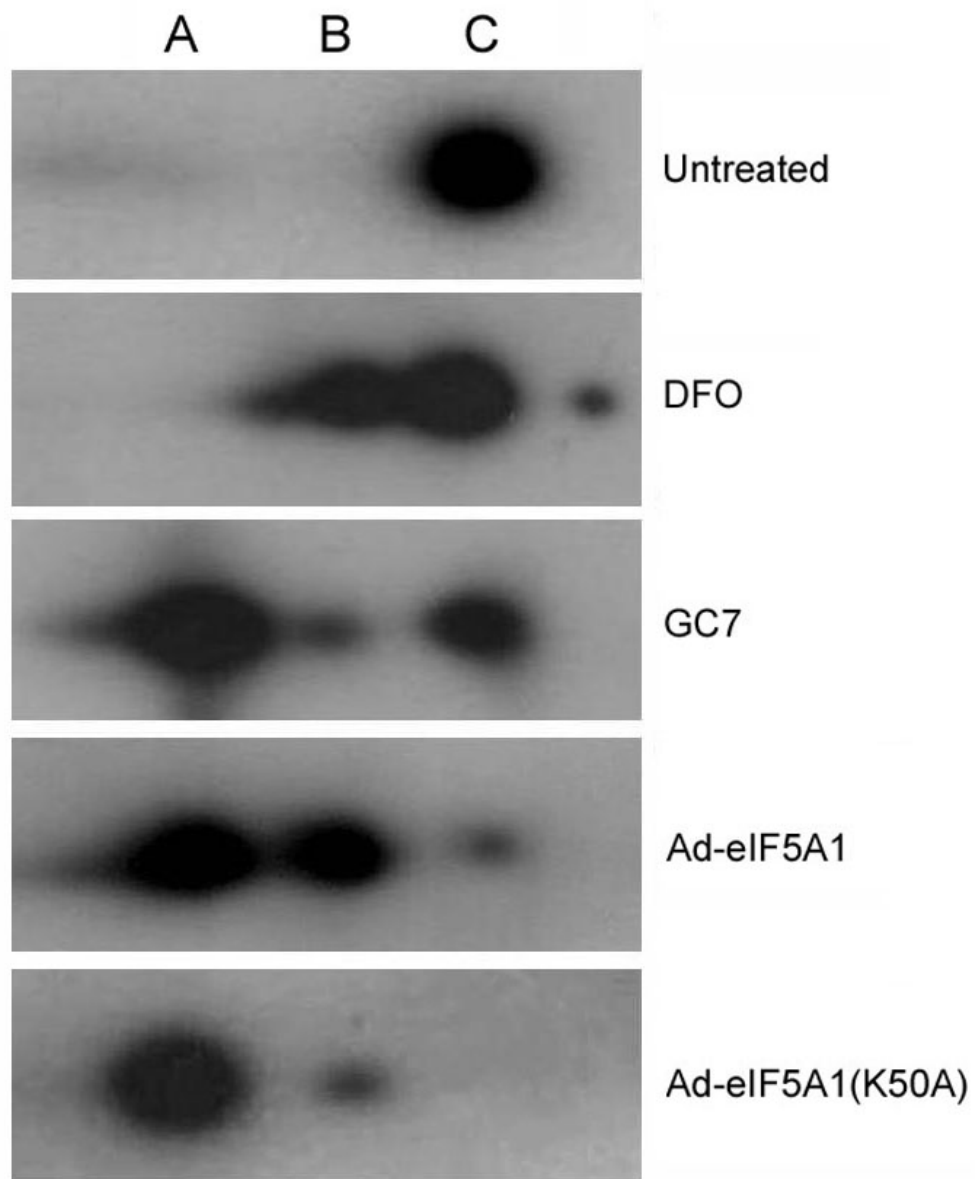
3.5.2 HA-tagged eIF5A1 is not hypusinated *in vitro*

That eIF5A1(K50A) is able to induce apoptosis as effectively as wild-type eIF5A1 suggests that eIF5A1-lysine$_{50}$ is the form of eIF5A that exhibits pro-apoptotic activity. Pertinent to this is the fact that, using the plasmid, pHM*eIF5A1*, Dominic Cliché from Dr. Thompson's laboratory found that expression of HA-tagged eIF5A1 in human colorectal carcinoma RKO cells also induced apoptosis (unpublished data). Indeed, this finding raised the possibility that the HA-tag prevents hypusination of eIF5A1.

In order to determine whether HA-tagged eIF5A1 protein is, in fact, incapable of being hypusinated, cells transfected with pHM*eIF5A1* were labeled with [^{3}H]-spermidine which gives rise to [^{3}H]-eIF5A during the hypusination reaction. Only hypusine-containing eIF5A can be labeled by [^{3}H]-spermidine since it is the only known protein to be modified by a structural contribution from spermidine (Clement et al., 2003). COS-7 cells were used for these experiments because they contain the SV40 T antigen that allows for episomal replication, and hence high expression, of pHM6 constructs containing the SV40 origin of DNA replication. Cells were transfected with pHM*eIF5A1*, pHM*eIF5A1(K50A)* or pHM*lacZ*6 (Figure 21), and then incubated in medium containing [^{3}H]-spermidine. During hypusination, the 4-aminobutyl moiety of [^{3}H]-spermidine is transferred to the lysine$_{50}$ residue of eIF5A1 by deoxyhypusine synthase (DHS), thereby labeling eIF5A1. At intervals of 48 and 72 hours after the introduction of [^{3}H]-spermidine, total cellular proteins were isolated and separated by SDS-PAGE. The fractionated proteins were visualized by autoradiography and also subjected to Western blot analysis using specific antibodies against eIF5A1 and HA. When the autoradiograms and the immunoblots for eIF5A1 and HA were aligned, it was clear that endogenous eIF5A1, but not trans-HA-tagged eIF5A1, was labeled (Figure 22A).

At face value, this can be interpreted as indicating that DHS does not react with HA-tagged eIF5A1 and that the HA-tagged protein does not become hypusinated. However, there are other possible interpretations. The result could, for example, reflect degradation of [^{3}H]-spermidine in the cells by diamine oxidase (DAO) (Holtta et al., 1975) or inadequate protein loading of the gel resulting in a radiolabeled signal that was not strong enough to be detected. To eliminate these possibilities, after the cells were transfected with pHM*eIF5A1* plasmid, aminoguanidine (AG), which is a specific and highly effective inhibitor of DAO (Gahl and Pitot, 1978), was added to the growth medium. In addition to its inhibitory effect on the degradation of spermidine, AG also completely inhibits serum-stimulated labeling of unrelated cellular proteins and has no effect on the labeling of eIF5A (Chen,

Figure 21: Maps of vectors used to express eIF5A1.

Human eIF5A1 and eIF5A1(K50A) were cloned between the *Hind* III and *Eco* RI sites of the empty pHM6 vector **(A)**, generating pHM*eIF5A1* and pHM*eIF5A1(K50A)* **(C)**, respectively, with hemagglutintin (HA) tagged at the N-terminus. pHM*lacZ*6 **(B)**, which contains an N-terminal HA epitope and a C-terminal His$_6$ tag, was used as the control vector. BGH pA, BGH polyadenylation signal; f1 ori, f1 origin; SV40 ori, SV40 promoter and origin; Neomycin, Neomycin-resistance gene; SV40 pA, SV40 polyadenylation signal; Col E1, Col E1 origin; Ampicillin, Ampicillin-resistance gene; P$_{CMV}$, CMV promoter.

A) pHM6 5450 bp

B) pHM*lacZ*6 8486 kb

C) pHM*eIF5A1* or pHM*eIF5A(K50A)* ~ 5900 bp

115
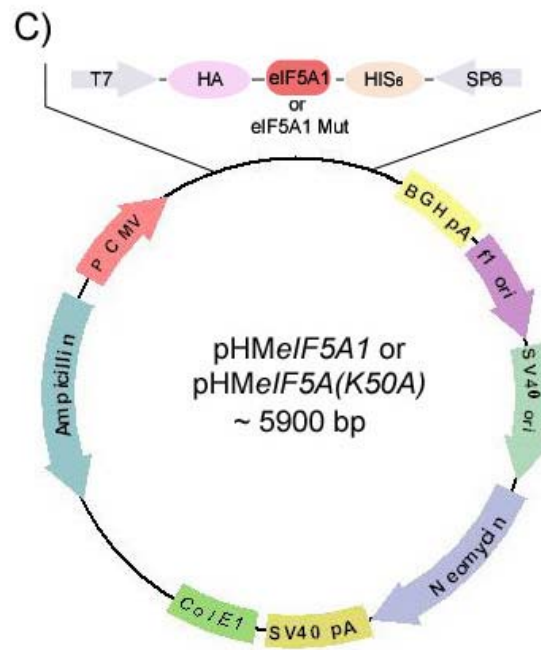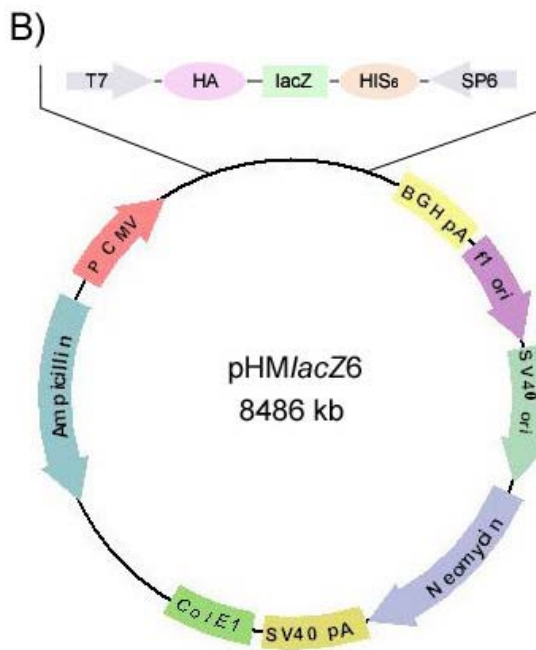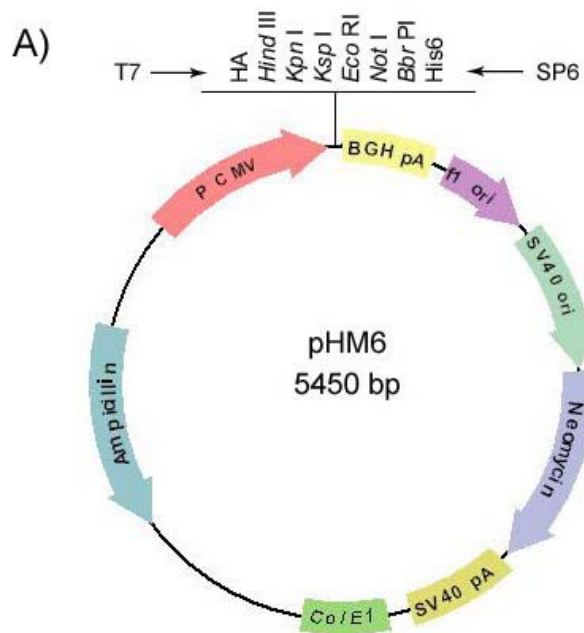
Figure 22: HA-tagged eIF5A1 is not hypusinated in COS-7 cells.

A) COS-7 cells were transfected with pHM*eIF5A1*, pHM*eIF5A1(K50A)* or pHM*lacZ*6 using electroporation and then incubated in complete medium containing 40 μCi/ml [^{3}H]-spermidine. Cell lysates were harvested 48 hours after transfection and proteins were fractionated by SDS-PAGE. [^{3}H]-labeled eIF5A1 was visualized by autoradiography. Endogenous and exogenous eIF5A1 were detected by incubating the same membrane with specific antibodies against eIF5A1 and HA, respectively. Lane 1: untreated; Lane 2: pHM*eIF5A1*; Lane 3: pHM*eIF5A1(K50A)*; Lane 4: pHM*lacZ*6. Results shown are representative of those obtained in 3 independent experiments. **B)** Cells were transfected with pHM*eIF5A1* using electroporation and then incubated in complete medium containing 40 μCi/ml [^{3}H]-spermidine and 1 mM aminoguanidine. At 48 hours (Lane 1) and 72 hours (Lane 2) after transfection, cell lysates were harvested and immunoprecipitated using specific antibody against eIF5A1. The IP products were fractionated by SDS-PAGE. [^{3}H]-labeled eIF5A1 was visualized by autoradiography. Endogenous and exogenous eIF5A1 were detected by incubating the same membrane with specific antibodies against eIF5A1 and HA, respectively. Results shown are representative of those obtained in 2 independent experiments.
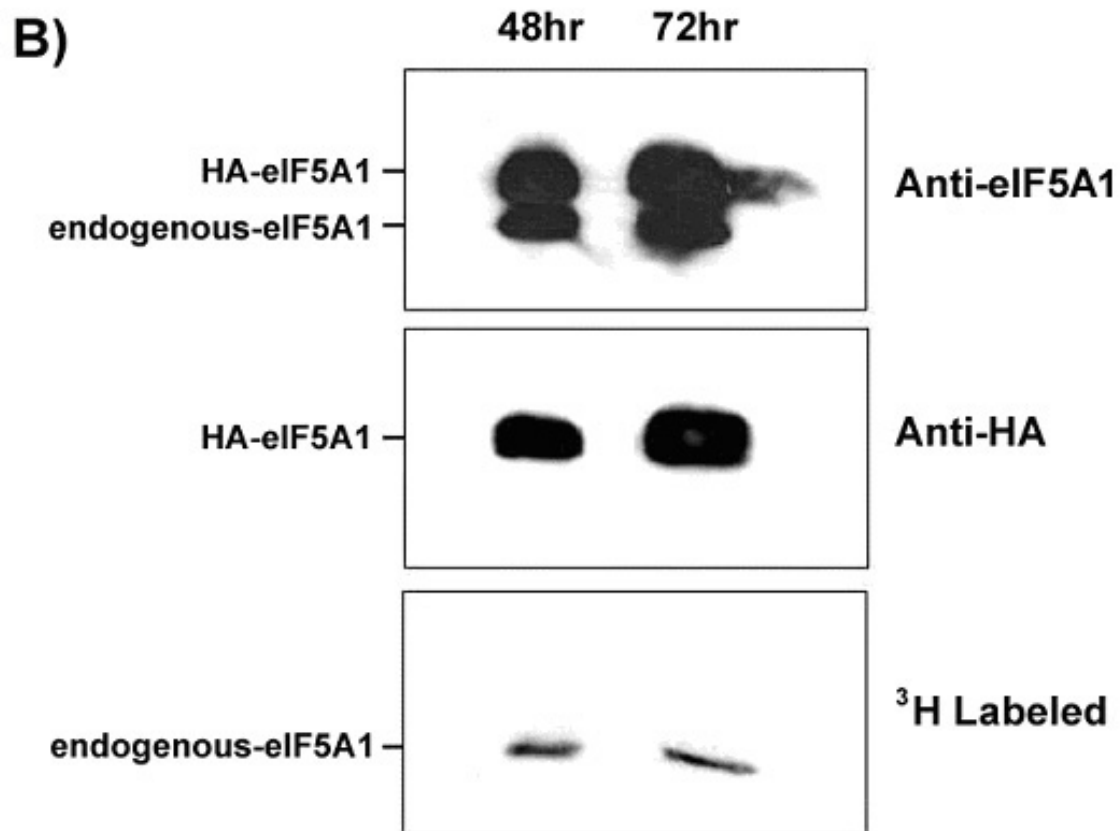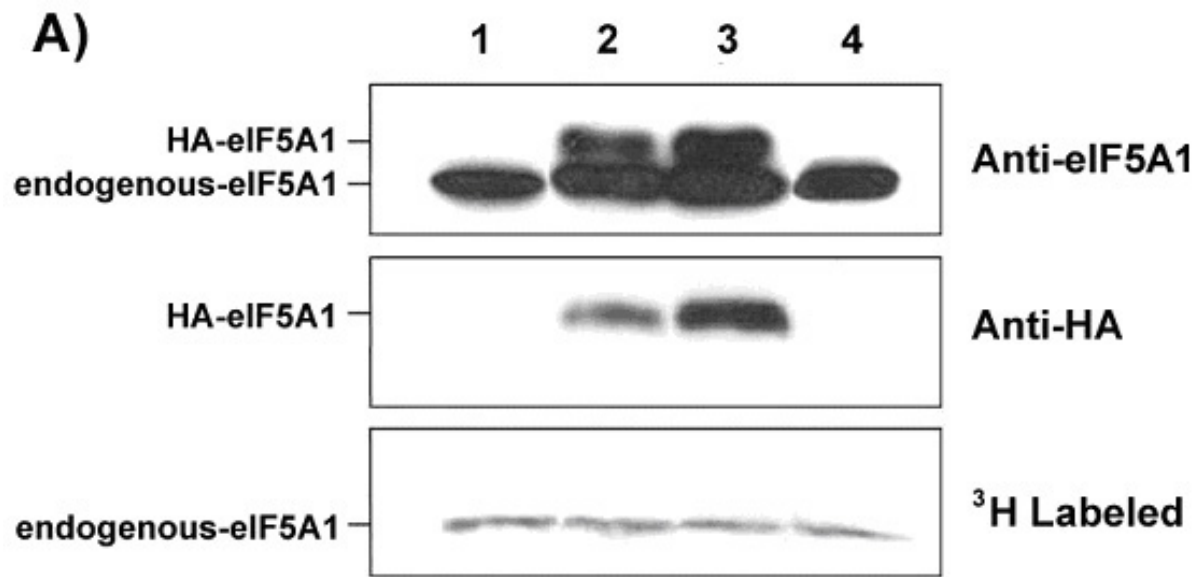
1983). As well, after the cell lysates were harvested, they were subjected to immunoprecipitation (IP) using specific antibody for eIF5A1 to concentrate both endogenous and exogenous eIF5A1 proteins. The IP product was in turn fractionated by SDS-PAGE, and the gel was then subjected to autoradiography and to immunoblotting using antibodies for eIF5A1 and HA. In this case as well, HA-tagged eIF5A1 was not labeled by tritium, while endogenous eIF5A1 was strongly labeled (Figure 22B).

There are two possible reasons why HA-tagged eIF5A1 is not hypusinated. First, addition of the HA-tag to the N-terminus of eIF5A1 may inhibit its interaction with DHS by masking, or rendering inaccessible, its DHS-binding site. Alternatively, the HA-tag may simply reduce the affinity of eIF5A1 for DHS and, by doing so, render HA-tagged eIF5A1 unable to compete successfully with endogenous eIF5A1 for a limited amount of DHS.

3.5.3 Lysine$_{50}$-containing eIF5A accumulates during SNP-induced apoptosis

With a view to further testing the possibility that it is the lysine$_{50}$-containing form of eIF5A that induces apoptosis, levels of the post-translationally modified forms of the protein in cells induced to undergo apoptosis by treatment with SNP, a source of NO, were examined. HT-29 cells were treated with 3 mM SNP. Total cell proteins were harvested 0.5, 1, 3.5, 6 and 24 hours later and subjected to 2D electrophoresis and Western blot analysis using eIF5A1-specific antibody. In the untreated cells, all of the eIF5A1 is in the form tentatively identified as hypusine-containing eIF5A1 (spot C), which is consistent with the contention that this form of eIF5A is not involved in the induction of apoptosis (Figure 23). Within 0.5 hours of treatment, deoxyhypusine-containing eIF5A1 (tentatively identified as spot B) began to accumulate, and within 3.5 hours there was also an accumulation of the spot tentatively identified as lysine$_{50}$-containing eIF5A (spot A) (Figure 23). This trend continued through 6 hours after treatment (Figure 23). These results are consistent with the contention that lysine$_{50}$-containing eIF5A1 is able to induce apoptosis, but don't rule out a role for deoxyhypusine-containing eIF5A1 as well.

Figure 23: Lysine$_{50}$-containing eIF5A1 accumulates after treatment with SNP.

HT-29 cells were treated with 3 mM SNP. Cell lysates were harvested at indicated times after the treatment and fractionated by 2D electrophoresis. The corresponding Western blot probed with anti-eIF5A1 is illustrated. A: acidic form (tentatively identified as lysine$_{50}$-containing eIF5A); B: intermediate form (tentatively identified as deoxyhypusine-containing eIF5A); C: basic form (tentatively identified as hypusine-containing eIF5A). The results shown are representative of those obtained in 3 independent experiments.
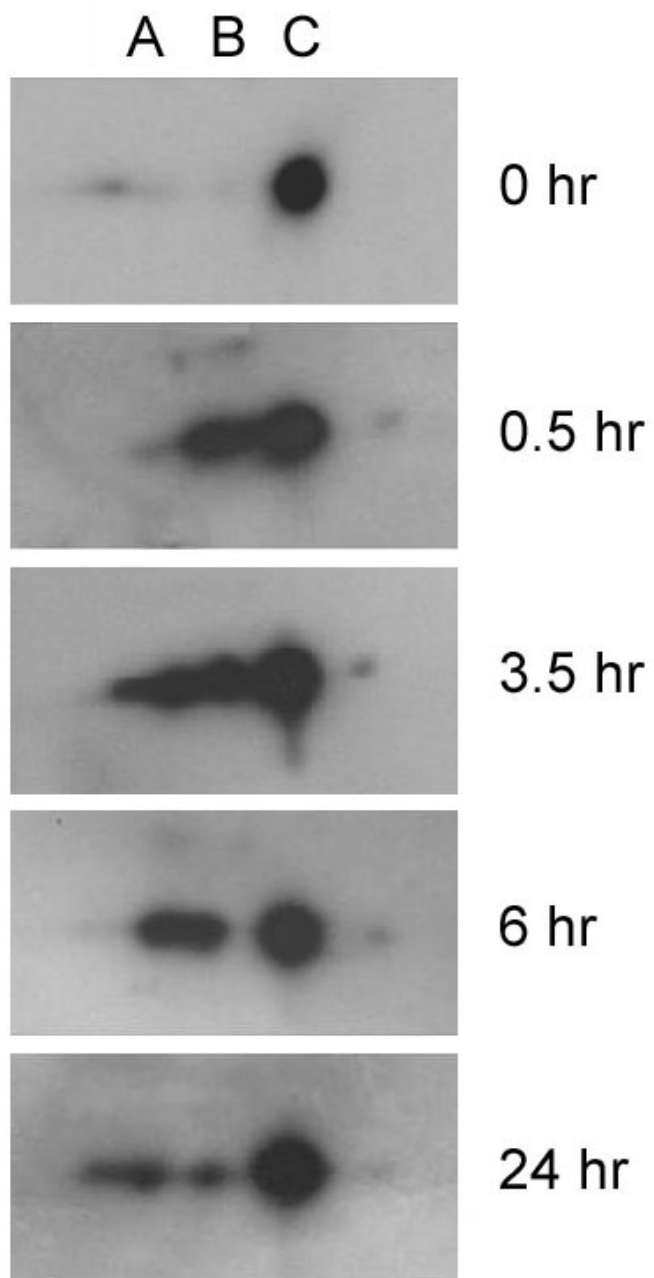
3.6 Identification of post-translationally modified forms of eIF5A1 by mass spectrometry

The post-translationally modified forms of eIF5A1 separated by 2D electrophesis were tentatively identified by Western blot analysis of changes in their relative levels in response to inhibitors of DHS and DHH and following transfection with eIF5A1(K50A), which is incapable of reacting with DHS. In order to obtain a more definitive identification, the separated spots reacting with eIF5A1 antibody in the 2D Western analysis were sequenced by MS and Tandem MS (MS/MS). This allowed identification of the post-translationally modified forms of eIF5A1 because the MS/MS fragmentation portrays weight differences between modified and unmodified amino acids. The mass spectrometry was performed by Zhenyu Cheng from Dr. McConkey's laboratory.

3.6.1 Lysine$_{47}$ and lysine$_{50}$ of eIF5A1 are both post-translationally modified

Hela S3 cell lysates were fractionated by 2D gel electrophoresis in preparation for mass spectrometric analysis. Representative gels stained with Coomassie blue for untreated Hela S3 cells and for Hela S3 cells infected with Ad-eIF5A1 for 48 hours are illustrated in Figure 24. eIF5A1 proteins are approximately 18 kDa in size and have a *pI* of ~5 (Park, 1988; Klier et al. 1995). Three proteins resolved in the 2D gels with molecular weights of ~18 kDa and *pI*s of 4.9, 5.0 and 5.1 (indicated by white arrows in Figure 24) were identified as eIF5A1 proteins by probing corresponding Western blots with anti-eIF5A1 (Figure 24). These proteins were cut out of the gels, and their identity as post-translationally modified forms of eIF5A1 was confirmed by mass spectrometry (Table 1 and Figure 25). In addition, however, MS and Tandem MS (MS/MS) revealed that lysine$_{47}$ and lysine$_{50}$ of eIF5A1 are both post-translationally modified. Lysine$_{47}$ becomes acetylated, and lysine$_{50}$ is converted to either deoxyhypusine or hypusine (Figure 25).

In untreated cells, the spot at *pI* 5.1 (designated as the basic form) was the most abundant eIF5A1 protein (Figures 24A and C). It was also present in Ad-eIF5A1-infected cells, but much less abundant (Figures 24B and C). Mass spectrometry of this protein revealed that lysine$_{50}$ is hypusinated, whereas lysine$_{47}$ is unmodified (Figure 25E). The identification score for this form of eIF5A1 was 75%, and the sequence coverage was 61% (Table 1).

Figure 24: 2-Dimensional gel electrophoresis of Hela S3 cell lysates.

Lysates of untreated Hela S3 cells **(A)** and Hela S3 cells infected with Ad-eIF5A1 for 48 hours **(B)** were harvested and fractionated by 2D electrophoresis. The gels were then stained with Coomassie blue. Protein spots indicated by white arrows were cut out of the gels and sequenced by mass spectrometry. The results shown are representative of those obtained in 3 independent experiments. **(C)** Protein spots indicated by white arrows in **A** and **B** were identified as eIF5A1 in corresponding Western blot probed with anti-eIF5A1. A: acidic form (tentatively identified as lysine$_{50}$-containing eIF5A); B: intermediate form (tentatively identified as deoxyhypusine-containing eIF5A); C: basic form (tentatively identified as hypusine-containing eIF5A). The results shown are representative of those obtained in 3 independent experiments.
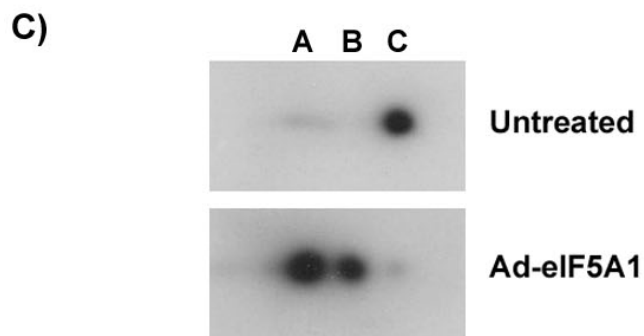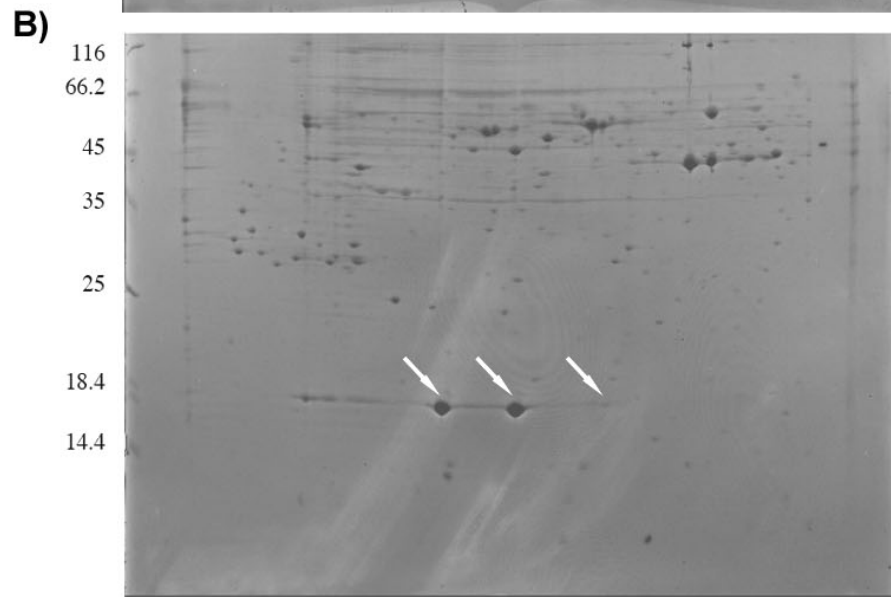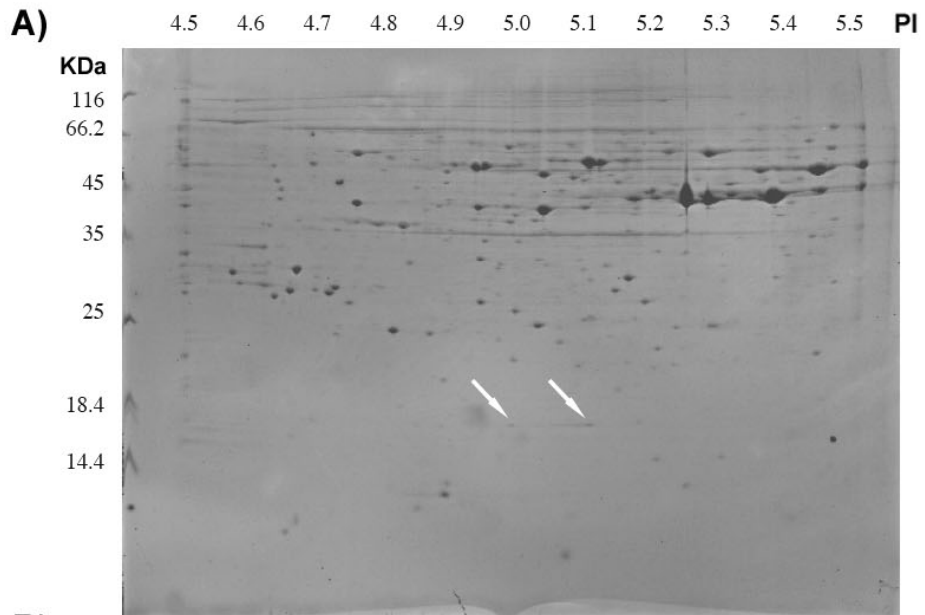
Table 1: Mass spectrometric identification of different isoelectric forms of eIF5A1 in Hela S3 cells.

* Identification scores are the number of PEAKS analysed using the MS/MS data from trypsin digests only.

** Sequence Coverage is the combined MS/MS data from both trypsin and Glu-C digests.

	pI	Identification score*	Sequence Coverge**
Acidic form in Ad-eIF5A1-infected cells	4.9	100%	100%
Intermediate form in Ad-eIF5A1-infected cells	5.0	100%	100%
Intermediate form in untreated cells	5.0	94%	70%
Basic form in both Ad-eIF5A1-infected and untreated cells	5.1	76%	62%

Figure 25: MS identification of the post-translational modification of lysine$_{47}$ and lysine$_{50}$ of eIF5A1 in Hela S3 cells.

The modifications of lysine$_{47}$ and lysine$_{50}$ were identified by MS (panels **C, D** and E) or Tandem MS (MS/MS) (panels A and B). **A)** Spectrum of the acidic form of eIF5A1 isolated from Ad-eIF5A1-infected cells (*pI* 4.9) illustrating that lysine$_{47}$ is acetylated, and that lysine$_{50}$ is unmodified. **B and D)** Spectra of the intermediate form of eIF5A1 (*pI* 5.0) isolated from untreated cells illustrating that it is a mixture of two different eIF5A1 proteins: one with lysine$_{47}$ acetylated and lysine$_{50}$ deoxyhypusinated; the other with lysine$_{47}$ acetylated and lysine$_{50}$ hypusinated. **C)** Spectrum of the intermediate form of eIF5A1 (*pI* 5.0) isolated from Ad-eIF5A1-infected cells illustrating that neither lysine$_{47}$ nor lysine$_{50}$ is modified. **E)** Spectrum of the basic form of eIF5A1 (*pI* 5.1) present in both untreated and Ad-eIF5A1-infected cells illustrating that lysine$_{47}$ is unmodified and lysine$_{50}$ is hypusinated. AcetK: acetylated lysine; HypuK: hypusinated lysine; DeHyK: deoxyhypusinated lysine; OxeM: oxidized methionine. X axis: ratio of mass to charge; Y axis: intensity relative to highest peak.
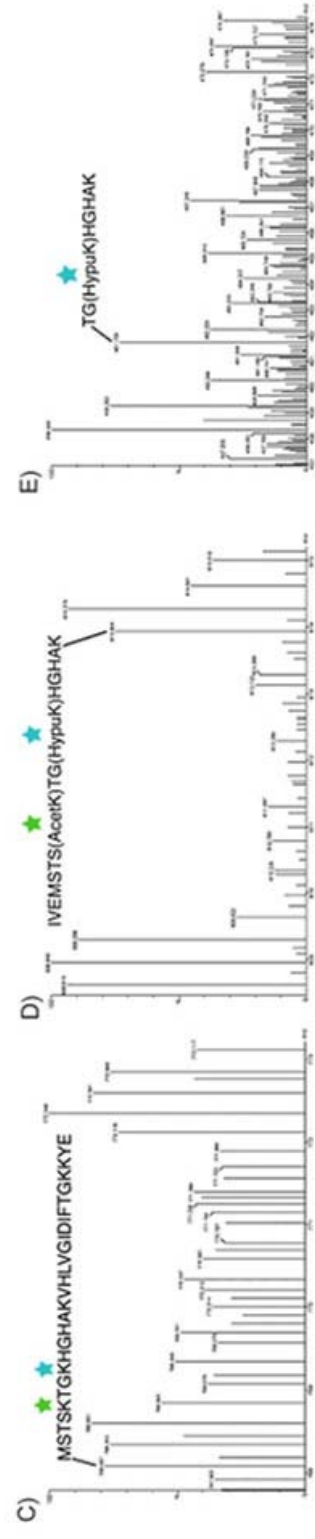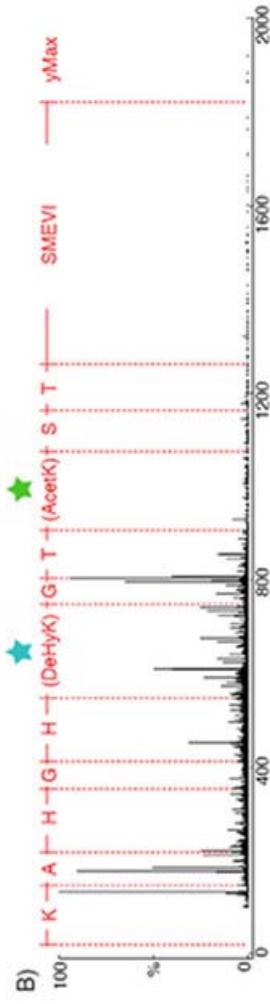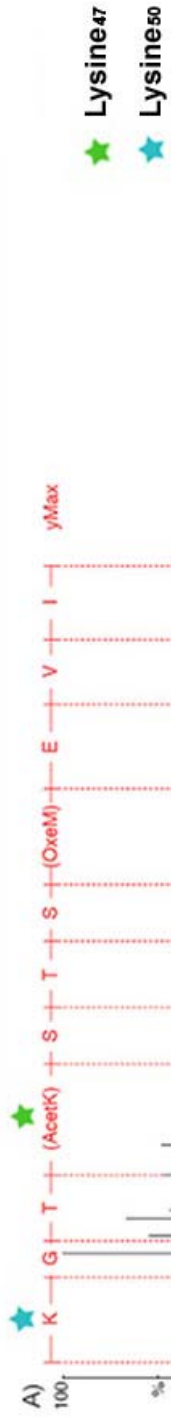
The eIF5A1 proteins migrating to *pI* 4.9 and *pI* 5.0 were most abundant in Ad-eIF5A1-infected cells (Figures 24B and C). These two spots were designated as the acidic form and the intermediate form, respectively. For the acidic form (*pI* 4.9), the identification score was 100%, and the sequence coverage was also 100% (Table 1). Analyses of the spectra for this protein indicated that lysine$_{47}$ is acetylated, and that lysine$_{50}$ is unmodified (Figure 25A). For the intermediate form (*pI* 5.0) isolated from Ad-eIF5A1-infected cells, the identification score was 100%, and sequence coverage was also 100% (Table 1), and in this case neither lysine$_{47}$ nor lysine$_{50}$ is modified (Figure 25C). For the intermediate form (*pI* 5.0) isolated from untreated cells, the identification score was 94% and the sequence coverage 70% (Table 1). However in this case there is a mixture of two different eIF5A1 proteins: one with lysine$_{47}$ acetylated and lysine$_{50}$ deoxyhypusinated; the other with lysine$_{47}$ acetylated and lysine$_{50}$ hypusinated (Figures 25B and D).

The post-translational modifications of lysine$_{47}$ and lysine$_{50}$ are summarized in Table 2.

3.6.2 Post-translational modification of other amino acid residues of eIF5A1

The mass spectrometric analysis indicated that for all of the isoelectric forms of eIF5A1 the starting methionine residue was cleaved, and the second alanine residue was acetylated (Figure 26A and Table 2). Since the first methionine is cleaved after translation, it is important to clarify that the numbering of amino acids used in this study is based on the amino acid sequence inferred from the cDNA sequence, with methionine in position 1. These modifications have been reported previously (Klier et al. 1995). Of particular interest, however, is the finding of an additional novel post-translation modification in the endo-proteinase digest which has not been previously reported. Specifically, for all of the isoelectric forms of eIF5A1, the asparagine residue at position 28 is deamidated (Figure 26 and Table 2). The deamidation of asparagine to aspartic acid or iaoaspartic acid is a two-step process involving a succinimide intermediate (Robinson and Rudd, 1974; Geiger and Clarke, 1987; Stephenson and Clarke, 1989). However, it is not possible to differentiate between aspartic acid and iaoaspartic acid by MS because their masses are identical (Schindler et al., 1996; Gonzalez et al., 2000; Lehmann et al., 2000; Luu et al., 2004; Cournover et al., 2005). Based on the intensities, the majority (70 to 85%) of peptides in the endo-proteinase digest contained aspartic acid or isoaspartic acid, and peptides containing the succinimide intermediate were also detectable (Figures 26B and C).

Figure 26: Post-translational modifications of other amino acids in eIF5A1 revealed by MS/MS.
A) Spectrum illustrating acetylation of the N-terminal alanine. **B)** Spectrum illustrating modification of asparagine$_{28}$ to a succinimide intermediate. **C)** Spectrum illustrating deamidation of asparagine$_{28}$ to aspartic acid or isoaspartic acid. AcetylA: Acetylated alanine. SuccN: succinimide intermediate. DeamN: deamidated asparagine. X axis: ratio of mass to charge; Y axis: intensity relative to highest peak.
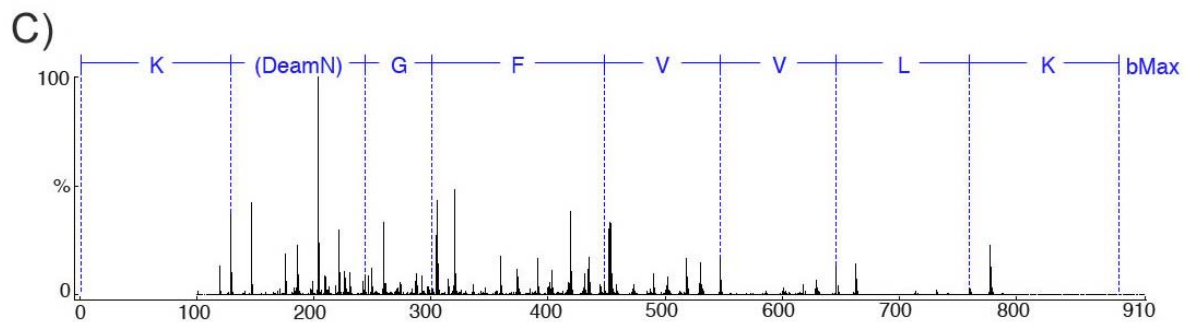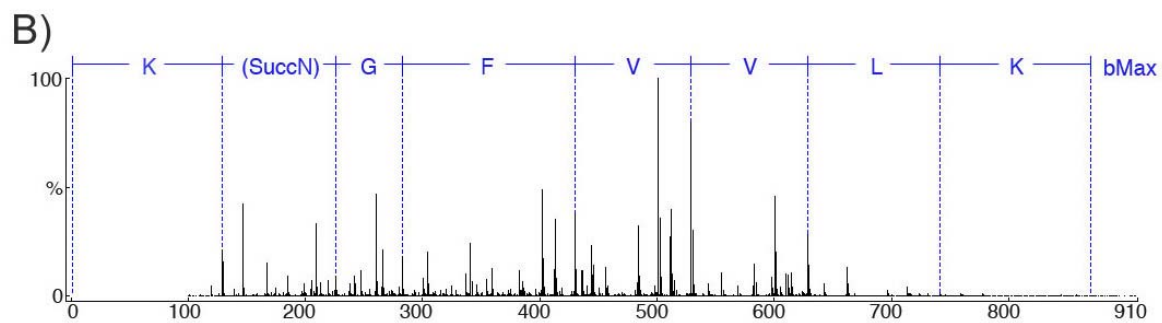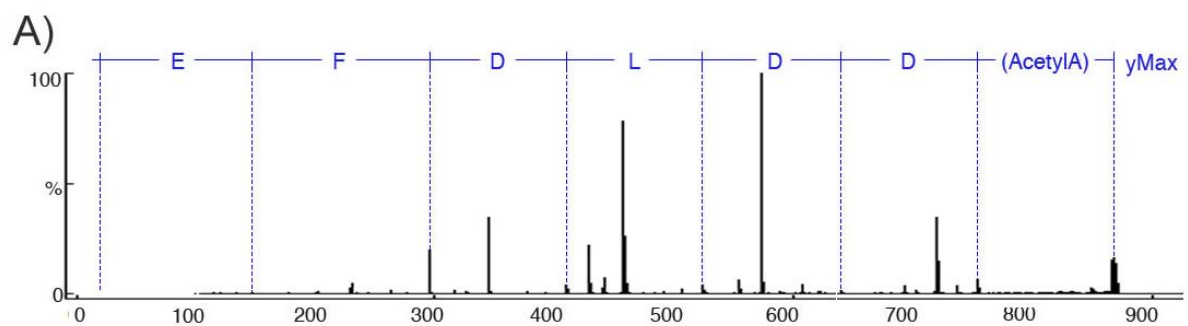
Table 2: Summary of the post-translational modifications of human eIF5A1.

"—": no modification. A2: alanine at position 2; N28: asparagine at position 28; K47: lysine at position 47; K50: lysine at position 50.

Residues / Forms	pI	A2	N28	K47	K50
Acid form in Ad-eIF5A1-infected cells	4.9	Acetylation	Deamidation	Acetylation	—
Intermediate form in Ad-eIF5A1-infected cells	5.0	Acetylation	Deamidation	—	—
Intermediate form in untreated cells	5.0	Acetylation	Deamidation	Acetylation	Hypusination or deoxyhypusination
Basic form in both Ad-eIF5A1-infected and untreated cells	5.1	Acetylation	Deamidation	—	Hypusination

3.7 The subcellular localization of eIF5A

Immunofluorescence staining and confocal microscopy of untreated Hela S3 cells indicated that eIF5A1 is mainly localized in the cytoplasm (Figure 27). Human eIF5A1 antibody and FITC-conjugated anti-mouse IgG were used to label eIF5A, MitoTracker Red CMXRos to label mitochondria, and Sytox blue to label nucleic acids (Figure 27C). In addition, cells were stained only with human eIF5A1 antibody and FITC-conjugated anti-mouse IgG to show that the staining of mitochondria and nucleic acids did not affect the location of eIF5A1 (Figure 27A). Cells were also stained only with FITC-conjugated anti-mouse IgG to show that the FITC-labeled proteins were truly eIF5A (Figure 27B).

Localization of eIF5A1 in the cell cytoplasm has been observed previously for both mammalian cells and yeast (Shi et al., 1996a & 1997; Valentini et al., 2002; Cracchiolo et al., 2004). However, when Hela S3 cells were infected with Ad-eIF5A1 or Ad-eIF5A1(K50A) to induce apoptosis, this localization changed. Specifically, although eIF5A1 was still in the cytoplasm, there was a distinct clustering of the protein in the vicinity of the plasma membrane within 24 hours of infection (Figure 28). By 48 hours after infection with Ad-eIF5A1 or Ad-eIF5A1(K50A), the clustering of eIF5A1 protein at the plasmalemma was more pronounced (Figure 29), whereas the localization pattern in cells infected with Ad-lacZ was indistinguishable from that for untreated cells (Figures 28 and 29).

It was not possible to distinguish between endogenous eIF5A1 and transgenic eIF5A1 in confocal microscopic images of cells infected with Ad-eIF5A1 or Ad-eIF5A1(K50A). Accordingly, the localization of eIF5A1 in Hela S3 cells induced to undergo apoptosis by treatment with Actinomycin D was also examined. The cells were treated with 0.5 μg/ml Actinomycin D for 12, 18 or 24 hours, and then stained to label eIF5A1, mitochondria and nucleic acids. Only endogenous eIF5A1 was present is these cells, and there was clear evidence for a progressive translocation of the protein from the cytoplasm to the nucleus coincident with the induction of apoptosis. eIF5A1 was localized in the cytoplasm in untreated cells, and it began to migrate into the nucleus within 12 hours after the treatment (Figure 30). This redistribution into the nucleus continued in a time-dependent manner such that by 18 hours after treatment, the intensity of eIF5A1 staining was approximately equal in the cytoplasm and in the nucleus, and by 24 hours, the concentration of eIF5A1 appeared to be higher in the nucleus than in the cytoplasm (Figure 30).

Figure 27: Visualization by laser scanning confocal microscopy of the subcellular localization of eIF5A1 in untreated Hela S3 cells.

A) Untreated Hela S3 cells were fixed and then treated with mouse polyclonal antibody against human eIF5A1 and FITC-conjugated anti-mouse IgG (green). Results shown are representative of those obtained in 2 independent experiments. **B)** Untreated Hela S3 cells were fixed and then treated only with FITC-conjugated anti-mouse IgG. Results shown are representative of those obtained in 2 independent experiments. **C)** Untreated Hela S3 cells were fixed and then treated with mouse polyclonal antibody against human eIF5A1 and FITC-conjugated anti-mouse IgG as well as with SYTOX Blue nucleic acid stain (blue) and Mitotracker Red CMXRos mitochondrial stain (red). Results shown are representative of those obtained in 4 independent experiments. Bar, 10 μm.
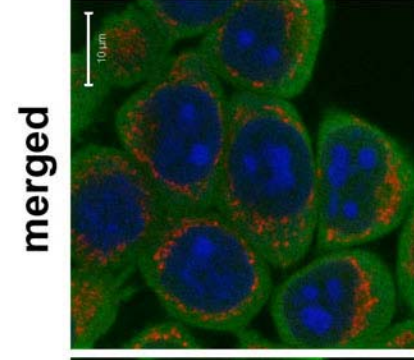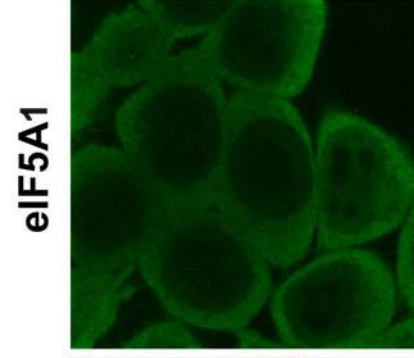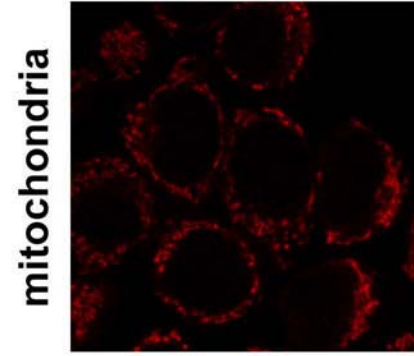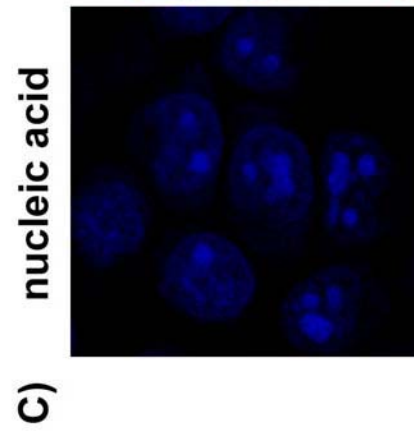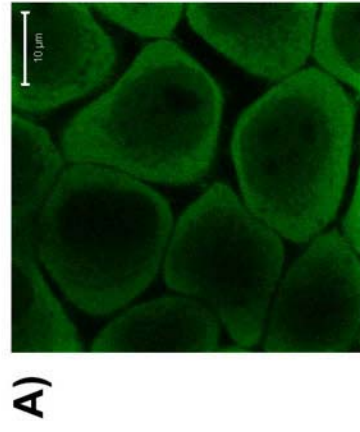
Figure 28: Visualization by laser scanning confocal microscopy of the subcellular localization of eIF5A1 in Hela S3 cells infected with adenovirus-eIF5A1 constructs for 24 hours.

Hela S3 cells were either untreated or infected with Ad-eIF5A1 or Ad-eIF5A1(K50A). After 24 hours, the cells were fixed and then treated with mouse polyclonal antibody against human eIF5A1 and FITC-conjugated anti-mouse IgG (green) as well as SYTOX Blue nucleic acid stain (blue) and Mitotracker Red CMXRos mitochondrial stain (red). Results shown are representative of those obtained in 3 independent experiments. Bar, 10 μm.
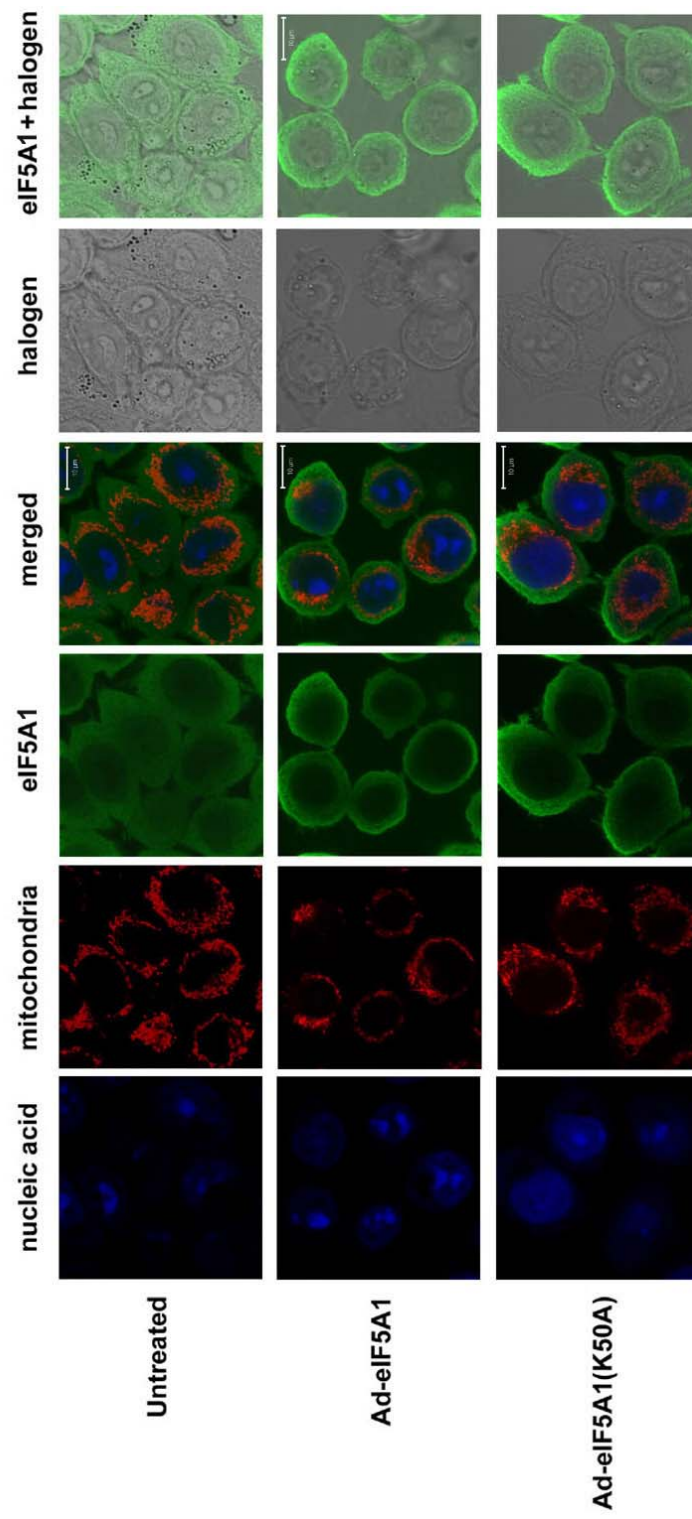
Figure 29: Visualization by laser scanning confocal microscopy of the subcellular localization of eIF5A1 in Hela S3 cells infected with adenovirus constructs for 48 hours.

Hela S3 cells were infected with Ad-eIF5A1, Ad-eIF5A1(K50A) or Ad-lacZ. After 48 hours, the cells were fixed and then treated with mouse polyclonal antibody against human eIF5A1 and FITC-conjugated anti-mouse IgG (green) as well as SYTOX Blue nucleic acid stain (blue) and Mitotracker Red CMXRos mitochondrial stain (red). Results shown are representative of those obtained in 3 independent experiments. Bar, 10 μm.
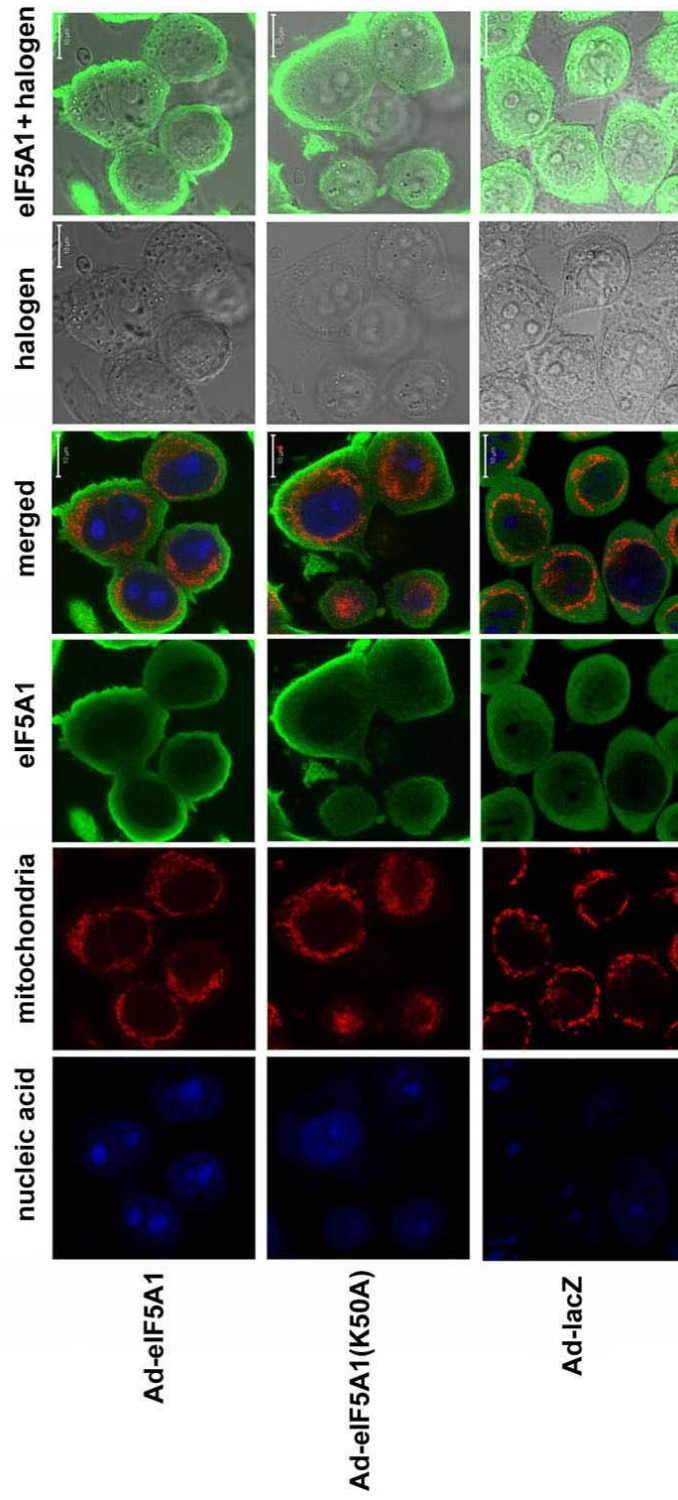
Figure 30: Visualization by laser scanning confocal microscopy of the subcellular localization of eIF5A1 in Actinomycin D-treated Hela S3 cells.

Hela S3 cells were either untreated or incubated with 0.5 µg/ml of Actinomycin D for 12, 18, or 24 hours. The cells were subsequently fixed and then treated with mouse polyclonal antibody against human eIF5A1 and FITC-conjugated anti-mouse IgG (green) as well as SYTOX Blue nucleic acid stain (blue) and Mitotracker Red CMXRos mitochondrial stain (red). Results shown are representative of those obtained in 3 independent experiments. Bar, 10 µm.

	nucleic acid	mitochondria	eIF5A1	merged
0 hr				
12 hr				
18 hr				
24 hr				
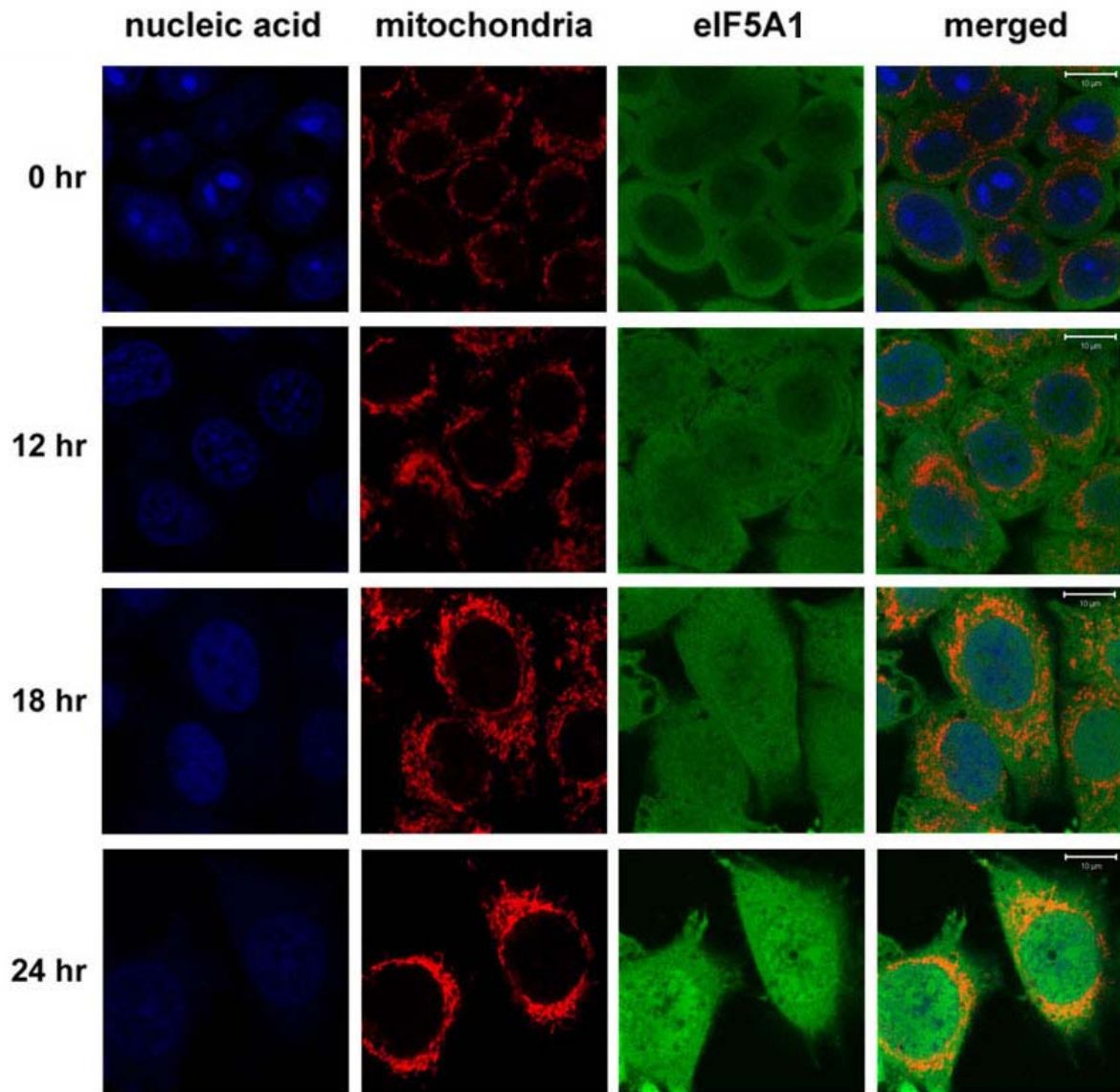
Chapter 4 Discussion

4.1 eIF5A1 is not required for mammalian cell growth

Although eIF5A was originally proposed to be a general translation initiation factor (Kemper et al., 1976; Benne et al., 1978; Schnier, et al., 1991), subsequent gene deletion experiments have indicated that it may not be required for global translation (Kang and Hershey, 1994; Zuk and Jacobson, 1998). Rather, based on the findings that eIF5A and its hypusine modification are required for growth of yeast (Schnier et al., 1991; Kang and Hershey, 1994; Sasaki et al., 1996; Park et al., 1998) and that inhibition of hypusine formation in mammalian cells causes growth arrest (Park et al., 1993b; Hanauske-Abel et al., 1994; Chen et al., 1996; Shi et al., 1996; Tome and Gerner, 1997), it has been suggested that eIF5A might be responsible for the translation of a subset of genes required for growth and proliferation (Kang and Hershey, 1994). However, in the present study when eIF5A1 siRNA was used to suppress the expression of eIF5A1 in human colon adenocarcinoma HT-29 cells and human cervical adenocarcinoma Hela S3 cells, cell growth was not affected.

Two methods were used to examine the effect of eIF5A1 siRNA on cell growth. One of them employed XTT, which measures cell viability by quantifying mitochondrial activity and, indirectly, reflects cell growth. In the second method, cell growth was scored using BrdU, which provides a measure of DNA synthesis commensurate with growth. Data from both assays indicated that inhibiting the expression of eIF5A1 by ~90% using eIF5A1 siRNA has no effect on mammalian cell growth. Further evidence supporting this contention came from experiments showing that although inhibition of hypusine formation using GC7 induces cell cycle arrest at the G1 phase, suppression of eIF5A1 using specific siRNA has no effect on cell cycle progression. GC7 is an inhibitor of DHS, the enzyme that mediates the first step leading to hypusination of eIF5A, and has been shown previously to induce G1 arrest (Park et al., 1993a & 1994; Shi et al., 1996b; Jansson et al., 2000). However, the findings of the present study raise the very real possibility that disruption of the cell cycle in the presence of CG7 is attributable to an effect of GC7 other than its ability to inhibit the formation of hypusinated eIF5A.

RNA interference (RNAi), which is an evolutionarily conserved process of gene silencing, has become a powerful tool for investigating gene function by reverse genetics (Reviewed by Caplen et al., 2001; Tuschl., 2001; Kawasaki et al., 2005). The eIF5A1 siRNA used in the present study proved to be a highly efficient suppressor of translation, reducing the level of eIF5A1 protein by up to

90%. However, since eIF5A1 is an abundant protein with a long half life (Benne et al., 1978; Gerner 1986; Nishimura et al., 2005), it is possible that even the remaining 10% of eIF5A1 protein is sufficient to guarantee its full function in the cells. Furthermore, transfection with synthesized siRNA oligonucleotides or siRNA-encoding plasmids to induce RNAi in mammalian cells only provides short-term gene silencing (Hao et al., 2005). Indeed, it has been suggested that RNAi is not equivalent to, and cannot be used as a replacement for, gene knockout approaches (McCaffrey and Kay, 2003). Thus, although the siRNA results obtained in the present study strongly indicate that eIF5A1 is not required for cell division, additional studies with an eIF5A1 gene knock-out cell line would provide important confirmation of this contention.

4.2 eIF5A1 is involved in the induction of apoptosis

Although suppression of eIF5A1 expression using siRNA had no discernible effect on cell proliferation, there was a pronounced inhibitory effect on apoptosis. This finding is in keeping with previous studies which, on the basis of different experimental approaches, also suggest an involvement of eIF5A in apoptosis (Caraglia et al., 1997, 1999, 2001 & 2003; Takeuchi et al., 2002; Jin et al., 2003; Li et al., 2004; Taylor et al., 2004 & 2007). In the present study, the efficacy of eIF5A1 siRNA as an inhibitor of apoptosis was tested using a variety of pro-apoptotic stimuli including sodium nitroprusside (SNP), Actinomycin D, a proteasome inhibitor and serum depletion, which induce programmed cell death in different ways. In every case, suppression of eIF5A1 expression provided considerable protection against the induction of programmed cell death.

SNP [$Na_2[Fe(CN)_5NO]_2H_2O$] is a "spontaneous" NO donor in that it releases NO (and NO^+) in aqueous solvents in the absence of enzymatic reduction or hydrolysis (Yamamoto and Bing, 2000; Al-Sa'doni and Ferro, 2006). However, degradation of SNP generates cyanide (CN^-) and free iron as well, which are also biologically active (Ramakrishna Rao and Cederbaum, 1996). Thus as noted previously (Kim et al., 2006), SNP should not be regarded simply as a NO donor. It is well established that, when used at mM concentration, SNP induces apoptosis in a variety of cell lines (Cui et al., 1994; Sumitani et al., 1997; Tsi et al., 2001; Olivier et al., 2005; Kwak et al., 2006; Gui et al., 2007; Wang et al., 2007). The fact that SNP is capable of triggering apoptosis is consistent with the ability of two of its products, iron and NO, to generate reactive oxygen and reactive nitrogen species (ROS and RNS), respectively, both of which induce DNA damage. Free iron released by SNP induces the formation of ROS through iron-catalyzed free radical formation known as the Fenton reaction

143

(Schroder, 2006). ROS are in turn capable of damaging several cellular components including DNA, lipids and proteins, thereby engendering both cytotoxic and genotoxic effects (Reviewed by Blokhina et al., 2003; Valko et al., 2005). The NO released from SNP not only directly damages cellular components, but also reacts with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) which is highly toxic (Wink et al., 1991; Liu and Hotchkiss, 1995). Although these RNS are capable of modifying multiple cellular signaling pathways through reversible S-nitrosation reactions (Reviewed by Akaike, 2000; Miersch and Mutus, 2005), they can also induce genotoxicity by causing direct DNA damage such as single strand breaks (Reviewed by Felley-Bosco, 1998). Through the induction of DNA damage, NO indirectly stimulates expression and accumulation of the tumor suppressor, p53, which is responsible for the transactivation of Bax and cyclin-dependent kinase inhibitor, p21, as well as the transrepression of Bcl-2 (Brockhaus and Brune, 1999; Pinsky et al., 1999; Kolb, 2000; Li et al., 2002) and thus serves as a strong inducer of apoptosis. SNP also activates the cAMP-PKA pathway, which is linked to the MAPK pathway, especially ERK1/2 and JNK (Kim et al., 2006).

The finding in the present study that eIF5A1 siRNA reduces SNP-induced cell death implicates eIF5A1 in ROS- and RNS-induced cytotoxicity and genotoxicity. It also complements a previous report from our laboratory that eIF5A1 is up-regulated during SNP-induced apoptosis in human colorectal cancer RKO cells (Taylor et al., 2007). Since eIF5A1 is upregulated in a p53-dependent manner in response to DNA damage and over-expression of eIF5A1 induces up-regulation of p53 and p53-dependent apoptosis, it has been suggested that eIF5A is involved in the p53-dependent apoptotic pathway (Li et al., 2004; Rahman-Roblick et al., 2007). At face value, this suggests that eIF5A1 involvement in SNP-induced apoptosis is through p53. However, the cell line, HT-29, used in these experiments only has mutant, non-functional p53, and thus apoptosis of HT-29 cells can only be p53-independent (Rodrigues et al., 1990; Violette et al., 2002). It seems clear, therefore, that the involvement of eIF5A1 in SNP-induced apoptosis is independent of p53. Indeed, it is known that NO can inhibit cell growth and induce apoptosis in p53-null cell lines (Messmer and Brune, 1996; Kawahara et al., 2001; Chao et al., 2004), and that ROS and RNS derived from SNP can directly induce mitochondria-mediated apoptosis through their inhibitory effect on respiration leading to reduction of ΔΨm, PT pore opening and the release of cytochrome *c* (Reviewed by Boyd and Cadenas, 2002; Brookes and Darley-Usmar, 2000; Moncada and Erusalimsky, 2002). In addition, NO induces release of ER Ca^{2+} resulting in ER stress and apoptosis through the induction of CHOP/GADD153, a C/EBP family transcription factor involved in the ER stress pathway (Kawahara et al., 2001; Oyadomari et al. 2001; Gotoh et al., 2002). Therefore, both mitochondrial dysfunction

144

and activation of the ER stress-CHOP pathway may account for p53-independent NO-induced cell death (Brune, 2003), and it is conceivable that eIF5A1 is involved in either or both of these apoptotic pathways.

The finding in the present study that eIF5A1 siRNA inhibits Actinomycin D-induced apoptosis in HT-29 cells in which p53 is defective further supports the contention that eIF5A1 protein is involved in mediating p53-independent apoptosis. Actinomycin D is an antineoplastic antibiotic that binds to DNA, inhibiting transcription and DNA replication (Guy and Taylor, 1978; Sobell, 1985) as well as inducing chromosomal aberrations and DNA strand breaks (Cherry and Hsu, 1982). Indeed, treatment with Actinomycin D is known to up-regulate p53 (Kastan et al., 1991) as well as enhance its DNA binding activity (Tishler et al., 1993). However, Actinomycin D also induces up-regulation of Bax, activation of the JNK pathway and apoptosis through a p53-independent mechanism (Kleeff et al., 2000). Furthermore, low concentrations of Actinomycin D can potentiate TRAIL-, Fas- and TNF-induced apoptosis, although this effect is cell type-dependent (Itoh and Nagata, 1993; Mori et al., 1999; Kim et al., 2002 & 2003; Park et al., 2003). It has been shown in another study from our laboratory that Actinomycin D induces apoptosis coincident with up-regulation of eIF5A1 and p53 in the human colorectal cancer cell line, RKO, which contains functional wild-type p53, and that siRNA-induced suppression of eIF5A1 inhibited Actinomycin D-induced up-regulation of p53 (Taylor et al., 2007). These observations collectively implicate eIF5A1 in Actinomycin D-induced apoptosis that is both p53-dependent and p53-independent.

The proteasome inhibitor, MG-132, which was also used as a pro-apoptotic agent in the present study, blocks the ubiquitin-proteasome (UP) pathway, the route by which a large number of proteins, including those involved in apoptosis, are degraded (Reviewed by Glickman and Ciechanover, 2002). Indeed, the use of proteasome inhibitors has recently emerged as an effective anticancer strategy since cancer cells are much more sensitive to proteasome inhibition than normal cells (Reviewed by Kisselev and Goldberg, 2001; Adams, 2002; Montagut et al., 2006). MG-132 not only induces cell cycle arrest at the G2/M interface, but also activates caspase 3, a key agent of apoptosis (Fujita et al., 1996; Drexler, 1997; Kitagawa et al., 1999; Wagenknecht et al., 1999; An et al., 2000; Naujokat et al., 2000; MacLaren et al., 2001; Sassone et al., 2006). In more general terms, MG-132 increases the lifespan of short-lived proteins which are often toxic and blocks the modification of proteins involved in signaling pathways, resulting in changes that inhibit cell survival and promote apoptosis (Reviewed by Drexler, 1998; Orlowski, 1999; Yang and Yu, 2003; Zhang et al., 2004; Vlahakis and Badley, 2006). The levels of p53, for example, are generally quite low in

normal cells due to its well-regulated degradation by the 26S proteasome (Chowdary et al., 1994; Meki et al., 1996), MG-132-induced inhibition of the 26S proteasome results in accumulation of p53 with all of its attendant downstream pro-apoptotic effects including transcriptional activation of Puma and Bim, and translocation of Bax and cytochrome *c* (Kitagawa et al., 1999; Lopes et al., 1997; MacLaren et al., 2001; Concannon et al., 2007; Ding et al., 2007). However, there is also evidence that UP inhibitors such as MG-132 are able to induce apoptosis through a p53-indendent pathway (An et al., 1998; Herrmann et al., 1998; Wagenknecht et al., 1999), possibly through the formation of ROS which then activate the mitochondrial apoptotic pathway leading to activation of the caspase cascade (Emanuele et al., 2002; Wu et al., 2002; Ling et al., 2003; Fribley et al., 2004; Yu et al., 2004; Lee et al., 2005; Nawrocki et al., 2005; Perez-Galan et al., 2006). The mechanism underlying ROS generation induced by UP inhibitors is not clear, but it has been proposed that they may do so by enhancing the ROS generating capability of mitochondria (Ling et al., 2003). In addition, however, UP inhibitors have been shown to engender the accumulation of misfolded proteins in the ER, thereby activating the ER stress pathway which not only initiates the caspase cascade directly, but also generates ROS leading to release of Ca^{2+} and a consequent activation of the mitochondrial apoptotic pathway (Fribley et al., 2004; Nawrocki et al., 2005; Perez-Galan et al., 2006). Inhibiting the UP pathway also decreases turnover of the pro-apoptotic proteins, Bim and Bik (Nikrad et al., 2005), as well as inhibitory κB (IκB) which inhibits the translocation and activation of nuclear factor kappa B (NF-κB), a key survival transcription activator (Liu et al., 1996; Maniatis, 1999; Chen et al., 2002). Therefore, treatment with a UP inhibitor such as MG-132 not only induces the accumulation of pro-apoptotic molecules, but also inhibits the pro-survival pathway (Liu et al., 1996; Maniatis, 1999; Chen et al., 2002; Nikrad et al., 2005).

The finding in the present study that eIF5A1 siRNA inhibits the induction of MG-132-induced apoptosis of HT-29 cells supports the contention that eIF5A1 is a key element of programmed cell death. It is also consistent with a previous report that treatment of Mo7e cells, a leukemia cell line, with UP inhibitors induces up-regulation of eIF5A1 (Jin et al., 2003). Interestingly, the protective effect of eIF5A1 siRNA against MG-132-induced apoptosis proved to be stronger at 72 hours after transfection than at 48 hours. This is consistent with the fact that the cellular eIF5A1 protein level is lower at 72 hour after transfection and suggests a correlation between the amount of eIF5A1 protein and the extent of apoptosis. Furthermore, cells transfected with eIF5A1 siRNA and treated with MG-132 for 24 hours had higher eIF5A1 protein levels than corresponding cells cultured in the absence of MG-132, indicating that eIF5A1 is degraded through the UP pathway.

Finally, eIF5A1 siRNA was also shown to inhibit cell death attributable to stress stemming from serum starvation. Serum, which is a commonly used supplement to cell culture media, contains a broad spectrum of molecules including hormones, growth factors, low-molecular-weight nutrients, carrier proteins for lipoid substances, trace elements as well as attachment and spreading factors (Reviewed by Griffiths, 1987). It has been shown that withdrawal of stimulatory growth factors present in serum, many of which play dual roles as mitogens and survival factors, induces cell cycle arrest and apoptosis in several cell types *in vitro* (Galli and Fratelli, 1993). Serum starvation causes cells to round-up leading to detachment and death and, at the molecular level, induces up-regulation of p53 (Koo et al., 2002). Indeed, it has been suggested that in the event of serum starvation p53 plays an essential role in the induction of G1 cell cycle arrest and in the activation of caspases 3, 6 and 12, key mediators of apoptosis (Kilic et al., 2002; Oya et al., 2003; You et al., 2006). However, serum deprivation also induces apoptosis in a p53-independent manner (Pützera et al., 2000; Takamatsu et al., 2001).

In the present study, untransfected HT-29 cells and HT-29 cells transfected with control siRNA exhibited typical nutrient stress symptoms when subjected to serum starvation including rounding-up and cessation of growth. By contrast, HT-29 cells transfected with eIF5A1 siRNA displayed strong resistance to serum starvation-induced stress. They continued to grow and divide normally, and were morphologically indistinguishable from cells cultured in media containing normal levels of FBS. Indeed, the finding that eIF5A1 siRNA inhibits the pro-apoptotic effects of serum starvation as well as apoptosis induced by SNP, Actinomycin D and MG-132 suggests that eIF5A1 protein is a key apoptotic regulator.

4.3 Molecular basis for the involvement of eIF5A in apoptosis

4.3.1 Over-expression of eIF5A induces p53-independent apoptosis

That suppression of eIF5A1 in HT-29 cells, which do not contain functional p53, inhibited cell death induced by serum deprivation as well as several pro-apoptotic agents suggests that eIF5A1 is able to respond to a number of apoptotic stimuli and to induce cell death in a p53-independent manner. To further test this concept, the effects of eIF5A1 over-expression on apoptosis were examined using adenovirus as an expression vehicle. Adenovirus constructs bearing wild-type eIF5A1 (Ad-eIF5A1), mutant eIF5A1 with lysine$_{50}$ switched to alanine which abolishes hypusine

modification [Ad-eIF5A(K50A)] and wild-type eIF5A2 (Ad-eIF5A2) were used for this purpose. The Ad-eIF5A(K50A) enabled assessment of whether hypusination of eIF5A1 is a prerequisite for its pro-apoptotic activity. Ad-lacZ was used as a negative control. Interestingly, Ad-eIF5A1, Ad-eIF5A1(K50A) and Ad-eIF5A2 all induced apoptosis in a time- and dose-dependent manner in HT-29 colon cancer cells and in Hela S3 cervical cancer cells. Moreover, neither of these cell lines have active p53. HT-29 cells contain mutant, non-functional p53 (Rodrigues et al., 1990; Violette et al., 2002), and Hela S3 cells are phenotypically null for p53 due to the presence of the HPV E6 oncogene (Scheffner et al., 1990 & 1991; Liang et al., 1993 & 1995; Thomas et al., 1996; Song et al., 1998; Talis et al., 1998).

Apoptosis is characterized by internucleosomal cleavage of DNA into fragments of 180-200 bp as a result of endonuclease activity as well as chromatin condensation, reduction of cell volume and formation of apoptotic bodies (Reviewed by Arends and Wyllie, 1991; Schwartzman and Cidlowski, 1993; Häcker, 2000). In this study, two methods were used to measure different aspects of apoptosis induced by Ad-eIF5A. One of these, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick-End Labeling (TUNEL) method, labels cells in which DNA fragmentation, a late event in apoptosis, has occurred (Gorczyca et al., 1993; Kasagi et al., 1994; Collins et al., 1997). Based on TUNEL assay results, it was clear that Ad-eIF5A1, Ad-eIF5A1(K50A) and Ad-eIF5A2 all strongly induce DNA fragmentation in HT-29 cells within 48 hour of infection. However, it has been suggested that DNA fragmentation also occurs during necrosis in a random instead of internucleosomal pattern (Gold et al., 1994; Mundle et al., 1995). Accordingly, detection of fragmented DNA using the TUNEL assay may fail to discriminate between necrosis and apoptosis (Grasl-Kraupp et al., 1995; Mundle et al., 1995). Furthermore, it has been shown that the morphological features of apoptosis can occur in the absence of internucleosomal DNA fragmentation (Falcieri et al., 1993; Cohen et al., 1992). Thus, using only TUNEL to measure apoptosis may not be sufficient.

To address this concern, the Annexin V/PI method was used to confirm that cells infected with different types of Ad-eIF5A constructs are undergoing apoptosis. During the course of apoptosis, phosphatidylserine, which is normally confined to the inner plasma membrane leaflet, is translocated to the outer leaflet of the plasma membrane (Martin et al., 1995b). This externalization of phosphatidylserine is an early signature of apoptosis that occurs before loss of cell membrane integrity and nuclear changes (Koopman et al., 1994; Martin et al., 1995). It can be detected using Annexin V, which binds to phosphatidylserine with high affinity (Andree et al., 1990; Koopman et

148

al., 1994). In the same assay, propidium iodide (PI), a cell-impermeable nucleic acid dye, is also used to distinguish between cells with intact and permeable plasma membranes (Taylor, 1980). As the cell membrane becomes increasingly permeable during the later stages of apoptosis, PI can readily move across the cell membrane and bind to cellular DNA, providing a means of identifying cells which have lost membrane integrity (Vermes et al., 1997; Herault et al., 1999). Thus, when cells are stained with fluorescein labeled Annexin V and PI and analyzed by flow cytometry, it is possible to distinguish viable cells, early stage-apoptotic cells, late stage-apoptotic cells and dead cells (Vermes et al., 1997; Herault et al., 1999). When Hela S3 cells were assayed by Annexin V/PI staining at different times after infection with different adenovirus constructs, it was apparent that for all of the constructs apoptosis was not initiated within the first 24 hours of infection. However, by 48 hours after infection 20 to 40 percent of the cells were at an early or late stage of apoptosis, and after an additional 24 hours most of the cells were dead. Thus, the Annexin V/PI data were consistent with the TUNEL results in indicating that within 48 hours of infection with Ad-eIF5A apoptosis had been initiated, and that it progressed in a time-dependent manner.

The cytotoxic effect of eIF5A on both HT-29 and Hela S3 cells was also measured using the XTT assay and Trypan blue staining. Consistent with what was observed using the TUNNEL assay and Annexin V/PI staining, both of these assays indicated a dramatic time-dependent decrease in the viability of cells over-expressing eIF5A. With Trypan blue, for example, which was used to distinguish cells containing intact and permeable plasma membrane, cells infected with adenovirus eIF5A constructs were still viable (intact plasma membrane) at 24 hours after infection, but between 24 and 48 hours viability started to decline, and by 72 hours most of the cells contained ruptured plasma membranes.

These findings collectively indicate that eIF5A1 and eIF5A2 are both capable of inducing apoptosis. Moreover, at least for HT-29 cells and Hela S3 cells, they do so through a p53-independent mechanism inasmuch as neither of these cell lines contain functional p53. However, these observations do not preclude the possibility that eIF5A is also able to effect apoptosis by working through p53. Indeed, other studies have demonstrated that the expression of p53 is regulated by eIF5A (Li et al., 2004; Taylor et al., 2007). As well, the finding that Ad-eIF5A1 and Ad-eIF5A1(K50A) proved to be essentially equivalent inducers of apoptosis in HT-29 and Hela S3 cells constitutes strong evidence that post-translational conversion of lysine$_{50}$ in eIF5A to either deoxyhypusine or hypusine is not required for the pro-apoptotic activity of this protein.

4.3.2 eIF5A activates caspases involved in apoptosis

The signaling events leading to apoptosis in effect comprise two major pathways, one involving death receptors (extrinsic pathway) and the other, changes in mitochondria (intrinsic pathway). The death receptor pathway is initiated upon the binding of extracellular death ligands to death receptors on the plasma membrane, leading to the formation of DISC that recruits and activates caspase 8 (Reviewed by Nagata, 1997; Ashkenazi and Dixit, 1998 & 1999). Caspase 8 then either directly cleaves and activates the effector caspases 3, 6, and 7 or induces mitochondrial dysfunction by generating the active BH3-only pro-apoptotic protein, tBid (Li et al., 1998; Luo et al., 1998). The mitochondrial pathway is activated by various cellular stresses through the regulation of Bcl-2 family proteins. Translocation of pro-apoptotic Bcl-2 family proteins to mitochondria induces permeabilization of the mitochondrial outer membrane and a consequent release of cytochrome *c* (Reviewed by Adams and Cory, 2001). Cytochrome *c* associates with Apaf-1 in the presence of ATP/dATP, forming the apoptosome which recruits and activates caspase 9. Caspase 9 in turn activates the effector caspases 3, 6 and 7 (Li et al., 1997; Hu et al., 1999; Rodriguez and Lazebnik, 1999).

The finding in the present study that over-expression of eIF5A1, eIF5A1(K50A) or eIF5A2 induces activation of the initiator caspases 8 and 9 as well as the effector caspase 3, all of which underpin the execution of apoptosis, indicates that eIF5A plays a central role in the induction of programmed cell death. Moreover, the activation of caspases by eIF5A proved to be time-dependent. Within 48 hours of infection with Ad-eIF5A, about 30% of infected cells exhibited activated caspases 3, 8, and 9, and by 72 hours the proportion of infected cells with activated caspases had increased to ~70%. Thus, at some point between 24 and 48 hours after infection, apoptosis signaling was initiated by the over-expression of eIF5A through a yet unknown mechanism, leading to the activation of death receptor pathway caspase 8 and mitochondrial pathway caspase 9, as well the effector caspase 3 which functions in both pathways. The key apoptotic indices including phosphatidylserine externalization, mitochondrial dysfunction, DNA fragmentation and plasma membrane permeabilization which were measured in the present study are known to be directly or indirectly attributable to the action of capases (Reviewed by Arends et al., 1990; Arends and Wyllie, 1991; Dragovich et al., 1998; Häcker, 2000; Hengartner, 2000).

4.3.3 eIF5A activates the mitochondrial pathway of apoptosis

4.3.3.1 Dissipation of mitochondrial ΔΨm and permeabilization of the mitochondrial outer membrane

The finding that over-expression of eIF5A induces activation of caspase 9 suggests its involvement in the mitochondrial pathway of apoptosis. This was confirmed by demonstrating that eIF5A plays a role in reduction of mitochondrial ΔΨm as well as permeabilization of the outer mitochondrial membrane, both key features of apoptosis.

Opening of the permeability transition (PT) pore in the inner mitochondrial membrane at an early stage of apoptosis disrupts the electrochemical gradient across the mitochondrial inner membrane, a gradient that is critical for normal mitochondrial function (Reviewed by Green and Reed, 1998). Indeed, dissipation of mitochondrial transmembrane potential (ΔΨm) is a general and early feature of apoptosis (Vayssiere et al., 1994; Petit et al., 1994; Zamzami et al., 1995; Marchetti et al., 1996), even earlier than phosphatidylserine exposure (Castedo et al., 1996). Loss of ΔΨm can be measured using cationic, lipophilic fluorescent dyes such as DiOC6(3) (Petit et al., 1994; Zamzami et al., 1995; Waterhouse et al., 2001), which was used for this purpose in the present study. Consistent with the Annexin V/PI-portrayal of the progress of apoptosis, cells infected with adenovirus eIF5A did not start to lose ΔΨm until at least 24 hours after infection. At 48 hours, 20-40% of the cells exhibited disrupted ΔΨm, and by 72 hours the ΔΨm had dissipated in most of the infected cells.

Loss of ΔΨm in the event of apoptosis occurs concomitantly with mitochondrial outer membrane permeabilization (MOMP) and the resultant release of cytochrome *c*, Smac/DIABLO, Htra2/Omi, AIF and endonuclease G, which are normally compartmentalized in the intermembrane mitochondrial space (IMS) (Vander Heiden et al., 1997; Heiskanen et al., 1999; Du et al., 2000; Verhagen et al., 2000; Suzuki et al., 2001; Waterhouse et al., 2001; Goldstein et al., 2005; Munoz-Pinedo et al., 2006). Indeed, cytochrome *c* plays an important role in the generation of ΔΨm, which is essential for various mitochondrial functions including the production of ATP by oxidative phosphorylation (Waterhouse et al., 2001). In the present study, it was clear from Western blot analysis of Hela S3 cell fractions that some cytochrome *c* had been released from the IMS into the cytosol ~48 hours after infection with Ad-eIF5A1, which coincided temporally with the dissipation of ΔΨm. However, by 55 hours after infection there was still a large amount of cytochrome *c* associated with mitochondria in Ad-eIF5A1-infected cells, reflecting incomplete release during the early stages of apoptosis. This has been reported previously (Waterhouse et al., 2001; Ott et al., 2002). In fact, it has been shown that BH3-only protein-mediated oligomerization of Bax and Bak at the mitochondrial

151

outer membrane only results in the release of a small percentage of cytochrome *c* (about 15%) stored in the intermembrane space (Scorrano and Korsmeyer, 2003; Tikhomirov and Carpenter, 2005). For release of the remaining 85%, there appears to be a requirement for a Bak and Bax-independent inner membrane remodelling suggesting that the release of cytochrome *c* is a two-step process (Scorrano and Korsmeyer, 2003). The release of cytochrome *c* observed following up-regulation of eIF5A in the present study did not appear to be biphasic, but the time required for complete release relative to the dissipation of ΔΨm is consistent with a two step process.

4.3.3.2 Translocation of Bax

Further evidence supporting the contention that eIF5A is involved in activating the mitochondrial pathway of apoptosis came from experiments demonstrating complete translocation of Bax from the cytosol to a membrane fraction containing mitochondria when Hela S3 human cervical cancer cells were infected with Ad-eIF5A1. In normal healthy cells, Bax resides primarily in the cytosol, with only a small portion loosely attached to the mitochondrial outer membrane (MOM) (Wolter et al., 1997; Suzuki et al., 2000; Schinzel et al., 2004). In response to stress signals that induce apoptosis, Bax undergoes a conformational change resulting in its self-oligomerization and insertion into the MOM (Wolter et al., 1997; Murphy et al., 1999; Putcha et al., 1999; Antonsson et al., 2001; Shou et al., 2003; Schinzel et al., 2004). This intracellular redistribution of Bax is thought to be an early feature of apoptosis since it precedes the release of cytochrome *c* and is coincident with loss of mitochondrial respiratory activity (Wolter et al., 1997; Murphy et al., 1999). Once localized on the MOM, Bax either interacts with components of the PT pore complex to promote opening of the pore and release of cytochrome *c* (Marzo et al., 1998; Narita et al., 1998; Shimizu et al., 1999), or induces the release of cytochrome *c* independently of the PT pore by forming a channel in the MOM (Eskes et al., 1998; Jurgensmeier et al., 1998). In the present study, although over-expression of eIF5A1 did not induce up-regulation of Bax, it did trigger the cytosol-to-mitochondria translocation of Bax, which is known to be both essential and sufficient for the activation of downstream signaling cascades inherent to apoptosis (Ghatan et al., 2000; He et al., 2000; Antonsson et al., 2001; Melino et al., 2004). Moreover, the translocation of Bax in response to up-regulation of eIF5A1 preceded the release of cytochrome *c*, which is consistent with reports that the redistribution of Bax leads to mitochondrial outer membrane permeablization (Jurgensmeier et al. 1998; Marzo et al., 1998; Narita et al., 1998; Shimizu et al., 1999).

4.3.3.3 Up-regulation of Bim

The BH3-only protein, Bim, is also pro-apoptotic. Indeed, it is referred to as a Bcl-2 interacting mediator of cell death, and it appears to function upstream of Bax and Bak inasmuch as constitutively active Bim in cells lacking both Bax and Bak fails to induce mitochondrial alterations associated with apoptosis (Zong et al., 2001). Recent data have prompted the suggestion that Bim is involved in oncogene-induced apoptosis. First, Bim has been shown to be transcriptionally activated by the oncogene, E2F-1, in the event of E2F-1-induced apoptosis (Hershko and Ginsberg, 2004; Zhao et al., 2004; Biswas et al., 2005). Second, Bim also mediates apoptosis induced by the oncogene, Myc, and loss of Bim has been shown to facilitate Myc-induced tumorigenesis (Egle et al., 2004; Hemann et al., 2005).

In keeping with the finding that eIF5A1 appears to regulate the translocation of Bax to mitochondria, enhanced expression of eIF5A1 in Hela S3 cells also induced up-regulation of Bim. The pro-apoptotic activity of Bim is regulated by association with Bcl-2-like proteins (O'Connor et al., 1998). There are three isoforms of Bim generated by alternative splicing: Bim_{EL}, Bim_L and Bim_S which is sometimes undetectable (O'Connor et al., 1998). Bim has been shown to be involved in apoptosis induced by several stimuli, including DNA damage, death receptor ligation, growth factor or cytokine deprivation and paclitaxel, an anticancer agent that interferes with microtubule dynamics (Bouillet et al., 1999; Putcha et al., 2001; Shinjyo et al., 2001; Biswas and Greene, 2002; Dijkers et al., 2002; Nijhawan et al., 2003; Opferman et al., 2003; Sunters et al., 2003; Zong et al., 2003; Chen and Zhou, 2004; Wang et al., 2004; Gomez-Bougie et al., 2005; Tan et al., 2005; Han et al., 2004, 2005 & 2006). Although all isoforms are able to induce apoptosis (O'Connor et al., 1998; Terradillos et al., 2002; Wilson-Annan et al., 2003), the shortest isoform, Bim_S, is thought to be the most potent (O'Connor et al., 1998). In healthy cells, all three major Bim isoforms are strongly associated with anti-apoptotic Bcl-2 family proteins localized on the mitochondrial outer membrane including Mcl-1, Bcl-2 and Bcl-xL. In addition, there is a small amount of Bim_{EL} and Bim_L weakly interacting with LC8, a component of the microtubule-associated dynein complex (Puthalakath et al., 1999; Chen and Zhou, 2004; Zhu et al., 2004; Gomez-Bougie et al., 2005; Liu et al., 2006). Among the complexes Bim forms with Bcl-2 proteins, the Mcl-1:Bim complex appears to be the most abundant and most critical for the initiation of apoptosis (Gomez-Bougie et al., 2005). Under non-apoptotic conditions, Bim is sequestered by Mcl-1, which effectively neutralizes its pro-apoptotic activity (Opferman et al.,

153

2003; Gomez-Bougie et al., 2004 & 2005; Han et al., 2004, 2005 & 2006). However, in response to pro-apoptotic stimuli such as UV, granzyme B, melphalan or TRAIL, Mcl-1 is degraded either proteasomally or by activated caspases, and Bim is released (Nijhawan et al., 2003; Opferman et al., 2003; Zong et al., 2003; Chen and Zhou, 2004; Gomez-Bougie et al., 2005; Han et al., 2004, 2005 & 2006). Released Bim is then thought to mediate apoptosis by inducing Bax activation (Gomez-Bougie et al., 2004; Zhu et al., 2004; Liu et al., 2006). However, since direct association between Bim and Bax has not yet been demonstrated (Gomez-Bougie et al., 2004; Zhu et al., 2004; Liu et al., 2006), it has been suggested that free Bim may exercise its pro-apoptotic function by participating in what has been called "hit and run" activation of Bax, (Wei et al., 2001; Gomez-Bougie et al., 2004; Zhu et al., 2004; Liu et al., 2006).

In addition to being sequestered by anti-apoptotic Bcl-2-like proteins, Bim is also regulated by phosphorylation. In healthy cells, Bim is phosphorylated by the ERK1/2 pathway which directly inhibits its pro-apoptotic function (Biswas and Greene, 2002) and also targets it for degradation by the ubiquitin-proteasome pathway (Weston et al., 2003; Ley et al., 2004 & 2005). In response to pro-apoptotic stimuli such as paclitaxel-treatment and growth factor or cytokine withdrawal, activation of the Forkhead transcription factor, FoxO3a, leads to elevated Bim expression coincident with dephosphorylation and activation of pre-existing Bim (Shinjyo et al., 2001; Dijkers et al., 2002; Stahl et al., 2002; Gilley et al., 2003; Sunters et al., 2003).

In the present study, up-regulation of both Bim_L and Bim_S was evident within 24 hours in Ad-eIF5A infected Hela S3 cells, and by 72 hours the up-regulation was more prominent with all three isoforms, Bim_{EL}, Bim_L and Bim_S being detectable. Bim is known not to be a p53 target (Villunger, et al., 2003). These findings are, therefore, consistent with the fact that eIF5A has been shown capable of inducing apoptosis in HT-29 cells and Hela-S3 cells, neither of which contain functional p53. Moreover, over-expression of eIF5A not only induced Bim up-regulation, but also appeared to facilitate alternative splicing of Bim, an intrinsic signal that promotes translocation of Bax and release of cytochrome *c*.

4.3.3.4 Puma, Bid and Bad

The Western blot analyses conducted in the present study indicated that Puma, Bid and Bad are not upregulated during eIF5A-induced apoptosis. This presumably reflects the fact that these proteins are p53 targets and that p53 is inactivated in Hela S3 cells (Sax et al., 2002; Villunger et al.,

2003; Melino et al., 2004; Jiang et al., 2006). In addition, however, there was no detectable cleavage of Bid in the eIF5A-up-regulated cells, which is surprising in light of the finding the eIF5A plays a role in the activation of caspase 8, the protease that cleaves Bid (Li et al., 1998; Luo et al., 1998). This is, however, consistent with previous reports indicating that activation of caspase 8 does not always correlate with strong, or even any, cleavage of Bid (Park et al., 2003; Sohn et al., 2005). Furthermore, there is evidence that the susceptibility of Bid to cleavage by caspase 8 is cell-type specific (Desagher et al., 2001; Degli Esposti et al., 2003). This finding, together with the fact that over-expression of eIF5A induces loss of mitochondrial transmembrane potential, release of cytochrome c into the cytosol and activation of caspase 9, suggests that activation of the mitochondrial apoptotic pathway by eIF5A is not initiated by Bid and activated caspase 8, but rather by up-regulation of Bim.

4.3.3.5 Cleavage of Bcl-2

Bcl-2, which is anti-apoptotic, is a ~26 kDa membrane-associated protein localized at multiple sites in the cell including the mitochondrial outer membrane, the endoplasmic reticulum (ER) and the nuclear envelope (Tsujimoto et al., 1987; Hockenbery et al., 1990; Alnemri et al., 1992). Mitochondrial Bcl-2 is known to abrogate the release of cytochrome c (Kluck et al., 1997; Adrain et al., 2001), whereas Bcl-2 on the ER and nuclear envelope functions to regulate cytosolic and nuclear calcium levels, thereby maintaining cellular calcium homeostasis (Marin et al., 1996). There have been several reports of effector caspase-mediated cleavage of Bcl-2 during apoptosis (Cheng et al., 1997; Fujita and Tsuruo, 1998; Fadeel et al., 1999; Hirotani et al., 1999). Specifically, effector caspases catalyze the cleavage of Bcl-2 at aspartate$_{34}$ resulting in loss of the N-terminal BH4 domain which is involved in the heterodimerization of Bcl-2 with Bax/Bak and thus essential for the anti-apoptotic activity of Bcl-2 (Cheng et al., 1997; Hirotani et al., 1999). Indeed, deletion of the BH4 domain by effector caspases not only inactivates the pro-survival activity of Bcl-2, but also releases a 22 to 23 kDa potent pro-apoptotic Bax-like fragment (Cheng et al., 1997). This Bax-like fragment cleaved from Bcl-2 acts as a death-agonist, inducing mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c into the cytosol, thereby amplifying the apoptotic cascade through a positive feedback loop of caspase activation (Cheng et al., 1997; Kirsch et al., 1999). Cleavage of Bcl-2 has been demonstrated in response to a variety of pro-apoptotic stimuli including Fas ligation, treatment with IL-2 or IL-3, GM-CSF withdrawal, alphavirus infection, anticancer drug

155

treatment, antimitotic agent treatment, oxidative stress and treatment with the DNA-damaging agent, cisplatin (Cheng et al., 1997; Fujita and Tsuruo, 1998; Grandgirard et al., 1998; Fadee et al., 1999; Hirotani et al., 1999; Lin et al., 2000; Del Bello et al., 2001; Fortney et al., 2002; Liang et al., 2002 & 2003; Mi et al., 2006). Moreover, HIV protease cleaves Bcl-2 at phenylalanine$_{112}$, yielding a 12-14 kDa proteolytic fragment which is also capable of inducing apoptosis (Strack et al., 1996).

The transcriptional activity of Bcl-2 has been reported to be very low in Hela cells resulting in low levels of Bcl-2 protein (Cleary et al., 1986; Liang et al., 1995; Liang et al., 1995; Odin et al., 2001; Hara et al., 2005). This was confirmed in the present study. However, over-expression of eIF5A2, but not eIF5A1 or eIF5A1(K50A), in Hela S3 cells resulted in the formation of a Bcl-2 protein distinguishable on the basis of its size (19-20 kDa) from the cleavage products of Bcl-2 formed by effector caspases and HIV protease. This cleaved Bcl-2 was clearly discernible within 24 hours of Ad-eIF5A2 infection, and was strongly up-regulated between 24 and 72 hours after infection. Previous reports have indicated that this 19-20 kDa Bcl-2-derived cleavage product is formed by a novel non-caspase cysteine protease acting at aspartate$_{64}$ of Bcl-2 (Yamamoto et al., 1998; Hoetelmans et al., 2003). The action of this protease thus removes the N-terminus of the Bcl-2 protein, which contains the BH4 domain, thereby inactivating the ability of Bcl-2 to suppress apoptosis (Yamamoto et al., 1998; Hoetelmans et al., 2003). Moreover, the resultant 19-20 kDa Bcl-2 fragment has been shown previously to be up-regulated following the induction of apoptosis and is thought to amplify the cell death program (Yamamoto et al., 1998). Of note with respect to the present study is that formation of this 19-20 kDa Bcl-2 fragment in Ad-eIF5A2-infected cells is accompanied by the release of mitochondrial cytochrome c, disruption of $\Delta\Psi$m and activation of caspases, which is consistent with the contention that removal of the BH4 domain of Bcl-2 abolishes its anti-apoptotic activity. In addition, however, the 19-20 kDa product of Bcl-2 cleavage proved to be strongly up-regulated during Ad-eIF5A2-induced apoptosis, to much higher amounts than the level of uncleaved Bcl-2 in untreated cells. This can be interpreted as indicating that in response to eIF5A2 up-regulation Hela S3 cells over-express Bcl-2 leading to enhanced levels of its 19-20 kDa cleavage product which in turn performs a pro-apoptotic function. It is not clear why Ad-eIF5A2 induces the over-expression of cleaved Bcl-2, whereas Ad-eIF5A1 and Ad-eIF5A1(K50A) do not.

4.3.4 eIF5A up-regulates p73

Hela cells, as well as many other human cervical carcinoma cell lines that are positive for high risk human papillomaviruses (HPV), contain inactive p53 due to the presence of HPV-encoded E6 (Scheffner et al., 1991). E6, together with E6-associated protein factor (E6AP) which is a cellular ubiquitin-protein ligase, binds p53. This not only ultimately results in p53 degradation through the ubiquitin-proteasome pathway, but also prevents p53 from entering the nucleus of cells to perform its tumor suppressor activity (Scheffner et al., 1990; Liang et al., 1993 & 1995; Thomas et al., 1996; Song et al., 1998; Talis et al., 1998). E6 has been shown to overcome p53-mediated apoptosis (Thomas et al., 1996) and also to suppress p53 induction by ionizing radiation (Song et al., 1998). Indeed, E6/E6AP-mediated p53 degradation is believed to be the major mechanism leading to cervical carcinomas associated with high risk HPVs (Mantovani and Banks, 2001). Accordingly, even though the Hela cell line contains wild-type p53, it is considered as a p53 null cell line. However, treatments of Hela cells that either prevent the association of E6 with p53 or repress the expression of E6 have been shown to induce up-regulation and nuclear accumulation of p53 and ensuing cell cycle arrest or apoptosis (Koivusalo et al., 2002; Wesierska-Gadek et al., 2002; Wei et al., 2005; Zhang et al., 2005).

In the present study, the lysates of cells infected with adenovirus-eIF5A constructs were fractionated by SDS-PAGE and examined by Western blot analysis using polyclonal p53 antibody. In keeping with the fact that these cells contain active E6/E6AP, there was little or no up-regulation of p53 in Hela S3 cells undergoing Ad-eIF5A-induced apoptosis. However, there was prominent over-expression of a protein with a MW of ~70-80 kDa which, because it cross-reacted with the p53-specfic antibody, is deemed to be p73, a p53-related protein that shares a high degree of sequence and structural homology with p53 (Jost et al., 1997; Kaghad et al., 1997). The homology between p53 and p73 is particularly extensive within the most conserved p53 domains. Indeed, p73 can be roughly divided into three domains: (1) the N-terminal transactivation domain (TAD) (29% identity between p53 and p73); (2) the sequence-specific DNA binding domain (DBD) (63% identity between p53 and p73); and (3) the oligomerization domain (OD) (42% identity between p53 and p73) (Kaghad et al., 1997). The high homology in the DBD between p73 and p53 has been interpreted as indicating that they bind to the same DNA sequences and transactivate the same genes (Levrero et al., 2000). Indeed, p73 has been shown to transactivate a number of p53 target genes including p21, p27, GADD45, 14-3-3σ, cyclin G, insulin-like growth factor-binding protein 3, Bax and Puma (Zhu et al., 1998; Melino et al., 2002). Although p73 and p53 have a number of target genes in common, p73 exhibits promoter

157

selectivity and has a number of unique target genes which are involved in cell cycle arrest, DNA repair or apoptosis and are not regulated by p53 (Fontemaggi et al., 2002). Thus, p73 is able to induce cell cycle arrest and apoptosis in response to genotoxic stress irrespective of the p53 status (Jost et al., 1997; Melino et al., 2002 & 2004).

Like p53, p73 is able to induce apoptosis through multiple mechanisms (Reviewed by Rossi et al., 2004; Ramadan et al., 2005). First, p73 can activate the mitochondrial apoptotic pathway via Puma transactivation and Bax translocation (Melino et al., 2004). Second, p73 can activate the death receptor pathway by transactivating Fas, thereby up-regulating its expression at the cell surface (Terrasson et al., 2005). In addition, p73 is able to induce ER stress by transcriptionally activating Scotin (Terrinoni et al., 2004), an ER protein that can induce apoptosis (Bourdon et al., 2002).

The molecular mechanism underlying the regulation of p73 activity is still poorly understood. There are reports that p73 is involved in DNA damage-induced apoptosis. However, it is only activated in response to ionizing-radiation and cisplatin, treatments that cross-link DNA (Agami et al., 1999; Gong et al., 1999; Yuan et al., 1999), and exhibits no response to treatment with Actinomycin D or methylmethane sulfonate or to UV irradiation (Kaghad et al., 1997; Levrero et al., 1999). There is evidence that when p73 is involved in DNA damage-induced apoptosis, it becomes phosphorylated and/or acetylated by modifying enzymes that include c-Abl tyrosine kinase, the mismatch repair protein, PMS2, p300 acetyltransferase, p38 stress-activated protein kinase and Yes-associated protein 1 (Yap1) (Gong et al., 1999; Agami et al., 1999; Yuan et al., 1999; Costanzo et al., 2002; Sanchez-Prieto et al., 2002; Shimodaira et al., 2003; Levy et al., 2007). These DNA damage-induced interactions between p73 and these proteins are thought to stabilize p73 and enhance its apoptotic activity.

p73 can also be activated by oncogenes through E2F1, the best-characterized member of the E2F family of transcription factors. E2F1 plays an essential role in cell cycle progression by regulating a large group of genes involved in G1/S transition including cyclin E, cdc25A and DNA polymerase α (Johnson et al., 1993; Sala et al., 1994; DeGregori et al., 1995; Ohtani et al., 1995). E2F1 is also able to induce apoptosis (Reviewed by Phillips and Vousden, 2001; Ginsberg, 2002; Pützer, 2007). Deregulation of E2F1 by mitogenic oncogenes including Ras, Myc, v-Abl and E1A triggers p53-dependent or p53-independent apoptosis, thus protecting the organism against oncogenic transformation (Qin et al., 1994; Palmero et al., 1998; Zindy et al., 1998; de Stanchina et al., 1999). In the p53-dependent pathway, E2F1 indirectly activates p53 through the tumour-suppressor, ARF, which interacts with Mdm2, thereby protecting p53 from degradation (Bates et al., 1998). Activated

158

p53 then regulates apoptosis either by transactivating pro-apoptotic molecules such as Puma, Noxa, Fas and DR5 or by directly translocating to the mitochondrial outer membrane to induce mitochondrial outer membrane permeabilization (Reviewed by Levine, 1997; El-Deiry, 1998; Marchenko et al., 2000; Sansome et al., 2001; Vousden and Lu, 2002; Sax and El-Deiry, 2003; Mihara and Moll, 2003; Erster et al., 2004; Endo et al., 2006). In the p53-independent pathway, E2F1 regulates apoptosis by inducing the expression of p73, Apaf-1, caspase proenzymes (e.g., procaspases 2, 3, 7, 8 and 9) and pro-apoptotic BH3-only proteins including Puma, Noxa, Bim, Bik and Hrk/DP5 through direct transcriptional activation (Stiewe and Putzer, 2000; Irwin et al., 2000; Furukawa et al., 2002; Nahle et al., 2002; Hershko and Ginsberg, 2004; Real et al., 2005). There are several reports on the activation of p73 expression by E2F1 or its activators including adenovirus-E1A and c-Myc (Stiewe and Putzer, 2000; Irwin et al., 2000; Lissy et al., 2000; Zaika et al., 2001; Kang et al., 2002; Seelan et al., 2002; Das et al., 2003; Matsumura et al., 2003; Flinterman et al., 2005). These data collectively indicate that induction of p73 is required for E2F1-induced apoptosis and have prompted the proposal that p73 activation by deregulated E2F1 activity might constitute a p53-independent, anti-tumorigenic safeguard mechanism (Dobbelstein et al., 2005).

The present study indicates that p73, a pro-apoptotic p53-like protein, is activated during eIF5A-induced apoptosis of Hela S3 cells. Of particular relevance to this finding is the fact that p73 is known to be functional in Hela cell lines because, unlike p53, it is not inactivated by the products of viral oncogenes such as SV40 T antigen and HPV E6 (Marin et al., 1998; Das et al., 2003a; Melino et al., 2004; Das and Somasundaram, 2006; Kim et al., 2006). In light of this, it seems reasonable to propose that up-regulation of p73 may be a key element of eIF5A1-induced signaling that invokes apoptosis. That p73 appears not to be up-regulated in Ad-eIF5A2-infected cells suggests that although eIF5A1 and eIF5A2 are both able to induce apoptosis and have many common targets of up-regulation, there are, nonetheless, distinctive elements in the signaling pathways they activate.

4.3.4.1 Possible mechanisms for the up-regulation of p73 by eIF5A1

The mechanism underlying the activation of p73 by eIF5A1 and eIF5A1(K50A) is unknown. It has been shown that p38 can directly phosphorylate p73, thereby stabilizing and activating the p73 protein (Sanchez-Prieto et al., 2002). It has also been shown that infection of A549 human lung adenocarcinoma cells with Ad-eIF5A1 induces phosphorylation of p38, thereby activating the p38

signaling pathway (Catherine Taylor, unpublished data). It is, therefore, possible that the activation of p38 by Ad-eIF5A1 is responsible for the up-regulation of p73.

A second possibility is that p73 may be transactivated by E2F1 in Ad-eIF5A1- and Ad-eIF5A1(K50A)-infected cells. Bim, another molecule that is up-regulated by Ad-eIF5A, is also a key mediator of oncogene-induced apoptosis and can be transactivated by E2F1 (Egle et al., 2004; Hershko and Ginsberg, 2004; Zhao et al., 2004; Biswas et al., 2005; Hemann et al., 2005). Therefore, E2F1 may play an important role in Ad-eIF5A1- and Ad-eIF5A1(K50A)-induced apoptosis by transactivating two key molecules, p73 and Bim, both of which are able to induce translocation of Bax and activation of the mitochondrial pathway, leading to the activation of caspase 9 and, subsequently, caspase 3 (Gomez-Bougie et al., 2004; Melino et al., 2004; Zhu et al., 2004; Liu et al., 2006). Furthermore, E2F1 can activate the p38 MAPK pathway by modulating the phosphorylation of p38 (Hershko et al., 2006), which could also account for the activation of p73 in Ad-eIF5A1-infected cells (Sanchez-Prieto et al., 2002; Catherine Taylor, unpublished data).

It is significant that Bim and p73, which are both involved in oncogene-induced apoptosis (Stiewe and Pützer, 2001; Zhao et al., 2004), are upregulated in Ad-eIF5A1- and Ad-eIF5A1(K50A)-infected Hela cells, for it raises the possibility that eIF5A1 itself may be an oncogene. Further to this, it has been shown that eIF5A1 is up-regulated not only in response to activation of the Myc oncogene *in vitro* (Coller et al., 2000; Boon et al., 2001), but also in lung adenocarcinomas compared to normal lung tissue (Chen et al., 2003). Higher levels of eIF5A1 protein are also a characteristic of lung tumors showing poor differentiation and have been associated with K-ras mutations, p53 nuclear accumulation and patients with low survival potential (Chen et al., 2003). It has already been proposed that eIF5A2, which unlike eIF5A1 is rarely expressed at detectable levels in healthy cells, is an oncogene based on its over-expression in several primary ovarian cancers and many ovarian cancer cell lines (Guan et al., 2001; Jenkins et al., 2001; Guan et al., 2004). It would appear from the present study that eIF5A1 may as well be oncogene, and that over-expression of eIF5A induces apoptosis by activating transcription factor E2F1 which in turn induces the expression of p73 and Bim, both of which are able to induce caspase activation leading to apoptosis.

The proposed oncogenic role of eIF5A is not in conflict with its pro-apoptotic function. It is well known that E2F1, which is not only an oncogene but also a tumor suppressor gene, has both positive and negative effects on tumorigenesis (Reviewed by Johnson, 2000; Bell and Ryan, 2004). There are reports that eIF5A also influences survival and death (Schnier et al., 1991; Park et al., 1993b; Hanauske-Abel et al., 1994; Kang and Hershey, 1994; Chen et al., 1996; Sasaki et al., 1996;

Shi et al., 1996; Caraglia et al., 1997, 1999, 2001 & 2003; Tome and Gerner, 1997; Park et al., 1998; Takeuchi et al., 2002; Jin et al., 2003; Li et al., 2004; Taylor et al., 2004 & 2007), which may be due to the fact that pathways controlling cell proliferation and apoptosis are coupled (Harrington et al., 1994). Thus eIF5A may have both oncogenic and tumor-suppressive properties, although how these two opposing functions are regulated remains to be elucidated.

4.3.4.1.1 Possible interaction among eIF5A, p73 and caspase 8

Although caspase 8 activation is classically linked to the activation of receptors of the TNF receptor family, some recent data suggest that caspase 8 is not restricted to apoptosis mediated by death receptors, and that it can be activated by staurosporine, anticancer drugs, cycloheximide, Shiga toxin and γ radiation in a death receptor-independent manner (Ferrari et al., 1998; Wesselborg et al., 1999; Ding et al., 2000; Engels et al., 2000; Ferreira et al., 2000; Tang et al., 2000; Jones et al., 2001; Fujii et al., 2003; Sohn et al., 2005). Several mechanisms are involved in the FADD-independent activation of caspase 8. First, caspase 8 can be cleaved and activated by other caspases including caspases 3, 6, and 9 in the mitochondrial pathway (Slee et al., 1999; Viswanath et al., 2001; Sohn et al., 2005) and subsequently involved in the amplification of caspase-mediated cell death. This entails either cleaving Bid, thereby enhancing the mitochondrial pathway (Engels et al., 2000; Tang et al., 2000; Viswanath et al., 2001), or directly translocating into the nucleus to cleave and inactivate poly(ADP-ribose) polymerase-2 (PARP-2) (Tang et al., 2000; Benchoua et al., 2002). PARP-2 plays a critical role in the maintenance of genomic integrity by modulating DNA repair and cell survival programs (Ame et al., 1999). Second, caspase 8 can be cleaved by non-caspase proteases such as HIV-1 protease and granzyme B (Medema et al., 1997; Nie et al., 2002). Indeed, activation of caspase 8 is thought to play a central role in mediating HIV-1- or granzyme B-induced apoptosis (Medema et al., 1997; Nie et al., 2002). Third, p53 can induce activation of caspase 8. It has been shown that p53 plays an important role in FADD-independent activation of caspase 8 by facilitating the assembly of a 600 kDa caspase 8-activating complex through protein-protein interactions (Ding et al., 2000). p53 can also transactivate Fas and/or KILLER/DR5, both of which belong to the TNF receptor family (Wu et al., 1997 & 1999; Muller et al., 1998). In some cases, over-expression of these death receptors alone is sufficient for the initiation of apoptosis (Papoff et al., 1999; Shinoura et al., 2000; Sax and El-Deiry, 2003).

Since Hela S3 cells, which contain inactive p53, were used in the present study, the possibility that eIF5A-induced activation of caspase 8 is achieved through p53 would appear to be ruled out. However, p73, a p53 homolog, which is functional in Hela cells and up-regulated following infection with Ad-eIF5A, can also transactivate Fas, thereby inducing up-regulation of Fas at the cell surface (Terrasson et al., 2005). Thus, in Ad-eIF5A-infected Hela S3 cells caspase 8 might be activated either directly by the upstream caspases 9 and 3, which are activated through the Bim-initiated mitochondrial pathway, or indirectly through p73 which can up-regulate Fas expression at the cell surface. Furthermore, caspase 8 may be activated in a FADD-dependent, but death receptor-independent manner, during Ad-eIF5A-induced apoptosis.

Both caspase 8 and caspase 9 are activated during eIF5A-induced apoptosis. Since there is no cleavage of Bid, caspase 8 may not be responsible for activation of the mitochondrial pathway. However, activation of caspases 9 and 3 may be responsible for the activation of caspase 8. Indeed, proteolytic processing of caspase 8 during death receptor-independent apoptosis has been proposed to be a positive post-mitochondrial feedback loop in many cell types (Slee et al., 1999; Ding et al., 2000; Besnault et al., 2001; Tang et al., 2002; Sohn et al., 2005).

4.4 Over-expression of eIF5A induces the accumulation of lysine$_{50}$ eIF5A

The finding that both Ad-eIF5A1 and Ad-eIF5A1(K50A) are able to induce apoptosis in HT-29 and Hela S3 human cancer cell lines raises the question of whether hypusination is required for the pro-apoptotic activity of eIF5A1. eIF5A1 is an abundant protein with a MW ~18 kDa and a *pI* of 5.1-5.5 (Cooper et al., 1982). It has been demonstrated previously that three distinct forms of human eIF5A1 can be separated by 2D electrophoresis on the basis of different *pI* values (Klier and Lottspeich, 1992; Wohl et al., 1995; Takeuchi et al., 2002; Jin et al., 2003; Dong et al., 2005; Nishimura et al., 2005). These previous studies indicated that one of these, which is hypusinated at lysine$_{50}$ and is referred to as the main form, comprises ~95% of the total eIF5A in untreated cells. An intermediate form has been shown to be acetylated at lysine$_{47}$ and either deoxyhypusinated or hypusinated at lysine$_{50}$. The third acidic form is acetylated at lysine$_{47}$ and unmodified at lysine$_{50}$ (Klier et al., 1995).

In the present study, 2D Western blotting and mass spectrometry were used to isolate and identify the post-translationally modified forms of eIF5A1. The three forms previously identified, the main form, the intermediate form and the acidic form were discernible. Of particular interest,

162

however, is the finding that over-expression of eIF5A1 using either wild-type eIF5A1 or mutant eIF5A1 adenovirus constructs failed to result in an accumulation of hypusinated eIF5A. Rather, the acidic form of eIF5A1 and, to a lesser extent, the intermediate form accumulated under these conditions. This can be interpreted as indicating that the high concentration of over-expressed precursor eIF5A1 overwhelms, and essentially inhibits, endogenous DHS. Similar results have also been demonstrated in yeast and human 293T cells. Specifically, over-expressed yeast HYP2, human eIF5A1 or human eIF5A2 proteins were not stoichiometrically deoxyhypusinated or hypusinated (Wohl et al., 1995; Clement et al., 2006), prompting the view that co-expression of DHS is needed for post-translational modification of all of the over-expressed eIF5A precursor protein (Clement et al., 2006).

It was also established in the present study that adding an influenza hemagglutin (HA) tag to the N-terminus of eIF5A1 renders it incapable of serving as a substrate for DHS. In these experiments, COS-7 African green monkey kidney cells were transfected with pHM*eIF5A1* plasmid to form HA-tagged recombinant eIF5A1 and then treated with $[H^3]$-spermidine to radiolabel deoxyhypusine formed by DHS. Whereas endogenous eIF5A1 was clearly labeled by $[H^3]$-spermidine indicating that it had participated as a substrate in the DHS reaction, the recombinant HA-tagged eIF5A1 protein was not labeled. Thus, as is the case for most enzymatic reactions, the association of DHS with at least one of its substrates, specifically eIF5A, appears to be dependent upon the structural integrity of the substrate. It is also likely that an over-abundance of either substrate for DHS, spermidine or eIF5A, impedes its ability to associate with its substrates in the correct stoichiometry, and that this underlies the inhibition of the reaction observed under conditions in which eIF5A1 is strongly over-expressed.

4.5 eIF5A1-lysine$_{50}$ appears to be an apoptogenic protein

Reports that inhibition of hypusine formation by inhibiting DHS or depleting the endogenous pool of spermidine causes cell cycle arrest and that the treated cells ultimately die have prompted the suggestion that hypusinated eIF5A1 is required for cell growth and proliferation (Schnier et al., 1991; Byers et al., 1992; Park et al., 1993b; Hanauske-Abel et al., 1994; Kang and Hershey, 1994; Chen et al., 1996; Sasaki et al., 1996; Shi et al., 1996; Tome and Gerner, 1997; Park et al., 1998; Choi et al., 2000; Kramer et al., 2001). However, GC7 and other inhibitors of DHS used in these studies have been shown to be non-specific (Lee et al., 1995; Chen et al., 1996; Nishimura et al., 2005). Moreover,

spermidine belongs to family of polyamines which participate in a large number of cellular reactions, including DNA synthesis and gene expression, and play a critical role in the proliferation, migration and differentiation of cells (Reviewed by Goyns, 1982; Pegg and McCann, 1982). Accordingly, it is not clear whether the suppression of cell proliferation in these types of experiments is attributable to inhibition of eIF5A hypusination or to a different unrelated effect of the treatment. This contention is supported by data obtained in the present study indicating suppression of eIF5A1 by up to 90% has no effect on cell proliferation.

The present study also provides strong evidence that eIF5A1-lysine$_{50}$ is able to induce apoptosis. In particular, over-expression of eIF5A1, which results in an accumulation of lysine$_{50}$-containing eIF5A1 was shown to be strongly pro-apoptotic. Further evidence supporting the contention that post-translational hypusination of lysine$_{50}$ in eIF5A1 is not required for its pro-apoptotic activity is the finding that eIF5A1(K50A), a mutated form of eIF5A1 in which lysine$_{50}$ is replaced by alanine, proved to be equally as effective at inducing apoptosis as wild-type eIF5A1. It thus seems reasonable to propose that eIF5A-lysine$_{50}$ is an apoptogenic protein.

Numerous functions for eIF5A have been proposed including involvement in protein translation initiation or elongation (Kang and Hershey, 1994; Lipowsky et al., 2000; Zanelli et al., 2006), nucleocytoplasmic shuttling of mRNA (Ruhl et al., 1993; Hanauske-Abel et al., 1995; Katahira et al., 1995; Liu and Tartakoff, 1997; Jin et al., 2003), mRNA turnover (Zuk and Jacobson, 1998; Zuk and Jacobson, 1998; Veress et al., 2000; Nishimura et al., 2002; Valentini et al., 2002; Schrader et al., 2006) and maintenance of actin cytoskeleton organization and microtubule stability (Weir and Yaffe, 2004; Zanelli and Valentini, 2005; Chatterjee et al., 2006). There have also been several reports suggesting that eIF5A1 is involved in apoptosis (Caraglia et al., 1997, 1999, 2001 & 2003; Takeuchi et al., 2002; Jin et al., 2003; Li et al., 2004; Taylor et al., 2004 & 2007). However, it is not clear from any of these reports whether hypusination of eIF5A is required for its pro-apoptotic activity. The finding in the present study that siRNA-mediated suppression of eIF5A1 inhibits apoptosis induced by serum deprivation or treatment with SNP, Actinomycin D or MG-132 further supports the contention that eIF5A is involved in apoptosis. Of particular interest, though, is the fact that the acidic isoform of eIF5A1, which contains lysine$_{50}$ as shown by mass spectrometry, accumulates within 3.5 hours of treatment with SNP. Moreover, eIF5A1 is known to be strongly up-regulated following treatment of cells with SNP (Taylor et al., 2007). These observations collectively suggest that lysine$_{50}$-containing eIF5A1 is a key facilitator of apoptosis induced by SNP. This possibility is consistent with the additional finding in the present study that over-expression of

eIF5A1(K50A), which cannot be either deoxyhypusinated or hypusinated, activates both the mitochondrial and death receptor pathways of apoptosis.

These data collectively support the contention that lysine$_{50}$-containing eIF5A1 is a pro-apoptotic protein. Of particular note in this context is the fact that hypusinated eIF5A is by far the dominant form of eIF5A in normal healthy cells, comprising ~95% of the total eIF5A. In the event of exposure to an apoptotic stimulus, there is new synthesis of eIF5A, and it seems reasonable to propose that this newly synthesized eIF5A is not post-translationally modified at lysine$_{50}$ and, as such, is able to induce apoptosis. Whether pre-existing hypusinated eIF5A can be converted back to lysine$_{50}$ in response to an apoptotic stimulus is not clear.

4.6 eIF5A1 is post-translationally modified at lysine$_{47}$ and lysine$_{50}$

An initially perplexing feature of experiments in the present study in which apoptosis was induced by infection with Ad-eIF5A was the finding that over-expression of eIF5A1(K50A), which cannot serve as a substrate for DHS, resulted not only in an accumulation of the acidic form of eIF5A1 (eIF5A1-lysine$_{50}$), but also, to a lesser extent, of the intermediate form thought to be deoxyhypusinated eIF5A1 (eIF5A1-deoxyhypusine$_{50}$). This apparent paradox was resolved when the various forms of post-translationally modified eIF5A1 were analyzed by mass spectrometry. Specifically, the three forms of eIF5A1 protein, the acidic form (*pI* 4.9), the intermediate form *(pI* 5.0) and the dominant form (*pI* 5.1), were isolated from Ad-eIF5A1-infected cells and from untreated cells and sequenced by mass spectrometry. This analysis indicated that lysine$_{47}$ and lysine$_{50}$ of eIF5A are both post-translationally modified. Lysine$_{47}$ becomes acetylated, and lysine$_{50}$ is converted to deoxyhypusine or hypusine. The acidic form of eIF5A1 (*pI* 4.9) in Ad-eIF5A1-infected cells isolated by 2D electrophoresis proved to be acetylated at lysine$_{47}$ and unmodified at lysine$_{50}$. The predominant form of eIF5A1 (*pI* 5.1) in both untreated and Ad-eIF5A1-infected cells proved to be unmodified at lysine$_{47}$ and hypusinated at lysine$_{50}$. The intermediate form (*pI* 5.0) in Ad-eIF5A1-infected cells was unmodified at both lysine$_{47}$ and lysine$_{50}$, whereas the intermediate form isolated from untreated control cells proved to be acetylated at lysine$_{47}$ and either dexoyhypusinated or hypusinated at lysine$_{50.}$

Thus, the finding that eIF5A1 acetylated at lysine$_{47}$ and unmodified at lysine$_{50}$ has a *pI* of 4.9 and that eIF5A1 unmodified at both lysine$_{47}$ and lysine$_{50}$ has a *pI* of 5.0 explains why over-expression of eIF5A1(K50A), which cannot serve as a substrate for DHS, gave rise to two forms of eIF5A1 in

2D Western blots. Moreover, the 2D Western blots are consistent with the view that eIF5A1-lysine$_{50}$ is the form of eIF5A1 that is apoptogenic. eIF5A1 unmodified at both lysine$_{47}$ and lysine$_{50}$ has not previously been detected in cells and would appear to be the precursor form of eIF5A1 that through post-translational modification becomes acetylated at lysine$_{47}$ and deoxyhypusinated or hypusinated at lysine$_{50}$. A possible pathway by which these modifications may occur is illustrated in Figure 31. This pathway is consistent with the post-translationally modified forms of eIF5A1 detected by mass spectrometry in cells infected with Ad-eIF5A1 and purports that: (1) newly synthesized eIF5A1 containing unmodified lysine$_{47}$ and lysine$_{50}$ is first post-translationally modified to eIF5A1-acetylated lysine$_{47}$-lysine$_{50}$; (2) lysine$_{50}$ is then converted to deoxyhypusine by DHS and to hypusine by DHH while lysine$_{47}$ remains acetylated; and (3) lysine 47 is de-acetylated and lysine 50 remains hypusinated. The proposed pathway is also consistent with the observation that the intermediate form of eIF5A1 on 2D gels (*pI* 5.0), determined to be eIF5A1-lysine$_{47}$-lysine$_{50}$ by mass spectrometry, accumulates within 30 minutes of treatment with SNP and before accumulation of the acidic form of eIF5A1 on 2D gels (*pI* 4.9) determined to be to eIF5A1-acetylated lysine$_{47}$-lysine$_{50}$ by mass spectrometry. However, the possibility that precursor eIF5A1 can be modified at lysine$_{50}$ by DHS in the absence of lysine$_{47}$ acetylation is not precluded. Indeed, it has been suggested that acetylation of lysine$_{47}$ might reduce the rate of hypusine synthesis (Klier et al., 1995). If this is so, acetylation of lysine$_{47}$ could constitute a means of regulating the pro-apoptotic function of eIF5A1. Specifically, it seems clear from experiments in the present study demonstrating essentially equivalent pro-apoptotic activities of Ad-eIF5A1(K50A) and Ad-eIF5A1 that eIF5A1-lysine$_{50}$ rather than eIF5A1-deoxyhypusine$_{50}$ or eIF5A1-hypusine$_{50}$ induces apoptosis.

Figure 31: Proposed post-translational modification scheme for eIF5A1.

The newly synthesized precursor eIF5A1 contains unmodified $lysine_{47}$ and $lysine_{50}$. In response to apoptotic stimuli (e.g., SNP), newly synthesized precursor eIF5A1 is acetylated on $lysine_{47}$, and remains unmodified on $lysine_{50}$, giving rise to the form of eIF5A that is apoptogenic [step (1)]. In normal cells that are not undergoing apoptosis, $lysine_{50}$ of eIF5A1-acetylated $lysine_{47}$-$lysine_{50}$ is quickly converted to deoxyhypusine and then to hypusine by deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DHH), respectively [step (2)]. Thereafter, $lysine_{47}$ is deacetylated giving rise to eIF5A1-$lysine_{47}$-$hypusine_{50}$ [step (3)]. Under normal conditions in healthy cells, the precursor eIF5A1 may also be directly post-translationally modified at $lysine_{50}$ to give rise to eIF5A1-$lysine_{47}$-$hypusine_{50}$ in keeping with the fact that the cells are not undergoing apoptosis [step (4)].

Acidic **Intermediate** **Basic**

pI   4.9   5.0   5.1

(1)   (2)   (3)   (4)

B

C

A

- Acetylated lysine
- Deoxyhypusinated lysine
- Unmodified lysine
- Hypusinated lysine
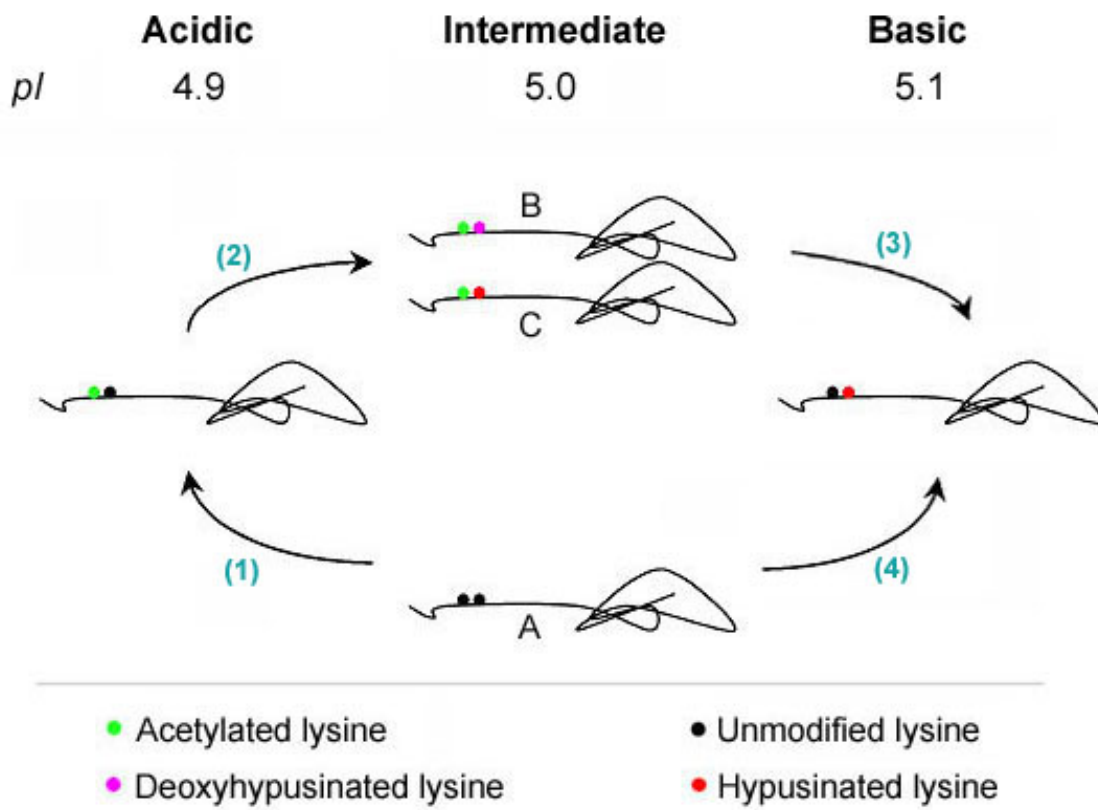
4.7 eIF5A1 is N-terminally modified

It was also established by mass spectrometry in the present study that eIF5A1 is N-terminally modified irrespective of its state of post-translational modification at lyinse$_{47}$ and lysine$_{50}$. Specifically, the methionine at position 1 is removed, and the alanine residue at position 2 is acetylated. Loss of the methionine at position 1 is not uncommon. Indeed, it has been estimated that 55-70% of eukaryotic cell proteins have lost this initial amino acid (Reviewed by Giglione et al., 2003). The process by which this methionine is removed is termed N-terminal methionine excision (NME). It occurs co-translationally and is mediated by methionine amino peptidase, leaving smaller residues such as alanine, cysteine, glycine, proline, serine, threonine and valine exposed at the N-terminus (Sherman et al., 1985; Frottin et al., 2006). As predicted from the N-end rule of protein stability, proteins possessing these small N-terminal residues, which are known as stabilizing residues, have relatively longer half-lives compared with proteins possessing bulky N-terminal residues such as arginine, lysine, leucine, phenylalanine, tyrosine and tryptophan, which are known as destabilizing residues (Gonda et al., 1989; Tobias et al., 1991). Furthermore, proteins with an N-terminal methionine are unstable and are targets for N-terminal ubiquitinylation (Meinnel et al., 2005). Therefore, it has been suggested that NME plays a crucial role by enhancing the half life of many proteins (Richo and Conner, 1994; Wakayama et al., 1995; Giglione et al., 2003; Liao et al., 2004).

NME is normally accompanied by acetylation of the newly exposed N-terminal amino acid, and this was found to be the case for eIF5A1 in the present study. For eIF5A1, NME gives rise to an N-terminal alanine (alanine$_2$), and for unmodified eIF5A as well as all of the post-translationally modified forms of eIF5A1 examined in the present study, alanine$_2$ proved to be acetylated. The process by which this occurs is termed N-α-acetylation (NAA). Like NME it occurs co-translationally and has been shown to occur in ~30% (by identity) and ~90% (by weight) of the mature proteins in the proteomes of some higher eukaryotes (Driessen et al., 1985; Polevoda and Sherman, 2003; Meinnel et al., 2005). NAA blocks the N-terminus of the protein, making it resistant to further modifications including amino peptidase-directed degradation and N-terminal ubiquitination (Meinnel et al., 2006). Thus, a major function of NAA appears to be protecting proteins from proteolysis. Indeed, it has been shown that the half lives of proteins co-translationally modified by NAA are typically about seven-fold longer than those of unmodified proteins (Meinnel et al., 2005 & 2006).

Co-translational N-terminal modification of proteins by NME and NAA are, therefore, important mechanisms for regulating protein stability. The fact that eIF5A1 undergoes these N-terminal modifications during its synthesis is consistent with reports that it has a comparatively long half life (Benne et al., 1978; Gerner 1986; Nishimura et al., 2005), and also imply that it has an important biological function.

4.8 eIF5A1 undergo deamidation

The mass spectrometric analysis of eIF5A1 indicated yet another post-translational modification of the protein not previously reported, specifically that asparagine$_{28}$ undergoes deamidation. Deamidation entails hydrolysis of the side-chain amide group on an asparagine or glutamine residue. The deamidation of asparagine generates succinimide as an intermediate, which is subsequently converted to aspartate or isoaspartate (Robinson and Rudd, 1974; Geiger and Clarke, 1987). The rate of deamidation is dependent on pH, temperature and tertiary structure ("nearest neighbor" effect) of the protein (Kossiakoff et al., 1988, Tyler-Cross and Schirch, 1991; Wright, 1991; Robinson and Robinson, 2001). For example, an asparagine followed by a glycine is highly susceptible to deamidation (Robinson and Rudd, 1974; Tyler-Cross and Schirch, 1991), and this is the case for human eIF5A1 in which asparagine$_{28}$ is followed by a glycine, glycine$_{29}$. In keeping with this, all of the forms of eIF5A1 resolvable by 2D Western blotting exhibited this modification of asparagine$_{28}$.

Deamidation results in the introduction of a negative charge at the site of modification, and this in turn is likely to alter the tertiary structure of the protein and affect its biological properties (Robinson and Rudd, 1974; Wright, 1991b). Indeed, for some proteins deamidation has been linked to loss of bioactivity (Wingfield et al., 1987; Friedman et al., 1991; Orru et al., 2000; Gupta and Srivastava, 2004b; Zomber et al., 2005). Moreover, deamidation at different sites in the same protein appears to have different effects on both structure and function (Gupta and Srivastava, 2004a). There is also evidence that deamidation is linked to protein degradation (Wingfield et al., 1987). Indeed, it has been hypothesized that deamidation functions as an internal clock, regulating the half-life of proteins (Robinson and Robinson, 2001 & 2004). The finding in the present study that for all resolvable forms of eIF5A1 asparagine$_{28}$ is deamidated suggests that deamidation of this asparagine may be a prerequisite either for eIF5A1 function or for subsequent post-translational modification such as acetylation or hypusination.

170

4.9 Subcellular localization of eIF5A1

p53 is known to localize on mitochondria in response to apoptotic stimuli and perform direct apoptogenic functions independently of its transactivation activity (Erster et al., 2004). It has been shown, for example, that in the event of apoptotis a fraction of the total p53 protein redistributes to the mitochondrial outer membrane and, by directly inducing Bax/Bak oligomerization and mitochondrial outer membrane permeablization, facilitates the release of cytochrome *c* leading to activation of caspases 9 and 3 (Mihara et al., 2003; Erster et al., 2004; Leu et al., 2004; Jiang et al., 2006). Since over-expression of eIF5A induces apoptosis by activating the mitochondrial pathway, the possibility that it also localizes in or on mitochondria was examined by confocal microscopy. However, this proved not to be the case. In untreated cells, endogenous eIF5A1 was predominantly in the cytoplasm, and there were smaller amounts of the protein in the nucleus. Following the induction of apoptosis by infection with Ad-eIF5A1, eIF5A1 was again detectable in the cytoplasm, but with time after infection it became increasingly clustered in the vicinity of the inner cellular face of the plasma membrane. It was not possible to distinguish endogenous eIF5A1 from trans-eIF5A1 in these experiments, but there is no apparent reason to assume that they would have different subcellular localizations. Nor was it possible to distinguish between the localization of eIF5A1-lysine$_{50}$ and eIF5A1-deoxyhypusine$_{50}$ or eIF5A1-hypusine$_{50}$ because, although the localization pattern of the protein was examined in both Ad-eIF5A1- and Ad-eIF5A1(K50A)-infected cells, eIF5A1-lysine$_{50}$ proved to be the predominant forms of eIF5A1 in both cases. Indeed, it is likely that the pattern of eIF5A1 distribution in both Ad-eIF5A1- and Ad-eIF5A1(K50A)-infected cells largely reflected that of eIF5A1-lysine$_{50.}$

There have been several previous reports on the localization of eIF5A discerned using both immunofluorescence and immunohistochemical staining or GFP-live imaging (Shi et al., 1996; Jao and Chen, 2002; Valentini et al., 2002; Jin et al., 2003; Cracchiolo et al., 2004; Taylor et al., 2007). The observation in the present study that eIF5A1 in untreated Hela cells is localized in the cytoplasm is consistent with most of these studies (Shi et al., 1996; Valentini et al., 2002; Jin et al., 2003; Cracchiolo et al., 2004; Taylor et al., 2007), although there is one report of a whole-cell distribution pattern for eIF5A1 (Jao and Chen, 2002). Similarly, the finding in the present study that some eIF5A1 is also detectable in the nucleus is in keeping with a previous proposal that eIF5A1 may accumulate in the nucleus by passive diffusion (Lipowsky et al., 2000; Jao and Chen, 2002). Jin et al (2003) reported that blocking the hypusination of eIF5A changed its distribution from cytoplasmic to a

whole-cell pattern. However, other groups have suggested that the hypusine residue contributes to the nuclear retention of eIF5A1 (Klier et al., 1995; Lipowsky, 2000).

The clustering of trans-eIF5A1 at the inner surface of the plasma membrane upon induction of apoptosis by infection of cells with Ad-eIF5A1 or Ad-eIF5A1(K50A) proved to be time-dependent suggesting that it contributes to the proapoptotic function of eIF5A. There have been several reports of ligand-independent death receptor clustering leading to subsequent recruitment of FADD and activation of death receptor pathway (Rehemtulla et al., 1997; Sheikh et al., 1998; Beltinger et al., 1999; Zhuang and Kochevar, 2003; Gajate et al., 2004; Gniadecki, 2004). In fact, it has been suggested that the activity of Fas is not only regulated by its ligand, but also by its association with lipid rafts, which are cholesterol-enriched lipid domains in the plasma membrane (Cremesti et al., 2001; Gajate et al., 2004; Lincoln et al., 2006). For example, Fas has been shown to translocate laterally through the plasma membrane into lipid rafts following antitumor chemotherapy, independently of association with FasL (Delmas et al., 2003; Huang et al., 2003; Lou et al., 2003; Lacour et al., 2004; Gajate and Mollinedo, 2005). Moreover lipid rafts also recruit apoptotic signaling molecules including FADD, procaspase 8, procaspase 10, JNK and Bid (Gajate et al., 2004; Gajate and Mollinedo, 2005). Therefore, lipid rafts not only concentrate Fas within the plane of the plasmalemma, but also serve as platforms for recruiting adaptor and effector proteins for Fas, thereby enhancing DISC formation (Mollinedo and Gajate, 2006). In light of this, it is conceivable that the clustering of eIF5A1 on the inside surface of the plasmalemma following the induction of apoptosis by its up-regulation may reflect involvement of eIF5A1 protein in the death receptor pathway of apoptosis. More specifically, the accumulation of eIF5A1 in the vicinity of the plasma membrane may, in a way that is not understood, facilitate the concentration of Fas into lipid rafts, thereby activating Fas-mediated downstream signaling. Involvement of eIF5A1 in the death receptor pathway is also consistent with the fact that infection with Ad-eIF5A induces up-regulation of the transcription factor, p73, which is known to regulate Fas (Terrasson et al., 2005), and also leads to activation of caspase 8, which is the initiator caspase for the extrinsic death receptor pathway (Varfolomeev et al., 1998).

It has been suggested that the FasL-independent recruitment of Fas into lipid rafts could be due to persistent activation of JNK (Reviewed by Mollinedo and Gajate, 2006). Relevant to this is other data from our laboratory showing that infection with Ad-eIF5A1 does in fact induce phosphorylation of JNK (Catherine Taylor, unpublished data). eIF5A has also been implicated in organization of the actin cytoskeleton (Zanelli and Valentini, 2005; Chatterjee et al., 2006), which in

turn, may regulate lipid raft-mediated signaling including that attributable to the clustering of Fas (Harder and Simons, 1999; Parlato et al., 2000; Lozupone et al., 2004; Fais et al., 2005; Gajate and Mollinedo, 2005). It is, therefore, conceivable that eIF5A1 impacts Fas aggregation in lipid rafts through an association with actin.

Of particular interest is the finding that when apoptotis was induced by treatment with Actinomycin D rather than by infection with Ad-eIF5A1 there was a notable translocation of endogenous eIF5A1 from the cytoplasm into the nucleus within 12 hours of treatment. Morover, the translocation increased with time through 18 to 24 hours. Permeabilization of the nuclear envelope is a key event during apoptosis (Robertson et al., 2000), and the time-dependent nature of eIF5A1 translocation into the nucleus following treatment with Actinomycin D is consistent with the notion that it gains access to the nucleus as the nuclear envelope becomes permeabilized. This contention is consistent with earlier reports that entry of eIF5A into the nucleus is insensitive to reagents that block the nuclear pore complex (NPC) (Lipowsky et al., 2000; Jao and Chen, 2002). However, by 24 hours after treatment with Actinomycin D, the concentration of eIF5A1 in the nucleus appeared to be higher than that in the cytoplasm suggesting that there was an active uptake of the protein from the cytosol. A similar redistribution of endogenous eIF5A1, but within a much shorter time (10 minutes), was noted when apoptosis was induced by treatment with TNFα (Taylor et al., 2007). That endogenous eIF5A1 plays a role in apoptotis is supported by the finding in the present study that eIF5A1 siRNA is a potent inhibitor of apoptotis induced by a number of pro-apoptotic stimuli including Actinomcyin D. The fact that endogenous eIF5A protein progressively localizes to the nucleus following the induction of apoptosis by Actinomycin D suggests that the apoptogenic effect of the protein may be dependent upon this migration.

It is noteworthy that the induction of apoptotis by treatment with Actinomcyin D on the one hand and infection with Ad-eIF5A1 on the other gave rise to quite different subcellular localizations of eIF5A1 protein. In the case of infection with Ad-eIF5A1, there was a distinct clustering of eIF5A1 protein at the inner surface of the plasmalemma and no evidence for localization in the nucleus. In contrast, when apoptosis was induced by treatment with Actinomycin D, endogenous eIF5A1 progressively translocated to the nucleus, and there was no apparent clustering of the protein at the cell surface. At face value, this suggests that eIF5A1 facilitates apoptosis in different ways by different mechanisms depending on the nature of the apoptotic stimulus. However, the possibility that it functions as an apoptogenic agent in multiple ways through multiple mechanisms no matter what

173

the apoptotic stimulus, and that, depending on the stimulus, a particular mechanism is dominant, is not precluded.

References

Abbruzzese, A., V. Liguori and M. H. Park (1988). Deoxyhypusine hydroxylase. Adv Exp Med Biol. 250: 459-466.

Abbruzzese, A., M. H. Park and J. E. Folk (1986). "Deoxyhypusine hydroxylase from rat testis. Partial purification and characterization." J Biol Chem 261(7): 3085-3089.

Adachi, K., M. Osaki, S. Kase, A. Takeda and H. Ito (2003). "Anti-Fas antibody-induced apoptosis and its signal transduction in human gastric carcinoma cell lines." Int J Oncol 23(3): 713-719.

Adams, J. (2002). "Proteasome inhibitors as new anticancer drugs." Curr Opin Oncol 14(6): 628-634.

Adams, J. M. and S. Cory (2001). "Life-or-death decisions by the Bcl-2 protein family." Trends Biochem Sci 26(1): 61-66.

Adjei, A. A. (2001). "Blocking oncogenic Ras signaling for cancer therapy." J Natl Cancer Inst 93(14): 1062-1074.

Adrain, C., E. M. Creagh and S. J. Martin (2001). "Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2." EMBO J 20(23): 6627-6636.

Agami, R., G. Blandino, M. Oren and Y. Shaul (1999). "Interaction of c-Abl and p73alpha and their collaboration to induce apoptosis." Nature 399(6738): 809-813.

Akaike, T. (2000). "Mechanisms of biological S-nitrosation and its measurement." Free Radic Res 33(5): 461-469.

Alnemri, E. S., N. M. Robertson, T. F. Fernandes, C. M. Croce and G. Litwack (1992). "Over-expressed full-length human BCL2 extends the survival of baculovirus-infected Sf9 insect cells." Proc Natl Acad Sci U S A 89(16): 7295-7299.

Al-Sa'doni, H. H. and A. Ferro (2005). "Current status and future possibilities of nitric oxide-donor drugs: focus on S-nitrosothiols." Mini Rev Med Chem 5(3): 247-254.

Ame, J. C., V. Rolli, V. Schreiber, C. Niedergang, F. Apiou, P. Decker, S. Muller, T. Hoger, J. Menissier-De Murcia and G. De Murcia (1999). "PARP-2, A novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase." J Biol Chem 274(25): 17860-17868.

Amendola, A., M. L. Gougeon, F. Poccia, A. Bondurand, L. Fesus and M. Piacentini (1996). "Induction of "tissue" transglutaminase in HIV pathogenesis: evidence for high rate of apoptosis of CD4+ T lymphocytes and accessory cells in lymphoid tissues." Proc Natl Acad Sci U S A 93(20): 11057-11062.

Amendola, A., G. Lombardi, S. Oliverio, V. Colizzi and M. Piacentini (1994). "HIV-1 gp120-dependent induction of apoptosis in antigen-specific human T cell clones is characterized by 'tissue' transglutaminase expression and prevented by cyclosporin A." FEBS J 339(3): 258-264.

Amendola, A., C. Rodolfo, A. D. Caro, F. Ciccosanti, L. Falasca and M. Piacentini (2001). ""Tissue" transglutaminase expression in HIV-infected cells: an enzyme with an antiviral effect?" Ann N Y Acad Sci 946: 108-120.

Amundson, S. A., T. G. Myers and A. J. Fornace (1998). "Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress." Oncogene 17(25): 3287-3299.

An, B., R. H. Goldfarb, R. Siman and Q. P. Dou (1998). "Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts." Cell Death Differ 5(12): 1062-1075.

An, W. G., S. G. Hwang, J. B. Trepel and M. V. Blagosklonny (2000). "Protease inhibitor-induced apoptosis: accumulation of wt p53, p21WAF1/CIP1, and induction of apoptosis are independent markers of proteasome inhibition." Leukemia 14(7): 1276-1283.

Andree, H. A., C. P. Reutelingsperger, R. Hauptmann, H. C. Hemker, W. T. Hermens and G. M. Willems (1990). "Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers." J Biol Chem 265(9): 4923-4928.

Andrus, L., P. Szabo, R. W. Grady, A. R. Hanauske, T. Huima-Byron, B. Slowinska, S. Zagulska and H. M. Hanauske-Abel (1998). "Antiretroviral effects of deoxyhypusyl hydroxylase inhibitors: a

hypusine-dependent host cell mechanism for replication of human immunodeficiency virus type 1 (HIV-1)." Biochem Pharmacol 55(11): 1807-18.

Antonsson, B., F. Conti, A. Ciavatta, S. Montessuit, S. Lewis, I. Martinou, L. Bernasconi, A. Bernard, J. J. Mermod, G. Mazzei, K. Maundrell, F. Gambale, R. Sadoul and J. C. Martinou (1997). "Inhibition of Bax channel-forming activity by Bcl-2." Science 277(5324): 370-372.

Antonsson, B., S. Montessuit, B. Sanchez and J. C. Martinou (2001). "Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells." J Biol Chem 276(14): 11615-11623.

Aoki, H., K. Dekany, S. L. Adams and M. C. Ganoza (1997). "The gene encoding the elongation factor P protein is essential for viability and is required for protein synthesis." J. Biol. Chem. 272(51): 32254-32259.

Arends, M. J., R. G. Morris and A. H. Wyllie (1990). "Apoptosis. The role of the endonuclease." Am J Pathol 136(3): 593-608.

Arends, M. J. and A. H. Wyllie (1991). "Apoptosis: mechanisms and roles in pathology." Int Rev Exp Pathol 32: 223-254.

Arita, D., M. Kambe, C. Ishioka and R. Kanamaru (1997). "Induction of p53-independent apoptosis associated with G2M arrest following DNA damage in human colon cancer cell lines." Jpn J Cancer Res 88(1): 39-43.

Arvanitis, C. and D. W. Felsher (2006). "Conditional transgenic models define how MYC initiates and maintains tumorigenesis." Semin Cancer Biol 16(4): 313-317.

Ashkenazi, A. and V. M. Dixit (1998). "Death Receptors: Signaling and Modulation." Science 281(5381): 1305 - 1308.

Ashkenazi, A. and V. M. Dixit (1999). "Apoptosis control by death and decoy receptors." Curr Opin Cell Biol 11(2): 255-260.

Ashkenazi, A., P. C. Pai, S. Fong, S. Leung, D. A. Lawrence, S. A. Marsters, C. Blackie, L. Chang, A. E. Mcmurtrey, A. Hebert, L. Deforge, I. L. Koumenis, D. Lewis, L. Harris, J. Bussiere, H. Koeppen, Z. Shahrokh and R. H. Schwall (1999). "Safety and antitumor activity of recombinant soluble Apo2 ligand." J Clin Invest 104(2): 155-162.

Bai, J., H. Nakamura, S. Ueda, Y. W. Kwon, T. Tanaka, S. Ban and J. Yodoi (2004). "Proteasome-dependent degradation of cyclin D1 in 1-methyl-4-phenylpyridinium ion (MPP+)-induced cell cycle arrest." J Biol Chem 279(37): 38710-38714.

Baldini, P. M., A. Lentini, P. Mattioli, B. Provenzano, P. De Vito, D. Vismara and S. Beninati (2006). "Decrease of polyamine levels and enhancement of transglutaminase activity in selective reduction of B16-F10 melanoma cell proliferation induced by atrial natriuretic peptide." Melanoma Res 16(6): 501-507.

Bartig, D., K. Lemkemeier, J. Frank, F. Lottspeich and F. Klink (1992). "The archaebacterial hypusine-containing protein. Structural features suggest common ancestry with eukaryotic translation initiation factor 5A." J Chromatogr 606(1): 43-48.

Bartig, D., H. Schümann and F. Klink (1990). "The unique post-translational modification leading to deoxyhypusine or hypusine is a general feature of the archaebacterial kingdom." Syst Appl Microbiol 13: 112-116.

Bates, S., A. C. Phillips, P. A. Clark, F. Stott, G. Peters, R. L. Ludwig and K. H. Vousden (1998). "p14ARF links the tumour suppressors RB and p53." Nature 395(6698): 124-125.

Beg, A. A. and D. Baltimore (1996). "An Essential Role for NF-B in Preventing TNF--alpha Induced Cell Death." Science 274(5288): 782 - 784.

Bell, L. A. and K. M. Ryan (2004). "Life and death decisions by E2F-1." Cell Death Differ 11(2): 137-142.

Beltinger, C., S. Fulda, T. Kammertoens, E. Meyer, W. Uckert and K. M. Debatin (1999). "Herpes simplex virus thymidine kinase/ganciclovir-induced apoptosis involves ligand-independent death receptor aggregation and activation of caspases." Proc Natl Acad Sci U S A 96(15): 8699-8704.

178

Benchoua, A., C. Couriaud, C. Guegan, L. Tartier, P. Couvert, G. Friocourt, J. Chelly, J. Menissier-De Murcia and B. Onteniente (2002). "Active caspase-8 translocates into the nucleus of apoptotic cells to inactivate poly(ADP-ribose) polymerase-2." J Biol Chem 277(37): 34217-34222.

Beninati, S., V. Gentile, M. Caraglia, A. Lentini, P. Tagliaferri and A. Abbruzzese (1998). "Tissue transglutaminase expression affects hypusine metabolism in BALB/c 3T3 cells." FEBS Lett 437(1-2): 34-38.

Beninati, S., L. Nicolini, J. Jakus, A. Passeggio and A. Abbruzzese (1995). "Identification of a substrate site for transglutaminases on the human protein synthesis initiation factor 5A." Biochem J. 305(Pt 3): 725-728.

Benne, R., M. L. Brown-Luedi and J. W. Hershey (1978). "Purification and characterization of protein synthesis initiation factors eIF-1, eIF-4C, eIF-4D, and eIF-5 from rabbit reticulocytes." J Biol Chem 253(9): 3070-3077.

Benne, R. and J. W. Hershey (1978). "The mechanism of action of protein synthesis initiation factors from rabbit reticulocytes." J Biol Chem 253(9): 3078-3087.

Bergeron, R. J., W. R. Weimar, R. Muller, C. O. Zimmerman, B. H. Mccosar, H. Yao and R. E. Smith (1998). "Effect of polyamine analogues on hypusine content in JURKAT T-cells." J Med Chem 41(20): 3901-3908.

Bernardi, P. (1999). "Mitochondrial transport of cations: channels, exchangers, and permeability transition." Physiol Rev 79(4): 1127-1155.

Bernardi, P., K. M. Broekemeier and D. R. Pfeiffer (1994). "Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane." J Bioenerg Biomembr 26(5): 509-517.

Besnault, L., N. Schrantz, M. T. Auffredou, G. Leca, M. F. Bourgeade and A. Vazquez (2001). "B cell receptor cross-linking triggers a caspase-8-dependent apoptotic pathway that is independent of the death effector domain of Fas-associated death domain protein." J Immunol 167(2): 733-740.

Bevec, D. and J. Hauber (1997). "Eukaryotic initiation factor 5A activity and HIV-1 Rev function." Biol Signals 6(3): 124-133.

Bevec, D., H. Jaksche, M. Oft, T. Wohl, M. Himmelspach, A. Pacher, M. Schebesta, K. Koettnitz, M. Dobrovnik, R. Csonga, F. Lottspeich and J. Hauber (1996). "Inhibition of HIV-1 replication in lymphocytes by mutants of the Rev cofactor eIF-5A." Science 271(5257): 1858-1860.

Bevec, D., H. Klier, W. Holter, E. Tschachler, P. Valent, F. Lottspeich, T. Baumruker and J. Hauber (1994). "Induced gene expression of the hypusine-containing protein eukaryotic initiation factor 5A in activated human T lymphocytes." Proc Natl Acad Sci U S A 91(23): 10829-10833.

Biswas, S. C. and L. A. Greene (2002). "Nerve growth factor (NGF) down-regulates the Bcl-2 homology 3 (BH3) domain-only protein Bim and suppresses its pro-apoptotic activity by phosphorylation." J Biol Chem 277(51): 49511-49516.

Biswas, S. C., D. X. Liu and L. A. Greene (2005). "Bim is a direct target of a neuronal E2F-dependent apoptotic pathway." J Neurosci 25(37): 8349-8358.

Blokhina, O., E. Virolainen and K. V. Fagerstedt (2003). "Antioxidants, oxidative damage and oxygen deprivation stress: a review." Ann Bot (Lond). 91(2): 179-194.

Boatright, K. M., M. Renatus, F. L. Scott, S. Sperandio, H. Shin, I. M. Pedersen, J. E. Ricci, E. W. A, D. P. Sutherlin, D. R. Green and G. S. Salvesen (2003). "A unified model for apical caspase activation." Mol Cell 11(2): 529-541.

Boatright, K. M. and G. S. Salvesen (2003). "Mechanisms of caspase activation." Curr Opin Cell Biol 15(6): 725-731.

Bogerd, H. P., A. Echarri, T. M. Ross and B. R. Cullen (1998). "Inhibition of human immunodeficiency virus Rev and human T-cell leukemia virus Rex function, but not Mason-Pfizer monkey virus constitutive transport element activity, by a mutant human nucleoporin targeted to Crm1." J Virol 72(11): 8627-8635.

Boldin, M. P., T. M. Goncharov, Y. V. Goltsev and D. Wallach (1996). "Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death." Cell 86(5): 803-15.

Boon, K., H. N. Caron, R. Van Asperen, L. Valentijn, M. C. Hermus, P. Van Sluis, I. Roobeek, I. Weis, P. A. Voute, M. Schwab and R. Versteeg (2001). "N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis." EMBO J 20(6): 1383-1393.

Borner, C. (2003). "The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions." Mol Immunol 39(11): 615-647.

Bossy-Wetzel, E., D. D. Newmeyer and D. R. Green (1998). "Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization." EMBO J 17(1): 37-49.

Bouillet, P., D. Metcalf, D. C. Huang, D. M. Tarlinton, T. W. Kay, F. Kontgen, J. M. Adams and A. Strasser (1999). "Pro-apoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity." Science 286(5445): 1735-1738.

Boukerche, H., Z. Z. Su, L. Emdad, P. Baril, B. Balme, L. Thomas, A. Randolph, K. Valerie, D. Sarkar and P. B. Fisher (2005). "mda-9/Syntenin: a positive regulator of melanoma metastasis." Cancer Res 65(23): 10901-10911.

Bouralexis, S., D. M. Findlay and A. Evdokiou (2005). "Death to the bad guys: targeting cancer via Apo2L/TRAIL." Apoptosis 10(1): 35-51.

Bourdon, J. C., J. Renzing, P. L. Robertson, K. N. Fernandes and D. P. Lane (2002). "Scotin, a novel p53-inducible pro-apoptotic protein located in the ER and the nuclear membrane." J Cell Biol 158(2): 235-246.

Boyd, C. S. and E. Cadenas (2002). "Nitric oxide and cell signaling pathways in mitochondrial-dependent apoptosis." Biol Chem 383(3-4): 411-423.

Bradshaw, R. A., W. W. Brickey and K. W. Walker (1998). "N-terminal processing: the methionine aminopeptidase and N alpha-acetyl transferase families." Trends Biochem Sci 23(7): 263-267.

Breckenridge, D. G. and D. Xue (2004). "Regulation of mitochondrial membrane permeabilization by BCL-2 family proteins and caspases." Curr Opin Cell Biol 16(6): 647-652.

Brockhaus, F. and B. Brune (1999). "p53 accumulation in apoptotic macrophages is an energy demanding process that precedes cytochrome c release in response to nitric oxide." Oncogene 18(47): 6403-6410.

Brookes, P. and V. M. Darley-Usmar (2002). "Hypothesis: the mitochondrial NO(*) signaling pathway, and the transduction of nitrosative to oxidative cell signals: an alternative function for cytochrome C oxidase." Free Radic Biol Med 32(4): 370-374.

Brune, B. (2003). "Nitric oxide: NO apoptosis or turning it ON?" Cell Death Differ 10(8): 864-869.

Byers, T. L., B. Ganem and A. E. Pegg (1992). "Cytostasis induced in L1210 murine leukaemia cells by the S-adenosyl-L-methionine decarboxylase inhibitor 5'-([(Z)-4-amino-2-butenyl]methylamino)-5'-deoxyadenosine may be due to hypusine depletion." Biochem J. 287(Pt 3): 717-24.

Byers, T. L., J. R. Lakanen, J. K. Coward and A. E. Pegg (1994). "The role of hypusine depletion in cytostasis induced by S-adenosyl-L-methionine decarboxylase inhibition: new evidence provided by 1-methylspermidine and 1,12-dimethylspermine." Biochem J. 303(Pt 2): 363-368.

Caelles, C., A. Helmberg and M. Karin (1994). "p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes." Nature 370(6486): 220-223.

Cai, J. and D. P. Jones (1998). "Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss." J Biol Chem 273(19): 11401-11404.

Candé, C., N. Vahsen, C. Garrido and G. Kroemer (2004). "Apoptosis-inducing factor (AIF): caspase-independent after all." Cell Death Differ 11(6): 591-595.

Caplen, N. J., S. Parrish, F. Imani, A. Fire and R. A. Morgan (2001). "Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems." Proc Natl Acad Sci U S A 98(17): 9742-9747.

Caponigro, G. and R. Parker (1996). "Mechanisms and control of mRNA turnover in Saccharomyces cerevisiae." Microbiol Rev 60(1): 233-249.

Caraglia, M., M. Marra, G. Giuberti, A. M. D'alessandro, A. Baldi, P. Tassone, S. Venuta, P. Tagliaferri and A. Abbruzzese (2003). "The eukaryotic initiation factor 5A is involved in the regulation of proliferation and apoptosis induced by interferon-alpha and EGF in human cancer cells." J Biochem (Tokyo). 133(6): 757-765.

Caraglia, M., M. Marra, G. Giuberti, A. M. D'alessandro, A. Budillon, S. D. Prete, A. Lentini, S. Beninati and A. Abbruzzese (2001). "The role of eukaryotic initiation factor 5A in the control of cell proliferation and apoptosis." Amino Acids 20: 91-104.

Caraglia, M., A. Passeggio, S. Beninati, A. Leardi, L. Nicolini, S. Improta, A. Pinto, A. R. Bianco, P. Tagliaferri and A. Abbruzzese (1997). "Interferon alpha2 recombinant and epidermal growth factor modulate proliferation and hypusine synthesis in human epidermoid cancer KB cells." Biochem J. 324(Pt 3): 737-741.

Caraglia, M., P. Tagliaferri, A. Budillon and A. Abbruzzese (1999). "Post-translational modifications of eukaryotic initiation factor-5A (eIF-5A) as a new target for anti-cancer therapy." Adv Exp Med Biol 472: 187-98.

Cartron, P. F., T. Gallenne, G. Bougras, F. Gautier, F. Manero, P. Vusio, K. Meflah, F. M. Vallette and P. Juin (2004). "The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA." Mol Cell 16(5): 807-818.

Castedo, M., T. Hirsch, S. A. Susin, N. Zamzami, P. Marchetti, A. Macho and G. Kroemer (1996). "Sequential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis." J Immunol 157(2): 512-521.

Chamot, D. and C. Kuhlemeier (1992). "Differential expression of genes encoding the hypusine-containing translation initiation factor, eIF-5A, in tobacco." Nucleic Acids Res 20(4): 665-659.

Chan, K. L., D. New, S. Ghandhi, F. Wong, C. M. Lam and J. T. Wong (2002). "Transcript levels of the eukaryotic translation initiation factor 5A gene peak at early G(1) phase of the cell cycle in the dinoflagellate Crypthecodinium cohnii." Appl Environ Microbiol 68(5): 2278-2284.

Chang, F., L. S. Steelman, J. G. Shelton, J. T. Lee, P. M. Navolanic, W. L. Blalock, R. Franklin and J. A. Mccubrey (2003). "Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway (Review)." Int J Oncol 22(3): 469-480.

Chang, H. C., T. H. Lee, L. Y. Chuang, M. H. Yen and W. C. Hung (1999). "Inhibitory effect of mimosine on proliferation of human lung cancer cells is mediated by multiple mechanisms." Cancer Lett 145(1-2): 1-8.

Chang, Y. C., Y. S. Lee, T. Tejima, K. Tanaka, S. Omura, N. H. Heintz, Y. Mitsui and J. Magae (1998). "mdm2 and bax, downstream mediators of the p53 response, are degraded by the ubiquitin-proteasome pathway." Cell Death Differ 9(1): 79-84.

Chao, D. T., G. P. Linette, L. H. Boise, L. S. White, C. B. Thompson and S. J. Korsmeyer (1995). "Bcl-XL and Bcl-2 repress a common pathway of cell death." J Exp Med 182(821-828).

Chao, J. I., P. C. Kuo and T. S. Hsu (2004). "Down-regulation of survivin in nitric oxide-induced cell growth inhibition and apoptosis of the human lung carcinoma cells." J Biol Chem 279(19): 20267-20276.

Chatterjee, I., S. R. Gross, T. G. Kinzy and K. Y. Chen (2006). "Rapid depletion of mutant eukaryotic initiation factor 5A at restrictive temperature reveals connections to actin cytoskeleton and cell cycle progression." Mol Genet Genomics 275(3): 264-276.

Chaudhary, P. M., M. Eby, A. Jasmin, A. Bookwalter, J. Murray and L. Hood (1997). "Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-kappaB pathway." Immunity 7(6): 821-830.

Chen, D. and Q. Zhou (2004). "Caspase cleavage of BimEL triggers a positive feedback amplification of apoptotic signaling." Proc Natl Acad Sci U S A 101(5): 1235-1240.

Chen, F., D. Chang, M. Goh, S. A. Klibanov and M. Ljungman (2000). "Role of p53 in Cell Cycle Regulation and Apoptosis following Exposure to Proteasome Inhibitors." Cell Death Differ 11(5): 239-246.

Chen, G., T. G. Gharib, D. G. Thomas, C. C. Huang, D. E. Misek, R. D. Kuick, T. J. Giordano, M. D. Iannettoni, M. B. Orringer, S. M. Hanash and D. G. Beer (2003). "Proteomic analysis of eIF-5A in lung adenocarcinomas." Proteomics 3(4): 496-504.

Chen, K. Y. (1983). "An 18000-dalton protein metabolically labeled by polyamines in various mammalian cell lines." Biochim Biophys Acta 756(3): 395-402.

Chen, K. Y. and D. L. Jao (1999). "Chemistry of hypusine formation on eukaryotic initiation factor 5A in Biological systems." J Chin Chem Soc 46(5): 727-734.

Chen, L., S. N. Willis, A. Wei, B. J. Smith, J. I. Fletcher, M. G. Hinds, P. M. Colman, C. L. Day, J. M. Adams and D. C. Huang (2005). "Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function." Mol Cell 17(3): 393-403.

Chen, M., H. He, S. Zhan, S. Krajewski, J. C. Reed and R. A. Gottlieb (2001). "Bid is cleaved by calpain to an active fragment *in vitro* and during myocardial ischemia/reperfusion." J Biol Chem 276(33): 30724-30728.

Chen, S., A. Fribley and C. Y. Wang (2002). "Potentiation of tumor necrosis factor-mediated apoptosis of oral squamous cell carcinoma cells by adenovirus-mediated gene transfer of NF-B inhibitor." J Dent Res 81(2): 98-102.

Chen, X., L. J. Ko, L. Jayaraman and C. Prives (1996). "p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells." Genes Dev 10(19): 2438-2451.

Chen, Y. and M. Z. Lai (2001). "c-Jun NH2-terminal kinase activation leads to a FADD-dependent but Fas ligand-independent cell death in Jurkat T cells." J Biol Chem 276(11): 8350-8357.

Chen, Z. P. and K. Y. Chen (1997a). "Dramatic attenuation of hypusine formation on eukaryotic initiation factor 5A during senescence of IMR-90 human diploid fibroblasts." J Cell Physiol 170(3): 248-254.

Chen, Z. P. and K. Y. Chen (1997b). "Marked elevation of hypusine formation activity on eukaryotic initiation factor 5A in v-HA-RAS transformed mouse NIH3T3 cells." Cancer Lett 115(2): 235-241.

Chen, Z. P., Y. P. Yan, Q. J. Ding, S. Knapp, J. A. Potenza, H. J. Schugar and K. Y. Chen (1996). "Effects of inhibitors of deoxyhypusine synthase on the differentiation of mouse neuroblastoma and erythroleukemia cells." Cancer Lett 105(2): 233-239.

Cheng, E. H., D. G. Kirsch, R. J. Clem, R. Ravi, M. B. Kastan, A. Bedi, K. Ueno and J. M. Hardwick (1997). "Conversion of Bcl-2 to a Bax-like death effector by caspases." Science 278(5345): 1966-1968.

Cheng, E. H., W. C. Wei, S. Weiler, R. A. Flavell, T. W. Mak, T. Lindsten and S. J. Korsmeyer (2001). "BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis." Mol Cell 8(3): 705-711.

Cherney, B. W., K. Bhatia and G. Tosato (1994). "A role for deregulated c-Myc expression in apoptosis of Epstein-Barr virus-immortalized B cells." Proc Natl Acad Sci U S A 91(26): 12967-12971.

Cherry, L. M. and T. C. Hsu (1982). "Restitution of chromatid and isochromatid breaks induced in the G2 phase by actinomycin D." Environ Mutagen 4(3): 259-265.

Chiarugi, A. (2005). ""Simple but not simpler": toward a unified picture of energy requirements in cell death." FASEB J 19: 1783-1788.

Chinnaiyan, A. M., K. O'rourke, M. Tewari and W. M. Dixit (1995). "FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis." Cell 81(4): 505-512.

Chinnaiyan, A. M., C. G. Tepper, M. F. Seldin, K. O'rourke, F. C. Kischkel, S. Hellbardt, P. H. Krammer, M. E. Peter and W. M. Dixit (1996). "FADD/MORT1 Is a Common Mediator of CD95 (Fas/APO-1) and Tumor Necrosis Factor Receptor-induced Apoptosis." J Biol Chem 271(9): 4961-4965.

Chittenden, T., C. Flemington, A. B. Houghton, R. G. Ebb, G. J. Gallo, B. Elangovan, G. Chinnadurai and R. J. Lutz (1995). "A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions." EMBO J 14(22): 5589-5596.

Chowdary, D. R., J. J. Dermody, K. K. Jha and H. L. Ozer (1994). "Accumulation of p53 in a mutant cell line defective in the ubiquitin pathway." Mol Cell Biol 14(3): 1997-2003.

Chuang, Y. Y., Q. Chen, J. P. Brown, J. M. Sedivy and H. L. Liber (1999). "Radiation-induced mutations at the autosomal thymidine kinase locus are not elevated in p53-null cells." Cancer Res 59(13): 3073-3076.

Chung, S. I., M. H. Park, J. E. Folk and M. S. Lewis (1991). "Eukaryotic initiation factor 5A: the molecular form of the hypusine-containing protein from human erythrocytes." Biochim Biophys Acta. 1076(3): 448-451.

Clark, A. M. and K. A. Ellem (1966). "A correlated morphological and functional study of the effects of actinomycin D on hela cells. II. The selective effects of low concentrations on the rapidly labeled types of RNA separated by column chromatography." Ann Med Exp Biol Fenn 44(2): 100-108.

Clarke, A. R., C. A. Purdie, D. J. Harrison, R. G. Morris, C. C. Bird, M. L. Hooper and A. H. Wyllie (1993). "Thymocyte apoptosis induced by p53-dependent and independent pathways." Nature 362(6423): 849-852.

Cleary, M. L., S. D. Smith and J. Sklar (1986). "Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation." Cell 47(1): 19-28.

Clem, R. J., E. H. Cheng, C. L. Karp, D. G. Kirsch, K. Ueno, A. Takahashi, M. B. Kastan, D. E. Griffin, W. C. Earnshaw, M. A. Veliuona and J. M. Hardwick (1998). "Modulation of cell death by Bcl-XL through caspase interaction." Proc Natl Acad Sci U S A 95(2): 554-559.

Clement, P. M., C. A. Henderson, Z. A. Jenkins, Z. Smit-Mcbride, E. C. Wolff, J. W. Hershey, M. H. Park and J. E. Johansson (2003). "Identification and characterization of eukaryotic initiation factor 5A-2." Eur J Biochem 270(21): 4254-4263.

Clement, P. M., H. E. Johansson, E. C. Wolff and M. H. Park (2006). "Differential expression of eIF5A-1 and eIF5A-2 in human cancer cells." FEBS J 273(6): 1102-1114.

Cochrane, A. W., A. Perkins and C. A. Rosen (1990). "Identification of sequences important in the nucleolar localization of human immunodeficiency virus Rev: relevance of nucleolar localization to function." J Virol 64(2): 881-885.

Cohen, G. M. (1997). "Caspases: the executioners of apoptosis." Biochem J. 326(Pt 1): 1-16.

Cohen, G. M., X. M. Sun, R. T. Snowden, D. Dinsdale and D. N. Skilleter (1992). "Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation." Biochem J 286(Pt 2): 331-334.

Coller, H. A., C. Grandori, P. Tamayo, T. Colbert, E. S. Lander, R. N. Eisenman and T. R. Golub (2000). "Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion." Proc Natl Acad Sci U S A 97(7): 3260-3265.

Collins, J. A., C. A. Schandi, K. K. Young, J. Vesely and M. C. Willingham (1997). "Major DNA fragmentation is a late event in apoptosis." J Histochem Cytochem 45(7): 923-934.

Concannon, C. G., B. F. Koehler, C. Reimertz, B. M. Murphy, C. Bonner, N. Thurow, M. W. Ward, A. Villunger, A. Strasser, D. Kogel and J. H. Prehn (2007). "Apoptosis induced by proteasome inhibition in cancer cells: predominant role of the p53/PUMA pathway." Oncogene 26(12): 1681-1692.

Cooper, H. L., M. H. Park, J. E. Folk, B. Safer and R. Braverman (1982). "Posttranslational formation of hypusine in a single major protein occurs generally in growing cells and is associated with activation of lymphocyte growth." Cell 29(3): 791-797.

Cooper, H. L., M. H. Park, J. E. Folk, B. Safer and R. Braverman (1983). "Identification of the hypusine-containing protein hy+ as translation initiation factor eIF-4D." Proc Natl Acad Sci U S A. 80(7): 1854-1857.

Costanzo, A., P. Merlo, N. Pediconi, M. Fulco, V. Sartorelli, P. A. Cole, G. Fontemaggi, M. Fanciulli, L. Schiltz, G. Blandino, C. Balsano and M. Levrero (2002). "DNA damage-dependent acetylation of p73 dictates the selective activation of apoptotic target genes." Mol Cell 9(1): 175-186.

Cournoyer, J. J., J. L. Pittman, V. B. Ivleva, E. Fallows, L. Waskell, C. E. Costello and P. B. O'connor (2005). "Deamidation: Differentiation of aspartyl from isoaspartyl products in peptides by electron capture dissociation." Protein Sci 14(2): 452-463.

Cowan, C. A., N. Yokoyama, L. M. Bianchi, M. Henkemeyer and B. Fritzsch (2002). "EphB2 guides axons at the midline and is necessary for normal vestibular function." Neuron 26(2): 417-430.

Cracchiolo, B. M., D. S. Heller, P. M. Clement, E. C. Wolff, M. H. Park and H. M. Hanauske-Abel (2004). "Eukaryotic initiation factor 5A-1 (eIF5A-1) as a diagnostic marker for aberrant proliferation in intraepithelial neoplasia of the vulva." Gynecol Oncol 94(1): 217-222.

Cremesti, A., F. Paris, H. Grassme, N. Holler, J. Tschopp, Z. Fuks, E. Gulbins and R. Kolesnick (2001). "Ceramide enables fas to cap and kill." J Biol Chem 276(26): 23954-23961.

Csonga, R., P. Ettmayer, M. Auer, C. Eckerskorn, J. Eder and H. Klier (1995). "Evaluation of the metal ion requirement of the human deoxyhypusine hydroxylase from HeLa cells using a novel enzyme assay." FEBS Lett 380(3): 209-214.

Cuconati, A., C. Mukherjee, D. Perez and E. White (2003). "DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells." Genes Dev 17(23): 2922-2932.

Cui, S., J. S. Reichner, R. B. Mateo and J. E. Albina (1994). "Activated murine macrophages induce apoptosis in tumor cells through nitric oxide-dependent or -independent mechanisms." Cancer Res 54(9): 2462-2467.

Cullen, B. R. and M. H. Malim (1991). "The HIV-1 Rev protein: prototype of a novel class of eukaryotic post-transcriptional regulators." Trends Biochem Sci 16(9): 346-350.

Dahm-Daphi, J., C. Sass and W. Alberti (2000). "Comparison of biological effects of DNA damage induced by ionizing radiation and hydrogen peroxide in CHO cells." Int J Radiat Biol 76(1): 67-75.

Daly, T. J., K. S. Cook, G. S. Gray, T. E. Maione and J. R. Rusche (1989). "Specific binding of HIV-1 recombinant Rev protein to the Rev-responsive element in vitro." Nature 342(6251): 816-9.

Danial, N. N. and S. J. Korsmeyer (2004). "Cell death: critical control points." Cell 116(2): 205-219.

Das, S., W. S. El-Deiry and K. Somasundaram (2003a). "Efficient growth inhibition of HPV 16 E6-expressing cells by an adenovirus-expressing p53 homologue p73beta." Oncogene 22(52): 8394-8402.

Das, S., W. S. El-Deiry and K. Somasundaram (2003b). "Regulation of the p53 homolog p73 by adenoviral oncogene E1A." J Biol Chem 278(20): 18313-18320.

Das, S. and K. Somasundaram (2006). "Therapeutic potential of an adenovirus expressing p73 beta, a p53 homologue, against human papilloma virus positive cervical cancer *in vitro* and in vivo." Cancer Biol Ther 5(2): 210-217.

De Laurenzi, V., A. Costanzo, D. Barcaroli, A. Terrinoni, M. Falco, M. Annicchiarico-Petruzzelli, M. Levrero and G. Melino (1998). "Two new p73 splice variants, gamma and delta, with different transcriptional activity." J Exp Med 188(9): 1763-1768.

De Laurenzi, V. D., M. V. Catani, A. Terrinoni, M. Corazzari, G. Melino, A. Costanzo, M. Levrero and R. A. Knight (1999). "Additional complexity in p73: induction by mitogens in lymphoid cells and identification of two new splicing variants epsilon and zeta." Cell Death Differ 6(5): 389-390.

De Stanchina, E., M. E. Mccurrach, F. Zindy, S. Y. Shieh, G. Ferbeyre, A. V. Samuelson, C. Prives, M. F. Roussel, C. J. Sherr and S. W. Lowe (1999). "E1A signaling to p53 involves the p19(ARF) tumor suppressor." Genes Dev 12(15): 2434-2442.

Deas, O., C. Dumont, M. Macfarlane, M. Rouleau, C. Hebib, F. Harper, F. Hirsch, B. Charpentier, G. M. Cohen and A. Senik (1998). "Caspase-Independent Cell Death Induced by Anti-CD2 or Staurosporine in Activated Human Peripheral T Lymphocytes." J Immunol 161: 3375-3383.

Degenhardt, K., G. Chen, T. Lindsten and E. White (2002b). "BAX and BAK mediate p53-independent suppression of tumorigenesis." Cancer Cell 2(3): 193-203.

Degenhardt, K., R. Sundararajan, T. Lindsten, C. Thompson and E. White (2002a). "Bax and Bak independently promote cytochrome C release from mitochondria." J Biol Chem 277(16): 14127-14134.

Degli Esposti, M., G. Ferry, P. Masdehors, J. A. Boutin, J. A. Hickman and C. Dive (2003). "Post-translational modification of Bid has differential effects on its susceptibility to cleavage by caspase 8 or caspase 3." J Biol Chem 278(18): 15749-15757.

Degli-Esposti, M. A., W. C. Dougall, P. J. Smolak, J. Y. Waugh, C. A. Smith and R. G. Goodwin (1997). "The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain." Immunity 7(6): 813-820.

Degregori, J., T. Kowalik and J. R. Nevins (1995). "Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes." Mol Cell Biol 15(8): 4215-4224.

Degterev, A., M. Boyce and J. Yuan (2003). "A decade of caspases." Oncogene 22(53): 8543-8567.

Del Bello, B., M. A. Valentini, F. Zunino, M. Comporti and E. Maellaro (2001). "Cleavage of Bcl-2 in oxidant- and cisplatin-induced apoptosis of human melanoma cells." Oncogene 20(33): 4591-4595.

Delmas, D., C. Rebe, S. Lacour, R. Filomenko, A. Athias, P. Gambert, M. Cherkaoui-Malki, B. Jannin, L. Dubrez-Daloz, N. Latruffe and E. Solary (2003). "Resveratrol-induced apoptosis is associated with Fas redistribution in the rafts and the formation of a death-inducing signaling complex in colon cancer cells." J Biol Chem 278(42): 41482-41490.

Demaurex, N. and C. Distelhorst (2003). "Cell biology. Apoptosis--the calcium connection." Science 300(5616): 65-67.

Deng, Y., X. Ren, L. Yang, Y. Lin and X. Wu (2003). "A JNK-Dependent Pathway Is Required for TNFa-Induced Apoptosis." Cell 115(1): 61-70.

Desagher, S., A. Osen-Sand, S. Montessuit, E. Magnenat, F. Vilbois, A. Hochmann, L. Journot, B. Antonsson and J. C. Martinou (2001). "Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8." Mol Cell 8(3): 601-611.

Deshmukh, M., K. Kuida and E. M. Johnson Jr (2000). "Caspase inhibition extends the commitment to neuronal death beyond cytochrome c release to the point of mitochondrial depolarization." J Cell Biol 150(1): 131-q43.

Deverman, B. E., B. L. Cook, S. R. Manson, R. A. Niederhoff, E. M. Langer, I. Rosova, L. A. Kulans, X. Fu, J. S. Weinberg, J. W. Heinecke, K. A. Roth and S. J. Weintraub (2002). "Bcl-xL deamidation is a critical switch in the regulation of the response to DNA damage." Cell 111(1): 51-62.

Dijkers, P. F., K. U. Birkenkamp, E. W. Lam, N. S. Thomas, J. W. Lammers, L. Koenderman and P. J. Coffer (2002). "FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity." J Cell Biol 156(3): 531-542.

Ding, H. F., Y. L. Lin, G. Mcgill, P. Juo, H. Zhu, J. Blenis, J. Yuan and D. E. Fisher (2000). "Essential role for caspase-8 in transcription-independent apoptosis triggered by p53." J Biol Chem 275(49): 38905-38911.

Ding, W. X., H. M. Ni, X. Chen, J. Yu, L. Zhang and X. M. Yin (2007). "A coordinated action of Bax, PUMA, and p53 promotes MG132-induced mitochondria activation and apoptosis in colon cancer cells." Mol Cancer Ther 6(3): 1062-9.

Dobbelstein, M., S. Strano, J. Roth and G. Blandino (2005). "p73-induced apoptosis: a question of compartments and cooperation." Biochem Biophys Res Commun 331(3): 688-693.

Donehower, L. A., M. Harvey, B. L. Slagle, M. J. Mcarthur, C. A. M. Jr, J. S. Butel and A. Bradley (1992). "Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours." Nature 356(6366): 215-221.

Dong, Z., R. J. Arnold, Y. Yang, M. H. Park, P. Hrncirova, Y. Mechref, M. V. Novotny and J. T. Zhang (2005). "Modulation of differentiation-related gene 1 expression by cell cycle blocker mimosine, revealed by proteomic analysis." Mol Cell Proteomics 4(7): 993-1001.

Dragovich, T., C. M. Rudin and C. B. Thompson (1998). "Signal transduction pathways that regulate cell survival and cell death." Oncogene 17(25): 3207-3213.

Dresselhaus, T., S. Cordts and H. Lorz (1999). "A transcript encoding translation initiation factor eIF-5A is stored in unfertilized egg cells of maize." Plant Mol Biol 39(5): 1063-1071.

Drexler, H. C. (1997). "Activation of the cell death program by inhibition of proteasome function." Proc Natl Acad Sci U S A 94(3): 855-860.

Drexler, H. C. (1998). "Programmed cell death and the proteasome." Apoptosis 3(1): 1-7.

Driessen, H. P., W. W. De Jong, G. I. Tesser and H. Bloemendal (1985). "The mechanism of N-terminal acetylation of proteins." CRC Crit Rev Biochem 18(4): 281-325.

Du, C., M. Fang, Y. Li, L. Li and X. Wang (2000). "Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition." Cell 102(1): 33-42.

Dumont, C., A. Durrbach, N. Bidere, M. Rouleau, G. Kroemer, G. Bernard, F. Hirsch, B. Charpentier, S. A. Susin and A. Senik (2000). "Caspase-independent commitment phase to apoptosis in activated blood T lymphocytes: reversibility at low apoptotic insult." Blood 96(3): 1030-1038.

Dutta, J., Y. Fan, N. Gupta, G. Fan and C. Gelinas (2006). "Current insights into the regulation of programmed cell death by NF-kappaB." Oncogene 25(51): 6800-6816.

Earnshaw, W. C., L. M. Martins and S. H. Kaufmann (1999). "Mammalian caspases: structure, activation, substrates, and functions during apoptosis." Annu Rev Biochem 68: 383-424.

Eby, M. T., A. Jasmin, A. Kumar, K. Sharma and P. M. Chaudhary (2000). "TAJ, a Novel Member of the Tumor Necrosis Factor Receptor Family, Activates the c-Jun N-terminal Kinase Pathway and Mediates Caspase-independent Cell Death." J Biol Chem 275(20): 15336-15342.

Egle, A., A. W. Harris, P. Bouillet and S. Cory (2004). "Bim is a suppressor of Myc-induced mouse B cell leukemia." Proc Natl Acad Sci U S A 101(16): 6164-6169.

Eguchi, Y., S. Shimizu and Y. Tsujimoto (1997). "Intracellular ATP levels determine cell death fate by apoptosis or necrosis." Cancer Res 57(10): 1835-1840.

El-Deiry, W. S. (1998). "Regulation of p53 downstream genes." Semin Cancer Biol 8(5): 345-357.

El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler and B. Vogelstein (1993). "WAF1, a potential mediator of p53 tumor suppression." Cell 75(4): 817-825.

Elfgang, C., O. Rosorius, L. Hofer, H. Jaksche, J. Hauber and D. Bevec (1999). "Evidence for specific nucleocytoplasmic transport pathways used by leucine-rich nuclear export signals." Proc Natl Acad Sci U S A. 96(11): 6229-6234.

Eman, M. R., E. Regan-Klapisz, M. W. Pinkse, I. W. Koop, J. Haverkamp, A. J. Heck, A. J. Verkleij and J. A. Post (2006). "Protein expression dynamics during replicative senescence of endothelial cells studied by 2-D difference in-gel electrophoresis." Electrophoresis 27(8): 1669-1682.

Emanuele, S., G. Calvaruso, M. Lauricella, M. Giuliano, G. Bellavia, A. D'anneo, R. Vento and G. Tesoriere (2002). "Apoptosis induced in hepatoblastoma HepG2 cells by the proteasome inhibitor MG132 is associated with hydrogen peroxide production, expression of Bcl-XS and activation of caspase-3." Int J Oncol 21(4): 857-865.

Emery, J. G., P. Mcdonnell, M. B. Burke, K. C. Deen, S. Lyn, C. Silverman, E. Dul, E. R. Appelbaum, C. Eichman, R. Diprinzio, R. A. Dodds, J. E. James, M. Rosenberg, J. C. Lee and P. R. Young (1998). "Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL." J Biol Chem 273(23): 14363-14367.

Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu and S. Nagata (1998). "A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD." Nature 391(6662): 43-50.

Endo, H., H. Kamada, C. Nito, T. Nishi and P. H. Chan (2006). "Mitochondrial translocation of p53 mediates release of cytochrome c and hippocampal CA1 neuronal death after transient global cerebral ischemia in rats." J Neurosci 26(30): 7974-7983.

Engels, I. H., A. Stepczynska, C. Stroh, K. Lauber, C. Berg, R. Schwenzer, H. W. H, R. U. Janicke, A. G. Porter, C. Belka, M. Gregor, K. Schulze-Osthoff and S. Wesselborg (2000). "Caspase-8/FLICE functions as an executioner caspase in anticancer drug-induced apoptosis." Oncogene 19(40): 4563-4573.

Erster, S., M. Mihara, R. H. Kim, O. Petrenko and U. M. Moll (2004). "*In vivo* mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation." Mol Cell Biol 24(15): 6728-6741.

Eshaque, B. (2006). "Characterization of Eukaryotic Translation Initiation Factor 5A isoforms (eIF-5A1 & eIF-5A2) using human cell lines as a model system."

Eskes, R., B. Antonsson, A. Osen-Sand, S. Montessuit, C. Richter, R. Sadoul, G. Mazzei, A. Nichols and J. C. Martinou (1998). "Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg2+ ions." J Cell Biol 143(1): 217-224.

Eskes, R., S. Desagher, B. Antonsson and J. Martinou (2000). "Bid Induces the Oligomerization and Insertion of Bax into the Outer Mitochondrial Membrane." Mol Biol Cell 20(3): 929-935.

Evan, G. I., A. H. Wyllie, C. S. Gilbert, T. D. Littlewood, H. Land, M. Brooks, C. M. Waters, L. Z. Penn and D. C. Hancock (1992). "Induction of apoptosis in fibroblasts by c-myc protein." Cell 69(1): 119-128.

Facchiano, A. M., P. Stiuso, M. L. Chiusano, M. Caraglia, G. Giuberti, M. Marra, A. Abbruzzese and G. Colonna (2001). "Homology modelling of the human eukaryotic initiation factor 5A (eIF-5A)." Protein Eng. 14(11): 881-890.

Fadeel, B., Z. Hassan, E. Hellstrom-Lindberg, J. I. Henter, S. Orrenius and B. Zhivotovsky (1999). "Cleavage of Bcl-2 is an early event in chemotherapy-induced apoptosis of human myeloid leukemia cells." Leukemia 13(5): 719-728.

Fais, S., A. De Milito and F. Lozupone (2005). "The role of FAS to ezrin association in FAS-mediated apoptosis." Apoptosis 10(5): 941-947.

Falcieri, E., A. M. Martelli, R. Bareggi, A. Cataldi and L. Cocco (1993). "The protein kinase inhibitor staurosporine induces morphological changes typical of apoptosis in MOLT-4 cells without concomitant DNA fragmentation." Biochem Biophys Res Commun 193(1): 19-25.

Fei, P., E. J. Bernhard and W. S. El-Deiry (2002). "Tissue-specific induction of p53 targets in vivo." Cancer Res 62(24): 7316-7327.

Felley-Bosco, E. (1998). "Role of nitric oxide in genotoxicity: implication for carcinogenesis." Cancer Metastasis Rev 17(1): 25-37.

Fernandez-Larrea, J., A. Merlos-Suarez, J. M. Urena, J. Baselga and J. Arribas (1999). "A role for a PDZ protein in the early secretory pathway for the targeting of proTGF-alpha to the cell surface." Mol Cell 3(4): 423-433.

Ferrari, D., A. Stepczynska, M. Los, S. Wesselborg and K. Schulze-Osthoff (1998). "Differential regulation and ATP requirement for caspase-8 and caspase-3 activation during CD95- and anticancer drug-induced apoptosis." J Exp Med 188(5): 979-984.

Ferreira, C. G., S. W. Span, G. J. Peters, F. A. Kruyt and G. Giaccone (2000). "Chemotherapy Triggers Apoptosis in a Caspase-8-dependent and Mitochondria-controlled Manner in the Non-Small Cell Lung Cancer Cell Line NCI-H460." Cancer Res 60(24): 7133-7141.

Ferri, K. F. and G. Kroemer (2000). "Control of apoptotic DNA degradation." Nat Cell Biol 2(4): E63-64.

Fesus, L., V. Thomazy, F. Autuori, M. P. Ceru, E. Tarcsa and M. Piacentini (1989). "Apoptotic hepatocytes become insoluble in detergents and chaotropic agents as a result of transglutaminase action." FEBS Lett 245(1-2): 150-154.

Fesus, L., V. Thomazy and A. Falus (1987). "Induction and activation of tissue transglutaminase during programmed cell death." FEBS Lett 224(1): 104-108.

Finucane, D. M., E. Bossy-Wetzel, N. J. Waterhouse, T. G. Cotter and D. R. Green (1999). "Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL." J Biol Chem 274(4): 2225-2233.

Fischer, U., R. U. Janicke and K. Schulze-Osthoff (2003). "Many cuts to ruin: a comprehensive update of caspase substrates." Cell Death Differ 10(1): 76-100.

Fletcher, J. I. and D. C. Huang (2006). "BH3-only proteins: orchestrating cell death." Cell Death Differ 13(8): 1268-1271.

Flinterman, M., L. Guelen, S. Ezzati-Nik, R. Killick, G. Melino, K. Tominaga, J. S. Mymryk, J. Gaken and M. Tavassoli (2005). "E1A activates transcription of p73 and Noxa to induce apoptosis." J Biol Chem 280(7): 5945-5959.

Folk, J. E. and J. S. Finlayson (1977). "The epsilon-(gamma-glutamyl)lysine crosslink and the catalytic role of transglutaminases." Adv Protein Chem 31(1): 1-133.

Fontemaggi, G., I. Kela, N. Amariglio, G. Rechavi, J. Krishnamurthy, S. Strano, A. Sacchi, D. Givol and G. Blandino (2002). "Identification of direct p73 target genes combining DNA microarray and chromatin immunoprecipitation analyses." J Biol Chem 277(45): 43359-43368.

Fortney, J. E., B. M. Hall, L. Bartrug and L. F. Gibson (2002). "Chemotherapy induces bcl-2 cleavage in lymphoid leukemic cell lines." Leuk Lymphoma 43(11): 2171-2178.

Fribley, A., Q. Zeng and C. Y. Wang (2004). "Proteasome Inhibitor PS-341 Induces Apoptosis through Induction of Endoplasmic Reticulum Stress-Reactive Oxygen Species in Head and Neck Squamous Cell Carcinoma Cells." Mol Cell Biol 24(22): 9695-9704.

Friedman, A. R., A. K. Ichhpurani, D. M. Brown, R. M. Hillman, L. F. Krabill, R. A. Martin, H. A. Zurcher-Neely and D. M. Guido (1991). "Degradation of growth hormone releasing factor analogs in neutral aqueous solution is related to deamidation of asparagine residues. Replacement of asparagine residues by serine stabilizes." Int J Pept Protein Res 37(1): 14-20.

Frischmeyer, P. A. and H. C. Dietz (1999). "Nonsense-mediated mRNA decay in health and disease." Hum Mol Genet 8(10): 1893-1900.

Frottin, F., A. Martinez, P. Peynot, S. Mitra, R. C. Holz, C. Giglione and T. Meinnel (2006). "The proteomics of N-terminal methionine cleavage." Mol Cell Proteomics 5(12): 2336-2349.

Fujii, J., T. Matsui, D. P. Heatherly, K. H. Schlegel, P. I. Lobo, T. Yutsudo, G. M. Ciraolo, R. E. Morris and T. Obrig (2003). "Rapid apoptosis induced by Shiga toxin in HeLa cells." Infect Immun 7(5): 2724-2735.

Fujita, E., T. Mukasa, T. Tsukahara, K. Arahata, S. Omura and T. Momoi (1996). "Enhancement of CPP32-like activity in the TNF-treated U937 cells by the proteasome inhibitors." Biochem Biophys Res Commun 224(1): 74-79.

Fujita, N., A. Nagahashi, K. Nagashima, S. Rokudai and T. Tsuruo (1998). "Acceleration of apoptotic cell death after the cleavage of Bcl-XL protein by caspase-3-like proteases." Oncogene 17(10): 1295-1304.

Fujita, N. and T. Tsuruo (1998). "Involvement of Bcl-2 cleavage in the acceleration of VP-16-induced U937 cell apoptosis." Biochem Biophys Res Commun 246(2): 484-8.

Fukuda, M., S. Asano, T. Nakamura, M. Adachi, M. Yoshida, M. Yanagida and E. Nishida (1997). "CRM1 is responsible for intracellular transport mediated by the nuclear export signal." Nature 390(6657): 308-311.

Furukawa, Y., N. Nishimura, Y. Furukawa, M. Satoh, H. Endo, S. Iwase, H. Yamada, M. Matsuda, Y. Kano and M. Nakamura (2002). "Apaf-1 is a mediator of E2F-1-induced apoptosis." J Biol Chem 277(42): 39760-39768.

Fuster, J. J., S. M. Sanz-Gonzalez, U. M. Moll and V. Andres (2007). "Classic and novel roles of p53: prospects for anticancer therapy." Trends Mol Med.

Gahl, W. A. and H. C. Pittot (1978). "Reversal by aminoguandine of the inhibition of preliferation of human fibroblasts by spermidine and spermine." Chem. Biol. Interactions 22: 91-98.

Gajate, C., E. Del Canto-Janez, A. U. Acuna, F. Amat-Guerri, E. Geijo, A. M. Santos-Beneit, R. J. Veldman and F. Mollinedo (2004). "Intracellular triggering of Fas aggregation and recruitment of apoptotic molecules into Fas-enriched rafts in selective tumor cell apoptosis." J Exp Med 200(3): 353-365.

Gajate, C. and F. Mollinedo (2005). "Cytoskeleton-mediated death receptor and ligand concentration in lipid rafts forms apoptosis-promoting clusters in cancer chemotherapy." J Biol Chem 280(12): 11641-11647.

Galli, G. and M. Fratelli (1993). "Activation of apoptosis by serum deprivation in a teratocarcinoma cell line: inhibition by L-acetylcarnitine." Exp Cell Res 204(1): 54-60.

Garrido, C. and G. Kroemer (2004). "Life's smile, death's grin: vital functions of apoptosis-executing proteins." Curr Opin Cell Biol. 16(6): 639-646.

Geiger, T. and S. Clarke (1987). "Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation." J Biol Chem 262(2): 785-794.

Geijsen, N., I. J. Uings, C. Pals, J. Armstrong, M. Mckinnon, J. A. Raaijmakers, J. J. Lammers, L. Koenderman and P. J. Coffer (2001). "Cytokine-Specific Transcriptional Regulation Through an IL-5R Interacting Protein." Science 293(5532): 1136 - 1138.

Gélinas, C. and E. White (2005). "BH3-only proteins in control: specificity regulates MCL-1 and BAK-mediated apoptosis." Genes Dev 19(11): 1263-1268.

Gerloff, D. L., M. Joachimiak, F. E. Cohen, G. M. Cannarozzi, S. G. Chamberlin and S. A. Benner (1998). "Structure prediction in a post-genomic environment: a secondary and tertiary structural model for the initiation factor 5A family." Biochem Biophys Res Commun 251(1): 173-181.

Germain, M., J. P. Mathai and G. C. S. Gc (2002). "BH-3-only BIK functions at the endoplasmic reticulum to stimulate cytochrome c release from mitochondria." J Biol Chem 277(20): 18053-18060.

Gerner, E. W., P. S. Mamont, A. Bernhardt and M. Siat (1986). "Post-translational modification of the protein-synthesis initiation factor eIF-4D by spermidine in rat hepatoma cells." Biochem J 239(2): 379-386.

Gerschenson, M., K. L. Houmiel and R. L. Low (1995). "Endonuclease G from mammalian nuclei is identical to the major endonuclease of mitochondria." Nucleic Acids Res 23: 88-97.

Ghatan, S., S. Larner, Y. Kinoshita, M. Hetman, L. Patel, Z. Xia, R. J. Youle and R. S. Morrison (2000). "p38 MAP kinase mediates bax translocation in nitric oxide-induced apoptosis in neurons." J Cell Biol 150(2): 335-347.

Giglione, C., A. Boularot and T. Meinnel (2004). "Protein N-terminal methionine excision." Cell Mol Life Sci 61(12): 1455-1474.

Giglione, C., O. Vallon and T. Meinnel (2003). "Control of protein life-span by N-terminal methionine excision." EMBO J 22(11): 13-23.

Gilley, J., P. J. Coffer and J. Ham (2003). "FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons." J Cell Biol 162(4): 613-622.

Ginsberg, D. (2002). "E2F1 pathways to apoptosis." FEBS Lett 529(1): 122-125.

Glick, B. R. and M. C. Ganoza (1975). "Identification of a soluble protein that stimulates peptide bond synthesis." Proc Natl Acad Sci U S A 72(11): 4257-4260.

Glick, B. R. and M. C. Ganoza (1976). "Characterization and site of action of a soluble protein that stimulates peptide-bond synthesis." Eur J Biochem 71(2): 483-491.

Glickman, M. H. and A. Ciechanover (2002). "The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction." Physiol Rev 82(2): 373-428.

Gniadecki, R. (2004). "Depletion of membrane cholesterol causes ligand-independent activation of Fas and apoptosis." Biochem Biophys Res Commun 320(1): 165-169.

Gold, R., M. Schmied, G. Giegerich, H. Breitschopf, H. P. Hartung, K. V. Toyka and H. Lassmann (1994). "Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques." Lab Invest 71(2): 219-225.

Goldstein, J. C., C. Munoz-Pinedo, J. E. Ricci, S. R. Adams, A. Kelekar, M. Schuler, R. Y. Tsien and D. R. Green (2005). "Cytochrome c is released in a single step during apoptosis." Cell Death Differ 12(5): 453-462.

Goldstein, J. C., N. J. Waterhouse, P. Juin, G. I. Evan and D. R. Green (2000). "The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant." Nat Cell Biol 2(3): 156-162.

Gomez-Bougie, P., R. Bataille and M. Amiot (2004). "The imbalance between Bim and Mcl-1 expression controls the survival of human myeloma cells." Eur J Immunol 34(11): 3156-3164.

Gomez-Bougie, P., R. Bataille and M. Amiot (2005). "Endogenous association of Bim BH3-only protein with Mcl-1, Bcl-xL and Bcl-2 on mitochondria in human B cells." Eur J Immunol 35(3): 971-976.

Gomez-Bougie, P., L. Oliver, S. Le Gouill, R. Bataille and M. Amiot (2005). "Melphalan-induced apoptosis in multiple myeloma cells is associated with a cleavage of Mcl-1 and Bim and a decrease in the Mcl-1/Bim complex." Oncogene 24(54): 8076-8079.

Gonda, D. K., A. Bachmair, I. Wunning, J. W. Tobias, W. S. Lane and A. Varshavsky (1989). "Universality and structure of the N-end rule." J Biol Chem 264(28): 16700-16712.

Gong, J. G., A. Costanzo, H. Q. Yang, G. Melino, W. G. Kaelin Jr, M. Levrero and J. Y. Wang (1999). "The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage." Nature 399(6738): 806-9.

González, L. J., T. Shimizu, Y. Satomi, L. Betancourt, V. Besada, G. Padrón, R. Orlando, T. Shirasawa, Y. Shimonishi and T. Takao (2000). "Differentiating alpha- and beta-aspartic acids by electrospray ionization and low-energy tandem mass spectrometry." Rapid Commun Mass Spectrom 14(22): 2092-2102.

Gopalakrishna, R., Z. H. Chen and U. Gundimeda (1993). "Nitric oxide and nitric oxide-generating agents induce a reversible inactivation of protein kinase C activity and phorbol ester binding." J Biol Chem 268(36): 27180-27185.

Gorczyca, W., J. Gong and Z. Darzynkiewicz (1993). "Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays." Cancer Res 53(8): 1945-1951.

Gordon, E. D., R. Mora, S. C. Meredith, C. Lee and S. L. Lindquist (1987a). "Eukaryotic initiation factor 4D, the hypusine-containing protein, is conserved among eukaryotes." J Biol Chem 262(34): 16585-16589.

Gordon, E. D., R. Mora, S. C. Meredith and S. L. Lindquist (1987b). "Hypusine formation in eukaryotic initiation factor 4D is not reversed when rates or specificity of protein synthesis is altered." J Biol Chem 262(34): 16590-16595.

Gorlich, D. and U. Kutay (1999). "Transport between the cell nucleus and the cytoplasm." Annu Rev Cell Dev Biol. 15: 607-660.

Gotoh, T., S. Oyadomari, K. Mori and M. Mori (2002). "Nitric oxide-induced apoptosis in RAW 264.7 macrophages is mediated by endoplasmic reticulum stress pathway involving ATF6 and CHOP." J Biol Chem 277(14): 12343-12350.

Goyns, M. H. (1982). "The role of polyamines in animal cell physiology." J Theor Biol 97(4): 577-589.

Grandgirard, D., E. Studer, L. Monney, T. Belser, I. Fellay, C. Borner and M. R. Michel (1998). "Alphaviruses induce apoptosis in Bcl-2-over-expressing cells: evidence for a caspase-mediated, proteolytic inactivation of Bcl-2." EMBO J 17(5): 1268-1278.

Grasl-Kraupp, B., B. Ruttkay-Nedecky, H. Koudelka, K. Bukowska, W. Bursch and R. Schulte-Hermann (1995). "In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note." Hepatology 21(5): 1465-1468.

Green, D. R. and J. C. Reed (1998). "Mitochondria and apoptosis." Science 281(5381): 1309-1312.

Griffiths, G. J., L. Dubrez, C. P. Morgan, N. A. Jones, J. Whitehouse, B. M. Corfe, C. Dive and J. A. Hickman (1999). "Cell damage-induced conformational changes of the pro-apoptotic protein Bak *in vivo* precede the onset of apoptosis." J Cell Biol 144(5): 903-914.

Griffiths, J. B. (1987). "Serum and growth factors in cell culture media--an introductory review." Dev Biol Stand 66: 155-160.

Gromov, P., G. L. Skovgaard, H. Palsdottir, I. Gromova, M. Ostergaard and J. E. Celis (2003). "Protein profiling of the human epidermis from the elderly reveals up-regulation of a signature of interferon-gamma-induced polypeptides that includes manganese-superoxide dismutase and the p85beta subunit of phosphatidylinositol 3-kinase." Mol Cell Proteomics 2(2): 70-84.

Grootjans, J. J., P. Zimmermann, G. Reekmans, A. Smets, G. Degeest, J. Durr and G. David (1997). "Syntenin, a PDZ protein that binds syndecan cytoplasmic domains." Proc Natl Acad Sci U S A 94(25): 13683-13688.

Guan, X. Y., J. M. Fung, N. F. Ma, S. H. Lau, L. S. Tai, D. Xie, Y. Zhang, L. Hu, Q. L. Wu, Y. Fang and J. S. Sham (2004). "Oncogenic role of eIF-5A2 in the development of ovarian cancer." Cancer Res 64(12): 4197-4200.

Guan, X. Y., J. S. Sham, T. C. Tang, Y. Fang, K. K. Huo and J. M. Yang (2001). "Isolation of a novel candidate oncogene within a frequently amplified region at 3q26 in ovarian cancer." Cancer Res 61(9): 3806-3809.

Gui, J., Y. Song, N. L. Han and F. S. Sheu (2007). "Characterization of transcriptional regulation of neurogranin by nitric oxide and the role of neurogranin in SNP-induced cell death: implication of neurogranin in an increased neuronal susceptibility to oxidative stress." Int J Biol Sci 3(4): 212-224.

Gupta, R. and O. P. Srivastava (2004a). "Effect of deamidation of asparagine 146 on functional and structural properties of human lens alphaB-crystallin." Invest Ophthalmol Vis Sci 45(1): 206-214.

Gupta, R. and O. P. Srivastava (2004b). "Deamidation affects structural and functional properties of human alphaA-crystallin and its oligomerization with alphaB-crystallin." J Biol Chem 279(43): 44258-44369.

Gustafsson, A. B. and R. A. Gottlieb (2007). "Bcl-2 family members and apoptosis, taken to heart." Am J Physiol Cell Physiol 292(1): C45-51.

Guy, A. L. and J. H. Taylor (1978). "Actinomycin D Inhibits Initiation of DNA Replication in Mammalian Cells." Proc Natl Acad Sci U S A 75(12): 6088-6092.

Häcker, G. (2000). "The morphology of apoptosis." Cell and Tissue Research 301(1): 5-17.

Hahn, H. P., M. Pang, J. He, J. D. Hernandez, R. Y. Yang, L. Y. Li, X. Wang, F. T. Liu and L. G. Baum (2004). "Galectin-1 induces nuclear translocation of endonuclease G in caspase- and cytochrome c-independent T cell death." Cell Death Differ 11(12): 1277-1286.

Hainaut, P. and M. Hollstein (2000). "p53 and human cancer: the first ten thousand mutations." Adv Cancer Res. 77: 81-137.

Haitel, A., H. G. Wiener, U. Baethge, M. Marberger and M. Susani (2000). "mdm2 expression as a prognostic indicator in clear cell renal cell carcinoma: comparison with p53 over-expression and clinicopathological parameters." Clin Cancer Res 6(5): 1840-1844.

Han, J., J. A. Goldstein, B. R. Gastman, C. J. Froelich, X. M. Yin and H. Rabinowich (2004). "Degradation of Mcl-1 by granzyme B: implications for Bim-mediated mitochondrial apoptotic events." J Biol Chem 279(21): 22020-22029.

Han, J., L. A. Goldstein, B. R. Gastman, A. Rabinovitz and H. Rabinowich (2005). "Disruption of Mcl-1.Bim complex in granzyme B-mediated mitochondrial apoptosis." J Biol Chem 280(16): 16383-16392.

Han, J., L. A. Goldstein, B. R. Gastman and H. Rabinowich (2006). "Interrelated roles for Mcl-1 and BIM in regulation of TRAIL-mediated mitochondrial apoptosis." J Biol Chem 281(15): 10153-10163.

Hanauske-Abel, H. M., M. H. Park, A. R. Hanauske, A. M. Popowicz, M. Lalande and J. E. Folk (1994). "Inhibition of the G1-S transition of the cell cycle by inhibitors of deoxyhypusine hydroxylation." Biochim Biophys Acta 1221(2): 115-124.

Hanauske-Abel, H. M., B. Slowinska, S. Zagulska, R. C. Wilson, L. Staiano-Coico, A. R. Hanauske, T. Mccaffrey and P. Szabo (1995). "Detection of a sub-set of polysomal mRNAs associated with modulation of hypusine formation at the G1-S boundary. Proposal of a role for eIF-5A in onset of DNA replication." FEBS Lett 366(2-3): 92-98.

Hao, D. L., C. M. Liu, W. J. Dong, H. Gong, X. S. Wu, D. P. Liu and C. C. Liang (2005). "Knockdown of human p53 gene expression in 293-T cells by retroviral vector-mediated short hairpin RNA." Acta Biochim Biophys Sin (Shanghai). 37(11): 779-783.

Hara, T., M. Omura-Minamisawa, C. Chao, Y. Nakagami, M. Ito and T. Inoue (2005). "Bcl-2 inhibitors potentiate the cytotoxic effects of radiation in Bcl-2 over-expressing radioresistant tumor cells." Int J Radiat Oncol Biol Phys 61(2): 517-528.

Harder, T. and K. Simons (1999). "Clusters of glycolipid and glycosylphosphatidylinositol-anchored proteins in lymphoid cells: accumulation of actin regulated by local tyrosine phosphorylation." Eur J Immunol 29(2): 556-562.

Harrington, E. A., A. Fanidi and G. I. Evan (1994). "Oncogenes and cell death." Curr Opin Genet Dev 4(1): 120-129.

Harris, M. H. and C. B. Thompson (2003). "The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability." Cell Death Differ 7(12): 1182-1191.

Hart, R. A., J. N. Billaud, S. J. Choi and T. R. Phillips (2002). "Effects of 1,8-diaminooctane on the FIV Rev regulatory system." Virology. 304(1): 97-104.

Harvey, D., P. Brokstein, L. Hong, M. Evans-Holm, C. Su, G. Tsang, S. Lewis and G. M. Rubin (1997). "BDGP/HHMI Drosophila EST Project. GenBank Accession numbers AI06678, AI106907, AI108365, AI113383 and AI 135193."

Harvey, M., M. J. Mcarthur, C. A. Montgomery Jr, J. S. Butel, A. Bradley and L. A. Donehower (1993). "Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice." Nat Genet 5(3): 225-229.

Hauber, I., D. Bevec, J. Heukeshoven, F. Kratzer, F. Horn, A. Choidas, T. Harrer and J. Hauber (2005). "Identification of cellular deoxyhypusine synthase as a novel target for antiretroviral therapy." J Clin Invest 115(1): 76-85.

Haupt, S., M. Berger, Z. Goldberg and Y. Haupt (2003). "Apoptosis - the p53 network." J Cell Sci 116(Pt 20): 4077-4085.

Hayflick, L. (1965). "The limited *in vitro* lifetime of huma diploid cell strains." Exp Cell Res 37: 614-636.

He, J., Y. Xiao, C. A. Casiano and L. Zhang (2000). "Role of mitochondrial cytochrome c in cocaine-induced apoptosis in coronary artery endothelial cells." J Pharmacol Exp Ther 295(3): 896-903.

Heibein, J. A., I. S. Goping, M. Barry, M. J. Pinkoski, G. C. Shore, D. R. Green and R. C. Bleackley (2000). "Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax." J Exp Med 192(10): 1391-1402.

Heiskanen, K. M., M. B. Bhat, H. W. Wang, J. Ma and A. L. Nieminen (1999). "Mitochondrial depolarization accompanies cytochrome c release during apoptosis in PC6 cells." J Biol Chem 274(9): 5654-5658.

Helmke, B. M., M. Polychronidis, A. Benner, M. Thome, J. Arribas and M. Deichmann (2004). "Melanoma metastasis is associated with enhanced expression of the syntenin gene." Oncol Rep 12(2): 221-8.

Helton, E. S. and X. C. X (2007). "p53 modulation of the DNA damage response." J Cell Biochem 100(4): 883-896.

Hemann, M. T., A. Bric, J. Teruya-Feldstein, A. Herbst, J. A. Nilsson, C. Cordon-Cardo, J. L. Cleveland, W. P. Tansey and S. W. Lowe (2005). "Evasion of the p53 tumour surveillance network by tumour-derived MYC mutants." Nature 436(7052): 807-811.

Henderson, B. R. and P. Percipalle (1997). "Interactions between HIV Rev and nuclear import and export factors: the Rev nuclear localisation signal mediates specific binding to human importin-beta." J Mol Biol 274(5): 693-707.

Hengartner, M. O. (2000). "The biochemistry of apoptosis." Nature 407(6805): 770-776.

Herault, O., P. Colombat, J. Domenech, M. Degenne, J. L. Bremond, L. Sensebe, M. C. Bernard and C. Binet (1999). "A rapid single-laser flow cytometric method for discrimination of early apoptotic cells in a heterogenous cell population." Br J Haematol 104(3): 530-537.

Herrmann, J. L., F. Briones Jr, S. Brisbay, C. J. Logothetis and T. J. Mcdonnell (1998). "Prostate carcinoma cell death resulting from inhibition of proteasome activity is independent of functional Bcl-2 and p53." Oncogene 17(22): 2889-2899.

Hershey, J. W. (1994). "Expression of initiation factor genes in mammalian cells." Biochimie 76(9): 847-852.

Hershko, T. and D. Ginsberg (2004). "Up-regulation of Bcl-2 homology 3 (BH3)-only proteins by E2F1 mediates apoptosis." J Biol Chem 279(10): 8627-8634.

Hershko, T., K. Korotayev, S. Polager and D. Ginsberg (2006). "E2F1 modulates p38 MAPK phosphorylation via transcriptional regulation of ASK1 and Wip1." J Biol Chem 281(42): 31309-31316.

Hidaka, M., J. Inoue, M. Yoshida and M. Seiki (1988). "Post-transcriptional regulator (rex) of HTLV-1 initiates expression of viral structural proteins but suppresses expression of regulatory proteins." EMBO J 7(2): 519-523.

Hirotani, M., Y. Zhang, N. Fujita, M. Naito and T. Tsuruo (1999). "NH2-terminal BH4 domain of Bcl-2 is functional for heterodimerization with Bax and inhibition of apoptosis." J Biol Chem 274(29): 20415-20420.

Hockenbery, D., G. Nunez, C. Milliman, R. D. Schreiber and S. J. Korsmeyer (1990). "Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death." Nature 348(6299): 334-6.

Hoetelmans, R. W., C. J. Van De Velde and J. H. Van Dierendonck (2003). "The presence of 19-kDa Bcl-2 in dividing cells." Cell Prolif 36(6): 293-306.

Hoffman, W. H., S. Biade, J. T. Zilfou, J. Chen and M. Murphy (2002). "Transcriptional repression of the anti-apoptotic survivin gene by wild type p53." J Biol Chem 277(5): 3247-3257.

Hofmann, W., B. Reichart, A. Ewald, E. Muller, I. Schmitt, R. H. Stauber, F. Lottspeich, B. M. Jockusch, U. Scheer, J. Hauber and M. C. Dabauvalle (2001). "Cofactor requirements for nuclear export of Rev response element (RRE)- and constitutive transport element (CTE)-containing retroviral RNAs. An unexpected role for actin." J Cell Biol 152(5): 895-910.

Holmberg, C., K. Helin, M. Sehested and O. Karlstrom (1998). "E2F-1-induced p53-independent apoptosis in transgenic mice." Oncogene 17(2): 143-155.

Holtta, E., P. Pulkkinen, K. Elfving and J. Janne (1975). "Oxidation of polymines by diamine oxidase from human seminal plasma." Biochem J 145(2): 373-378.

Honda, R., H. Tanaka and H. Yasuda (1997). "Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53." FEBS Lett 420(1): 25-27.

Hong, S. J., T. M. Dawson and V. L. Dawson (2004). "Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling." Trends Pharmacol Sci 25(5): 259-264.

Hou, Q. and Y. T. Hsu (2005). "Bax translocates from cytosol to mitochondria in cardiac cells during apoptosis: development of a GFP-Bax-stable H9c2 cell line for apoptosis analysis." Am J Physiol Heart Circ Physiol 289(1): H477-487.

Hsu, H., J. Huang, H. B. Shu, V. Baichwal and D. V. Goeddel (1996). "TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex." Immunity 4(4): 387-396.

Hsu, H., J. Xiong and D. W. Goeddel (1995). "The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation." Cell 81(4): 495-504.

Hu, Y., M. A. Benedict, L. Ding and G. Nunez (1999). "Role of cytochrome c and dATP/ATP hydrolysis in Apaf-1-mediated caspase-9 activation and apoptosis." EMBO J 18(13): 3586-3595.

Hu, Z., K. Chen, L. Wang and Q. Yao (2005). "Identification and characterization of Bombyx mori eIF5A gene through bioinformatics approaches." In Silico Biol 5(5-6): 573-580.

Huang, D. C., J. M. Adams and S. Cory (1998). "The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4." EMBO J 17(4): 1029-1039.

Huang, H. L., L. M. Fang, S. P. Lu, C. K. Chou, T. Y. Luh and M. Z. Lai (2003). "DNA-damaging reagents induce apoptosis through reactive oxygen species-dependent Fas aggregation." Oncogene 22(50): 8168-8177.

Huffaker, T. C., J. H. Thomas and D. Botstein (1988). "Diverse effects of beta-tubulin mutations on microtubule formation and function." J Cell Biol 106(6): 1997-2010.

Hunter, J. J. and T. G. Parslow (1996). "A peptide sequence from Bax that converts Bcl-2 into an activator of apoptosis." J Biol Chem 271(15): 8521-8524.

Imaoka, N. and T. Nakajima (1973). "Hypusine, N6-(4-amino-2-hydroxybutyl)-2,6-diaminohexanoic acid, in tissue proteins of mammals." Biochim Biophys Acta 320(1): 97-103.

In, J. G., K. Fujino and Y. Kikuta (1997). "Nucleotide sequence of five cDNAs (Accession Nos.AB004823-AB004827) encoding eukaryotic translation initiationfactor 5A (eIF-5A) from potato (PGR97-147)." Plant Physiol. 115: 864.

Irwin, M., M. C. Marin, A. C. Phillips, R. S. Seelan, D. I. Smith, W. Liu, E. R. Flores, K. Y. Tsai, T. Jacks, K. H. Vousden and W. G. Kaelin Jr (2000). "Role for the p53 homologue p73 in E2F-1-induced apoptosis." Nature 407(6804): 645-648.

Itoh, N. and S. Nagata (1993). "A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen." J Biol Chem 268(15): 10932-10937.

Jacobs, C. W., A. E. Adams, P. J. Szaniszlo and J. R. Pringle (1988). "Functions of microtubules in the Saccharomyces cerevisiae cell cycle." J Cell Biol 107(4): 1409-1426.

Jakus, J., E. C. Wolff, M. H. Park and J. E. Folk (1993). "Features of the spermidine-binding site of deoxyhypusine synthase as derived from inhibition studies. Effective inhibition by bis- and mono-guanylated diamines and polyamines." J Biol Chem 268(18): 13151-13159.

Jansson, B. P., L. Malandrin and H. E. Johansson (2000). "Cell cycle arrest in archaea by the hypusination inhibitor N(1)-guanyl-1,7-diaminoheptane." J Bacteriol 182(4): 1158-1161.

Jao, D. L. and K. Y. Chen (2002). "Subcellular localization of the hypusine-containing eukaryotic initiation factor 5A by immunofluorescent staining and green fluorescent protein tagging." J Cell Biochem 86(3): 590-600.

Jao, D. L. and Y. K, Chen (2006). "Tandem affinity purification revealed the hypusine-dependent binding of eukaryotic initiation factor 5A to the translating 80S ribosomal complex." J Cell Biochem 97(3): 583-98.

Jasiulionis, M. G., A. D. Luchessi, A. G. Moreira, P. P. Souza, A. P. Suenaga, M. Correa, C. A. Costa, R. Curi and C. M. Costa-Neto (2007). "Inhibition of eukaryotic translation initiation factor 5A (eIF5A) hypusination impairs melanoma growth." Cell Biochem Funct 25(1): 109-114.

209

Jenkins, Z. A., P. G. Hååg and H. E. Johansson (2001). "Human eIF5A2 on chromosome 3q25-q27 is a phylogenetically conserved vertebrate variant of eukaryotic translation initiation factor 5A with tissue-specific expression." Genomics 71(1): 101-109.

Jiang, P., W. Du, K. Heese and M. Wu (2006). "The Bad guy cooperates with good cop p53: Bad is transcriptionally up-regulated by p53 and forms a Bad/p53 complex at the mitochondria to induce apoptosis." Mol Cell Biol 26(23): 9071-9082.

Jin, B. F., K. He, M. R. Hu, M. Yu, B. F. Shen and X. M. Zhang (2003a). "The effect of eIF-5A on the G1-S in cell cycle regulation." Zhongguo Shi Yan Xue Ye Xue Za Zhi 11(4): 325-328.

Jin, B. F., K. He, H. X. Wang, J. Wang, T. Zhou, Y. Lan, M. R. Hu, K. H. Wei, S. C. Yang, B. F. Shen and X. M. Zhang (2003b). "Proteomic analysis of ubiquitin-proteasome effects: insight into the function of eukaryotic initiation factor 5A." Oncogene 22(31): 4819-4830.

Jin, Z. and W. S. El-Deiry (2006). "Distinct signaling pathways in TRAIL- versus tumor necrosis factor-induced apoptosis." Mol Biol Cell 26(21): 8136-8148.

Joe, Y. A., E. C. Wolff and M. H. Park (1995). "Cloning and expression of human deoxyhypusine synthase cDNA. Structure-function studies with the recombinant enzyme and mutant proteins." J Biol Chem 270(38): 22386-22392.

Johnson, D. G. (2000). "The paradox of E2F1: oncogene and tumor suppressor gene." Mol Carcinog 27(3): 151-157.

Johnson, D. G., J. K. Schwarz, W. D. Cress and J. R. Nevins (1993). "Expression of transcription factor E2F1 induces quiescent cells to enter S phase." Nature 365(6444): 349-352.

Jones, D. T., K. Ganeshaguru, A. E. Virchis, F. N. I, W. W. Lowdell, A. B. Mehta, H. G. Prentice, A. V. Hoffbrand and R. G. Wickremasinghe (2001). "Caspase 8 activation independent of Fas (CD95/APO-1) signaling may mediate killing of B-chronic lymphocytic leukemia cells by cytotoxic drugs or gamma radiation." Blood 98(9): 2800-2807.

Jost, C. A., M. C. Marin and W. G. Kaelin Jr (1997). "p73 is a simian [correction of human] p53-related protein that can induce apoptosis." Nature 389(6647): 191-194.

Junker, U., D. Bevec, C. Barske, C. Kalfoglou, S. Escaich, M. Dobrovnik, J. Hauber and E. Bohnlein (1996). "Intracellular expression of cellular eIF-5A mutants inhibits HIV-1 replication in human T cells: a feasibility study." Hum Gene Ther 7(15): 1861-1869.

Jurgensmeier, J. M., Z. Xie, Q. Deveraux, L. Ellerby, D. Bredesen and J. C. Reed (1998). "Bax directly induces release of cytochrome c from isolated mitochondria." Proc Natl Acad Sci U S A 95(9): 4997-5002.

Kaghad, M., H. Bonnet, A. Yang, L. Creancier, J. C. Biscan, A. Valent, A. Minty, P. Chalon, J. M. Lelias, X. Dumont, P. Ferrara, F. Mckeon and D. Caput (1997). "Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers." Cell 90(4): 809-819.

Kang, H. A. and J. W. Hershey (1994). "Effect of initiation factor eIF-5A depletion on protein synthesis and proliferation of Saccharomyces cerevisiae." J Biol Chem 269(6): 3934-3940.

Kang, H. A., H. G. Schwelberger and J. W. Hershey (1993). "Translation initiation factor eIF-5A, the hypusine-containing protein, is phosphorylated on serine in Saccharomyces cerevisiae." J Biol Chem 268(20): 14750-14756.

Kang, K. H., J. H. Lee, K. C. Kim, S. W. Ham, M. Y. Kim and K. H. Choi (2002). "Induction of p73beta by a naphthoquinone analog is mediated by E2F-1 and triggers apoptosis in HeLa cells." FEBS Lett 522(1-3): 161-167.

Kang, K. R., E. C. Wolff, M. H. Park, J. E. Folk and S. I. Chung (1995). "Identification of YHR068w in Saccharomyces cerevisiae chromosome VIII as a gene for deoxyhypusine synthase. Expression and characterization of the enzyme." J Biol Chem 270(31): 18408-18412.

Kannan, K., N. Kaminski, G. Rechavi, J. Jakob-Hirsch, N. Amariglio and D. Givol (2001). "DNA microarray analysis of genes involved in p53 mediated apoptosis: activation of Apaf-1." Oncogene 20(26): 3449-3455.

Kasagi, N., Y. Gomyo, H. Shirai, S. Tsujitani and H. Ito (1994). "Apoptotic cell death in human gastric carcinoma: analysis by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling." Jpn J Cancer Res 85(9): 939-945.

Kastan, M. B., O. Onyekwere, D. Sidransky, B. Vogelstein and R. W. Craig (1991). "Participation of p53 protein in the cellular response to DNA damage." Cancer Res 51(23 Pt 1): 6304-6311.

Katahira, J., T. Ishizaki, H. Sakai, A. Adachi, K. Yamamoto and H. Shida (1995). "Effects of translation initiation factor eIF-5A on the functioning of human T-cell leukemia virus type I Rex and human immunodeficiency virus Rev inhibited trans dominantly by a Rex mutant deficient in RNA binding." J Virol 69(5): 3125-3133.

Kaufmann, S. H., S. Desnoyers, Y. Ottaviano, N. E. Davidson and G. G. Poirier (1993). "Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis." Cancer Res 53(17): 3976-3985.

Kawahara, K., S. Oyadomari, T. Gotoh, S. Kohsaka, H. Nakayama and M. Mori (2001). "Induction of CHOP and apoptosis by nitric oxide in p53-deficient microglial cells." FEBS Lett 506(2): 135-139.

Kawasaki, H., K. Taira and K. V. Morris (2005). "siRNA induced transcriptional gene silencing in mammalian cells." Cell Cycle 4(3): 442-448.

Kemper, W. M., K. W. Berry and W. C. Merrick (1976). "Purification and properties of rabbit reticulovyte protein synthesis initiation factors M2Balpha and M2Bbeta." J. Biol, Chem 251(18): 5551-5557.

Kemper, W. M., W. C. Merrick, B. Redfield, C. K. Liu and H. Weissbach (1976). "Purification and properties of rabbit reticulocyte elongation factor 1." Arch Biochem Biophys 174(2): 603-12.

Kerr, J. F., A. H. Wyllie and A. R. Currie (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer 26(4): 239-257.

Kessis, T. D., R. J. Slebos, W. G. Nelson, M. B. Kastan, B. S. Plunkett, S. M. Han, A. T. Lorincz, L. Hedrick and K. R. Cho (1993). "Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage." Proc Natl Acad Sci U S A 90(9): 3988-3992.

Khor, L. Y., M. Desilvio, T. Al-Saleem, M. E. Hammond, D. J. Grignon, W. Sause, M. Pilepich, P. Okunieff, H. Sandler and A. Pollack (2005). "MDM2 as a predictor of prostate carcinoma outcome: an analysis of Radiation Therapy Oncology Group Protocol 8610." Cancer 104(5): 962-967.

Kilic, M., R. Schäfer, J. Hoppe and U. Kagerhuber (2002). "Formation of noncanonical high molecular weight caspase-3 and -6 complexes and activation of caspase-12 during serum starvation induced apoptosis in AKR-2B mouse fibroblasts." Cell Death Differ 9(2): 125-137.

Kim, H. A., Y. H. Kim and Y. W. Song (2003). "Facilitation of fas mediated apoptosis of human chondrocytes by the proteasome inhibitor and actinomycin D." J Rheumatol 30(3): 550-558.

Kim, H. A., K. B. Lee and S. C. Bae (2005). "The mechanism of low-concentration sodium nitroprusside-mediated protection of chondrocyte death." Arthritis Res Ther 7(3): R526-R535.

Kim, H. A. and Y. W. Song (2002). "TNF-alpha-mediated apoptosis in chondrocytes sensitized by MG132 or actinomycin D." Biochem Biophys Res Commun 295(4): 937-944.

Kim, H. J., I. Tsoy, M. K. Park, Y. S. Lee, J. H. Lee, H. G. Seo and K. C. Chang (2006). "Iron released by sodium nitroprusside contributes to heme oxygenase-1 induction via the cAMP-protein kinase A-mitogen-activated protein kinase pathway in RAW 264.7 cells." Mol Pharmacol 69(5): 1633-1640.

Kim, K. C., C. S. Jung and K. H. Choi (2006). "Over-expression of p73 enhances cisplatin-induced apoptosis in HeLa cells." Arch Pharm Res 29(2): 152-158.

Kim, K. K., L. W. Hung, H. Yokota, R. Kim and S. H. Kim (1998). "Crystal structures of eukaryotic translation initiation factor 5A from Methanococcus jannaschii at 1.8 A resolution." Proc Natl Acad Sci U S A 95(18): 10419-24.

Kim, Y. S., K. R. Kang, E. C. Wolff, J. K. Bell, P. Mcphie and M. H. Park (2006). "Deoxyhypusine hydroxylase is a Fe(II)-dependent, HEAT-repeat enzyme. Identification of amino acid residues critical for Fe(II) binding and catalysis [corrected]." J Biol Chem 281(19): 13217-13225.

King, K. L. and J. A. Cidlowski (1998). "Cell cycle regulation and apoptosis." Annu Rev Physiol 60: 601-617.

Kirsch, D. G., A. Doseff, B. N. Chau, D. S. Lim, N. C. De Souza-Pinto, R. Hansford, M. B. Kastan, Y. A. Lazebnik and J. M. Hardwick (1999). "Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c." J Biol Chem 274(30): 21155-21161.

213

Kisselev, A. F. and A. L. Goldberg (2001). "Proteasome inhibitors: from research tools to drug candidates." Chem Biol 8(8): 739-758.

Kitagawa, H., E. Tani, H. Ikemoto, I. Ozaki, A. Nakano and S. Satoshi Omura (1999). "Proteasome inhibitors induce mitochondria-independent apoptosis in human glioma cells." FEBS Lett 443(2): 181-186.

Kleeff, J., M. Kornmann, H. Sawhney and M. Korc (2000). "Actinomycin D induces apoptosis and inhibits growth of pancreatic cancer cells." Int J Cancer 86(3): 399-407.

Klein, S. L., R. L. Strausberg, L. Wagner, J. Pontius, S. W. Clifton and P. Richardson (2002). "Genetic and genomic tools for Xenopus research: The NIH Xenopus initiative." Dev Dyn. 225(4): 384-391.

Klier, H., R. Csonga, H. C. Joao, C. Eckerskorn, M. Auer, F. Lottspeich and J. Eder (1995). "Isolation and structural characterization of different isoforms of the hypusine-containing protein eIF-5A from HeLa cells." Biochemistry 34(45): 14693-14702.

Klier, H. and F. Lottspeich (1992). "Detection of the hypusine-containing protein (HP = eIF-5A) in crude yeast extracts by two-dimensional western blots." Electrophoresis 13(9-10): 732-735.

Kluck, R. M., E. Bossy-Wetzel, D. R. Green and D. D. Newmeyer (1997). "The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis." Science 275(5303): 1132-1136.

Koettnitz, K., T. Wohl, B. Kappel, F. Lottspeich, J. Hauber and D. Bevec (1995). "Identification of a new member of the human eIF-5A gene family." Gene 159(2): 283-284.

Koivusalo, R., E. Krausz, P. Ruotsalainen, H. Helenius and S. Hietanen (Chemoradiation of cervical cancer cells: targeting human papillomavirus E6 and p53 leads to either augmented or attenuated apoptosis depending on the platinum carrier ligand). "2002." Cancer Res 62(24): 7364-7371.

Kokontis, J. M., A. J. Wagner, M. O'leary, S. Liao and N. Hay (2001). "A transcriptional activation function of p53 is dispensable for and inhibitory of its apoptotic function." Oncogene 20(6): 659-668.

Kolb, J. P. (2000). "Mechanisms involved in the pro- and anti-apoptotic role of NO in human leukemia." Leukemia 14(9): 1685-1694.

Koo, H. N., H. J. Jeong, S. H. Hong, J. H. Choi, N. H. An and H. M. Kim (2002). "High molecular weight water-soluble chitosan protects against apoptosis induced by serum starvation in human astrocytes." J Nutr Biochem 13(4): J Nutr Biochem.

Koo, T. H., J. J. Lee, E. M. Kim, K. W. Kim, H. D. Kim and J. H. Lee (2002). "Syntenin is over-expressed and promotes cell migration in metastatic human breast and gastric cancer cell lines." Oncogene 21(26): 4080-4088.

Koopman, G., C. P. Reutelingsperger, G. A. Kuijten, R. M. Keehnen, S. T. Pals and M. H. Van Oers (1994). "Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis." Blood 84(5): 1415-1420.

Koroll, M., F. G. Rathjen and H. Volkmer (2001). "The neural cell recognition molecule neurofascin interacts with syntenin-1 but not with syntenin-2, both of which reveal self-associating activity." J Biol Chem 276(14): 10646-10654.

Kossiakoff, A. A. (1988). "Tertiary structure is a principal determinant to protein deamidation." Science 240(4849): 191-194.

Kroemer, G. (2003). "Mitochondrial control of apoptosis: an introduction." Biochem Biophys Res Commun 304(3): 433-435.

Kroemer, G., L. Galluzzi and C. Brenner (2007). "Mitochondrial membrane permeabilization in cell death." Physiol Rev 87(1): 99-163.

Kulp, K. S. and P. R. Vulliet (1996). "Mimosine blocks cell cycle progression by chelating iron in asynchronous human breast cancer cells." Toxicol Appl Pharmacol 139(2): 356-364.

Kutay, U. and S. Guttinger (2005). "Leucine-rich nuclear-export signals: born to be weak." Trends Cell Biol 15(3): 121-124.

Kuwana, T., L. Bouchier-Hayes, J. E. Chipuk, C. Bonzon, B. A. Sullivan, D. R. Green and D. D. Newmeyer (2005). "BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly." Mol Cell 17(4): 525-535.

Kuwana, T., M. R. Mackey, G. Perkins, M. H. Ellisman, M. Latterich, R. Schneiter, D. R. Green and D. D. Newmeyer (2002). "Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane." Cell 111(3): 331-342.

Kuwana, T. and D. D. Newmeyer (2003). "Bcl-2-family proteins and the role of mitochondria in apoptosis." Curr Opin Cell Biol 15(6): 691-699.

Kwak, H. J., K. M. Park, S. Lee, H. J. Lim, S. H. Go, S. M. Eom and H. Y. Park (2006). "Preconditioning with low concentration NO attenuates subsequent NO-induced apoptosis in vascular smooth muscle cells via HO-1-dependent mitochondrial death pathway." Toxicol Appl Pharmacol 217(2): 176-184.

Kyrpides, N. C. and C. R. Woese (1998). "Universally conserved translation initiation factors." Proc Natl Acad Sci U S A 95(1): 224-228.

Lacour, S., A. Hammann, S. Grazide, D. Lagadic-Gossmann, A. Athias, O. Sergent, G. Laurent, P. Gambert, E. Solary and M. T. Dimanche-Boitrel (2004). "Cisplatin-induced CD95 redistribution into membrane lipid rafts of HT29 human colon cancer cells." Cancer Res 64(10): 3593-3598.

Lavin, M. F. and N. Gueven (2006). "The complexity of p53 stabilization and activation." Cell Death Differ 13(6): 941-950.

Lazebnik, Y. (2001). "Why do regulators of apoptosis look like bacterial toxins?" Curr Biol 11(19): R767-768.

Leach, F. S., T. Tokino, P. Meltzer, M. Burrell, J. D. Oliner, S. Smith, D. E. Hill, D. Sidransky, K. W. Kinzler and B. Vogelstein (1993). "p53 Mutation and MDM2 amplification in human soft tissue sarcomas." Cancer Res 53(10 Suppl): 2231-2234.

Leblanc, H. N. and A. Ashkenazi (2003). "Apo2L/TRAIL and its death and decoy receptors." Cell Death Differ 10(1): 66-75.

Lee, C. S., E. S. Han, Y. S. Han and H. Bang (2005). "Differential effect of calmodulin antagonists on MG132-induced mitochondrial dysfunction and cell death in PC12 cells." Brain Res Bull 67(3): 225-234.

Lee, Y., H. K. Kim, H. E. Park, M. H. Park and Y. A. Joe (2002). "Effect of N1-guanyl-1,7-diaminoheptane, an inhibitor of deoxyhypusine synthase, on endothelial cell growth, differentiation and apoptosis." Mol Cell Biochem 237(1-2): 69-76.

Lee, Y. B. and J. E. Folk (1998). "Branched-chain and unsaturated 1,7-diaminoheptane derivatives as deoxyhypusine synthase inhibitors." Bioorg Med Chem 6(3): 253-270.

Lee, Y. B., Y. A. Joe, E. C. Wolff, E. K. Dimitriadis and M. H. Park (1999). "Complex formation between deoxyhypusine synthase and its protein substrate, the eukaryotic translation initiation factor 5A (eIF5A) precursor." Biochem J. 340(Pt 1): 273-281.

Lehmann, W. D., A. Schlosser, G. Erben, R. Pipkorn, D. Bossemeyer and V. Kinzel (2000). "Analysis of isoaspartate in peptides by electrospray tandem mass spectrometry." Protein Sci 9(11): 2260-2268.

Lemasters, J. J., T. Qian, C. A. Bradham, D. A. Brenner, W. E. Cascio, L. C. Trost, Y. Nishimura, A. L. Nieminen and B. Herman (1999). "Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death." J Bioenerg Biomembr 31(4): 305-319.

Letai, A., M. C. Bassik, L. D. Walensky, M. D. Sorcinelli, S. Weiler and S. J. Korsmeyer (2002). "Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics." Cancer Cell 2(3): 183-192.

Leu, J. I., P. Dumont, M. Hafey, M. E. Murphy and D. L. George (2004). "Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex." Nat Cell Biol 6(5): 443-450.

Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." Cell 88(3): 323-331.

Levrero, M., V. De Laurenzi, A. Costanzo, J. Gong, G. Melino and J. Y. Wang (1999). "Structure, function and regulation of p63 and p73." Cell Death Differ 6(12): 1146-1153.

Levrero, M., V. De Laurenzi, A. Costanzo, J. Gong, J. Y. Wang and G. Melino (2000). "The p53/p63/p73 family of transcription factors: overlapping and distinct functions." J Cell Sci 113(Pt 10): 1661-1670.

Levy, D., Y. Adamovich, N. Reuven and Y. Shaul (2007). "The Yes-associated protein 1 stabilizes p73 by preventing Itch-mediated ubiquitination of p73." Cell Death Differ 14(4): 743-751.

Ley, R., K. E. Ewings, K. Hadfield and S. J. Cook (2005). "Regulatory phosphorylation of Bim: sorting out the ERK from the JNK." Cell Death Differ 12(8): 1008-1014.

Ley, R., K. E. Ewings, K. Hadfield, E. Howes, K. Balmanno and S. J. Cook (2004). "Extracellular signal-regulated kinases 1/2 are serum-stimulated "Bim(EL) kinases" that bind to the BH3-only protein Bim(EL) causing its phosphorylation and turnover." J Biol Chem 279(10): 8837-8847.

Li, A. L., J. Y. Li, B. F. Jin, Q. N. Ye, T. Zhou, S. D. Yu, X. Pan, J. H. Man, K. He, M. Yu, M. R. Hu, J. Wang, S. C. Yang, B. F. Shen and X. M. Zhang (2004). "A novel eIF5A complex functions as a regulator of p53 and p53-dependent apoptosis." J Biol Chem 279(47): 49251-49258.

Li, C. Q., L. J. Trudel and G. N. Wogan (2002). "Nitric oxide-induced genotoxicity, mitochondrial damage, and apoptosis in human lymphoblastoid cells expressing wild-type and mutant p53." Proc Natl Acad Sci U S A 99(16): 10364-10369.

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

Li, J., S. Zelenin, A. Aperia and O. Aizman (2006). "Low doses of ouabain protect from serum deprivation-triggered apoptosis and stimulate kidney cell proliferation via activation of NF-kappaB." J Am Soc Nephrol 17(7): 1848-1857.

Li, L. Y., X. Luo and X. Wang (2001). "Endonuclease G is an apoptotic DNase when released from mitochondria." Nature 412(6842): 95-99.

Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri and X. Wang (1997). "Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade." Cell 91(4): 479-489.

Li, R., P. D. Sutphin, D. Schwartz, D. Matas, N. Almog, R. Wolkowicz, N. Goldfinger, H. Pei, M. Prokocimer and V. Rotter (1998). "Mutant p53 protein expression interferes with p53-independent apoptotic pathways." Oncogene 16(25): 3269-3277.

Li, Y., Y. Yamakita and R. M. Krug (1998). "Regulation of a nuclear export signal by an adjacent inhibitory sequence: the effector domain of the influenza virus NS1 protein." Proc Natl Acad Sci U S A 95: 4864-4869.

Liang, X. H., S. Mungal, A. Ayscue, J. D. Meissner, P. Wodnicki, D. Hockenbery, S. Lockett and B. Herman (1995). "Bcl-2 protooncogene expression in cervical carcinoma cell lines containing inactive p53." J Cell Biochem 57(3): 509-521.

Liang, X. H., M. Volkmann, R. Klein, B. Herman and S. J. Lockett (1993). "Co-localization of the tumor-suppressor protein p53 and human papillomavirus E6 protein in human cervical carcinoma cell lines." Oncogene 8(10): 2645-2652.

Liang, Y., K. D. Nylander, C. Yan and N. F. Schor (2002). "Role of caspase 3-dependent Bcl-2 cleavage in potentiation of apoptosis by Bcl-2." Mol Pharmacol 61(1): 142-149.

Liang, Y., C. Yan, K. D. Nylander and N. F. Schor (2003). "Early events in Bcl-2-enhanced apoptosis." Apoptosis 8(6): 609-616.

Liao, D. I., E. C. Wolff, M. H. Park and D. R. Davies (1998). "Crystal structure of the NAD complex of human deoxyhypusine synthase: an enzyme with a ball-and-chain mechanism for blocking the active site." Structure 15(6): 23-32.

Liao, Y. D., J. C. Jeng, C. F. Wang, S. C. Wang and S. T. Chang (2004). "Removal of N-terminal methionine from recombinant proteins by engineered E. coli methionine aminopeptidase." Protein Sci 13(7): 1802-1810.

Liebermann, D. A., B. Hoffman and R. A. Steinman (1995). "Molecular controls of growth arrest and apoptosis: p53-dependent and independent pathways." Oncogene 11(1): 199-210.

Liebermann, D. A., B. Hoffman and D. Vesely (2007). "p53 induced growth arrest versus apoptosis and its modulation by survival cytokines." Cell Cycle 6(2): 166-170.

Lin, A. (2003). "Activation of the JNK signaling pathway: breaking the brake on apoptosis." Bioessays 25(1): 17-24.

Lin, H., X. M. Zhang, C. Chen and B. D. Chen (2000). "Apoptosis of Mo7e Leukemia Cells Is Associated with the Cleavage of Bcl-2 into a Shortened Fragment That Is Not Functional for Heterodimerization with Bcl-2 and Bax." Cell Death Differ 261(1): 180-186.

Lin, W., X. Wei, H. Xue, M. Kelimu, R. Tao, Y. Song and Z. Zhou (2000). "Study on DNA strand breaks induced by sodium nitroprusside, a nitric oxide donor, *in vivo* and in vitro." Mutat Res 466(2): 187-195.

Lincoln, J. E., M. Boling, A. N. Parikh, Y. Yeh, D. G. Gilchrist and L. S. Morse (2006). "Fas signaling induces raft coalescence that is blocked by cholesterol depletion in human RPE cells undergoing apoptosis." Invest Ophthalmol Vis Sci 47(5): 2172-2178.

Lindsten, T., A. J. Ross, A. King, W. X. Zong, J. C. Rathmell, H. A. Shiels, E. Ulrich, K. G. Waymire, P. Mahar, K. Frauwirth, Y. Chen, M. Wei, V. M. Eng, D. M. Adelman, M. C. Simon, A. Ma, J. A. Golden, G. Evan, S. J. Korsmeyer, G. R. Macgregor and C. B. Thompson (2000). "The combined functions of pro-apoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues." Mol Cell 6(6): 1389-1399.

Ling, Y. H., L. Liebes, Y. Zou and R. Perez-Soler (2003). "Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic response to Bortezomib, a novel proteasome inhibitor, in human H460 non-small cell lung cancer cells." J Biol Chem 278(36): 33714-33723.

Lipowsky, G., F. R. Bischoff, P. Schwarzmaier, R. Kraft, S. Kostka, E. Hartmann, U. Kutay and D. Gorlich (2000). "Exportin 4: a mediator of a novel nuclear export pathway in higher eukaryotes." EMBO J 19(16): 4362-4371.

Lissy, N. A., P. K. Davis, M. Irwin, W. G. Kaelin and S. F. Dowdy (2000). "A common E2F-1 and p73 pathway mediates cell death induced by TCR activation." Nature 407(6804): 642-645.

Liu, J., P. A. Lynch, C. Y. Chien, G. T. Montelione, R. M. Krug and H. M. Berman (1997). "Crystal structure of the unique RNA-binding domain of the influenza virus NS1 protein." Nat. Struct. Biol 4(11): 896-899.

Liu, R. H. and J. H. Hotchkiss (1995). "Potential genotoxicity of chronically elevated nitric oxide: a review." Mutat Res 339(2): 73-89.

Liu, X., C. N. Kim, J. Yang, R. Jemmerson and X. Wang (1996). "Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c." Cell 86(1): 147-157.

Liu, X., Y. Zhu, S. Dai, J. White, F. Peyerl, J. W. Kappler and P. Marrack (2006). "Bcl-xl does not have to bind Bax to protect T cells from death." J Exp Med 203(13): 2953-2961.

Liu, X., H. Zou, C. Slaughter and X. Wang (1997c). "DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis." Cell 89(2): 175-84.

Liu, Y. and A. M. Tartakoff (1997b). "A role for eIF5A in mRNA export." Mol Biol Cell 8 (suppl.): 426a.

Liu, Y. P., M. Nemeroff, Y. P. Yan and K. Y. Chen (1997a). "Interaction of eukaryotic initiation factor 5A with the human immunodeficiency virus type 1 Rev response element RNA and U6 snRNA requires deoxyhypusine or hypusine modification." Biol Signals 6(3): 166-174.

Liu, Z. G. (2004). "Molecular mechanism of TNF signaling and beyond." Cell Res 15(1): 24-27.

Liu, Z. G., H. Hsu, D. V. Goeddel and M. Karin (1996). "Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death." Cell 87(3): 565-576.

Lockwood, A. H., P. Sarkar and U. Maitra (1972). "Release of Polypeptide Chain Initiation Factor IF-2 During Initiation Complex Formation." Proc Natl Acad Sci U S A 69(12): 3602-3605.

Lopes, U. G., P. Erhardt, R. Yao and G. M. Cooper (1997). "p53-dependent Induction of Apoptosis by Proteasome Inhibitors." J Biol Chem 272(20): 12893-12896.

Lorenzo, H. K., S. A. Susin, J. Penninger and G. Kroemer (1999). "Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death." Cell Death Differ 6(6): 516-524.

Loughran, O. and N. B. La Thangue (2000). "Apoptotic and growth-promoting activity of E2F modulated by MDM2." Mol Cell Biol 20(6): 2186-2197.

Lowe, S. W., E. M. Schmitt, S. W. Smith, B. A. Osborne and T. Jacks (1993). "p53 is required for radiation-induced apoptosis in mouse thymocytes." Nature 362(6423): 847-849.

Lozupone, F., L. Lugini, P. Matarrese, F. Luciani, C. Federici, E. Iessi, P. Margutti, G. Stassi, W. Malorni and S. Fais (2004). "Identification and relevance of the CD95-binding domain in the N-terminal region of ezrin." J Biol Chem 279(10): 9199-9207.

Lu, J., Z. P. Chen, Y. P. Yan, S. Knapp, H. Schugar and K. Y. Chen (2000). "Aminohexanoic hydroxamate is a potent inducer of the differentiation of mouse neuroblastoma cells." Cancer Lett 160(1): 59-66.

Lu, Z. and S. Xu (2006). "ERK1/2 MAP kinases in cell survival and apoptosis." IUBMB Life 58(11): 621-631.

Luetjens, C. M., N. T. Bui, B. Sengpiel, G. Munstermann, M. Poppe, A. J. Krohn, E. Bauerbach, J. Krieglstein and J. H. Prehn (2000). "Delayed mitochondrial dysfunction in excitotoxic neuron death: cytochrome c release and a secondary increase in superoxide production." J Neurosci 20(15): 5715-5723.

Luo, J., Y. Sun, H. Lin, Y. Qian, Z. Li, S. S. Leonard, C. Huang and X. Shi (2003). "Activation of JNK by vanadate induces a Fas-associated death domain (FADD)-dependent death of cerebellar granule progenitors in vitro." J Biol Chem 278(7): 4542-4551.

Luo, X., I. Budihardjo, H. Zou, C. Slaughter and X. Wang (1998). "Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors." Cell 94(4): 481-490.

Luu, N. C., S. Robinson, R. Zhao, R. Mckean and D. P. Ridge (2004). "Mass spectrometric differentiation of alpha- and beta-aspartic acid in a pseudo-tetrapeptide thrombosis inhibitor and its isomer." Eur J Mass Spectrom 10(2): 279-287.

Macfarlane, M., M. Ahmad, S. M. Srinivasula, T. Fernandes-Alnemri, G. M. Cohen and E. S. Alnemri (1997). "Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL." J Biol Chem 272(41): 25417-25420.

Maclaren, A. P., R. S. Chapman, A. H. Wyllie and C. J. Watson (2001). "p53-dependent apoptosis induced by proteasome inhibition in mammary epithelial cells." Cell Death Differ 8(3): 210-218.

Maddika, S., S. R. Ande, S. Panigrahi, T. Paranjothy, K. Weglarczyk, A. Zuse, M. Eshraghi, M. Manda, E. Wiechec and M. Los (2007). "Cell survival, cell death and cell cycle pathways are interconnected: Implications for cancer therapy." Drug Resist Updat.

Magdolen, V., H. Klier, T. Wohl, F. Klink, H. Hirt, J. Hauber and F. Lottspeich (1994). "The function of the hypusine-containing proteins of yeast and other eukaryotes is well conserved." Mol Gen Genet 244(6): 646-652.

Maiti, T. and U. Maitra (1997). "Characterization of translation initiation factor 5 (eIF5) from Saccharomyces cerevisiae. Functional homology with mammalian eIF5 and the effect of depletion of eIF5 on protein synthesis *in vivo* and in vitro." J Biol Chem 272(29): 18333-18340.

Maki, C. G., J. M. Huibregtse and P. M. Howley (1996). "*In vivo* ubiquitination and proteasome-mediated degradation of p53(1)." Cancer Res 56(11): 2649-2654.

Malim, M. H., S. Bohnlein, J. Hauber and B. R. Cullen (1989). "Functional dissection of the HIV-1 Rev trans-activator--derivation of a trans-dominant repressor of Rev function." Cell 85(1): 205-214.

Malim, M. H. and B. R. Cullen (1991). "HIV-1 structural gene expression requires the binding of multiple Rev monomers to the viral RRE: implications for HIV-1 latency." Cell 61(2): 241-248.

Malim, M. H. and B. R. Cullen (1993). "Rev and the fate of pre-mRNA in the nucleus: implications for the regulation of RNA processing in eukaryotes." Mol Cell Biol 13(10): 6180-6189.

Maniatis, T. (1999). "A ubiquitin ligase complex essential for the NF-B, Wnt/Wingless, and Hedgehog signaling pathways." Genes Dev 13(5): 505-510.

Mantovani, F. and L. Banks (2001). "The human papillomavirus E6 protein and its contribution to malignant progression." Oncogene 20(54): 7874-7887.

Marcellus, R. C., J. N. Lavoie, D. Boivin, G. C. Shore, G. Ketner and P. E. Branton (1998). "The early region 4 orf4 protein of human adenovirus type 5 induces p53-independent cell death by apoptosis." J Virol 72(9): 7144-53.

Marchenko, N. D., A. Zaika and U. M. Moll (2000). "Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling." J Biol Chem 275(21): 16202-16212.

Marchet, A., S. Mocellin, C. Belluco, A. Ambrosi, F. Demarchi, E. Mammano, M. Digito, A. Leon, A. D'arrigo, M. Lise and D. Nitti (2007). "Gene expression profile of primary gastric cancer: towards the prediction of lymph node status." Ann Surg Oncol 14(3): 1058-1064.

Marchetti, A., F. Buttitta, S. Girlando, P. Dalla Palma, S. Pellegrini, P. Fina, C. Doglioni, G. Bevilacqua and M. Barbareschi (1995). "mdm2 gene alterations and mdm2 protein expression in breast carcinomas." J Pathol 175(1): 31-38.

Marchetti, P., M. Castedo, S. A. Susin, N. Zamzami, T. Hirsch, A. Macho, A. Haeffner, F. Hirsch, M. Geuskens and G. Kroemer (1996). "Mitochondrial permeability transition is a central coordinating event of apoptosis." J Exp Med 184(3): 1155-1160.

Marin, M. C., A. Fernandez, R. J. Bick, S. Brisbay, L. M. Buja, M. Snuggs, D. J. Mcconkey, A. C. Von Eschenbach, M. J. Keating and T. J. Mcdonnell (1996). "Apoptosis suppression by bcl-2 is correlated with the regulation of nuclear and cytosolic Ca2+." Oncogene 12(11): 2259-2266.

Marin, M. C., C. A. Jost, M. S. Irwin, J. A. Decaprio, D. Caput and W. G. Kaelin Jr (1998). "Viral oncoproteins discriminate between p53 and the p53 homolog p73." Mol Cell Biol 18(11): 6316-6324.

Marsters, S. A., J. P. Sheridan, P. M. Pitti, A. Huang, M. Skubatch, D. Baldwin, J. Yuan, A. Gurney, A. D. Goddard, P. Godowski and A. Ashkenazi (1997). "A novel receptor for Apo2L/TRAIL contains a truncated death domain." Curr Biol 7(12): 1003-6.

224

Martin, S. J. (1993a). "Apoptosis: suicide, execution or murder?" Trends Cell Biol 3(5): 141-144.

Martin, S. J. (1993b). "Programmed cell death and AIDS." Science 262(5138): 1355-1357.

Martin, S. J., G. A. O'brien, W. K. Nishioka, A. J. Mcgahon, A. Mahboubi, T. C. Saido and D. R. Green (1995a). "Proteolysis of fodrin (non-erythroid spectrin) during apoptosis." J Biol Chem 270(12): 6425-6428.

Martin, S. J., C. P. Reutelingsperger, A. J. Mcgahon, J. A. Rader, R. C. Van Schie, D. M. Laface and D. R. Green (1995b). "Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by over-expression of Bcl-2 and Abl." J Exp Med 182(5): 1545-1556.

Marzo, I., C. Brenner, N. Zamzami, J. M. Jurgensmeier, S. A. Susin, H. L. Vieira, M. C. Prevost, Z. Xie, S. Matsuyama, J. C. Reed and G. Kroemer (1998). "Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis." Science 281(5385): 2027-2031.

Mashima, T., M. Naito, N. Fujita, K. Noguchi and T. Tsuruo (1995). "Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in VP-16-induced U937 apoptosis." Biochem Biophys Res Commun. 217(3): 1185-1192.

Mathai, J. P., M. Germain, R. C. Marcellus and G. C. Shore (2002). "Induction and endoplasmic reticulum location of BIK/NBK in response to apoptotic signaling by E1A and p53." Oncogene 21(16): 2534-2544.

Mathai, J. P., M. Germain and G. C. Shore (2005). "BH3-only BIK regulates BAX,BAK-dependent release of Ca2+ from endoplasmic reticulum stores and mitochondrial apoptosis during stress-induced cell death." J Biol Chem 277(20): 18053-18060.

Matsumura, I., H. Tanaka and Y. Kanakura (2003). "E2F1 and c-Myc in cell growth and death." Cell Cycle 2(4): 333-338.

Mattaj, I. W. and L. Englmeier (1998). "Nucleocytoplasmic transport: the soluble phase." Annu Rev Biochem 67: 265-306.

Mayer, B. and R. Oberbauer (2003). "Mitochondrial regulation of apoptosis." News Physiol Sci 18: 89-94.

Mcbride, H. M., M. Neuspiel and S. Wasiak (2006). "Mitochondria: more than just a powerhouse." Curr Biol 16(14): R551-560.

Mccaffrey, A. P. and M. A. Kay (2003). "RNA interference gene therapy: RNA interference gets infectious." Gene Ther 10(15): 1205-1205.

Mccarthy, N. J., W. M. K, C. S. Gilbert and G. I. Evan (1997). "Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak." J Cell Biol 136(1): 215-227.

Medema, J. P., R. E. Toes, C. Scaffidi, T. S. Zheng, R. A. Flavell, C. J. Melief, M. E. Peter, R. Offringa and P. H. Krammer (1997). "Cleavage of FLICE (caspase-8) by granzyme B during cytotoxic T lymphocyte-induced apoptosis." Eur J Immunol 27(12): 3492-3498.

Mehta, K. D., D. Leung, L. Lefebvre and M. Smith (1990). "The ANB1 locus of Saccharomyces cerevisiae encodes the protein synthesis initiation factor eIF-4D." J Biol Chem 265(15): 8802-8807.

Meinnel, T., P. Peynot and C. Giglione (2005). "Processed N-termini of mature proteins in higher eukaryotes and their major contribution to dynamic proteomics." Biochimie 87(8): 701-712.

Meinnel, T., A. Serero and C. Giglione (2006). "Impact of the N-terminal amino acid on targeted protein degradation." Biol chem 387(7): 839-851.

Melino, G., M. Annicchiarico-Petruzzelli, L. Piredda, E. Candi, V. Gentile, P. J. Davies and M. Piacentini (1994). "Tissue transglutaminase and apoptosis: sense and antisense transfection studies with human neuroblastoma cells." Mol Cell Biol 14(10): 6584-6596.

Melino, G., F. Bernassola, M. Ranalli, K. Yee, W. X. Zong, M. Corazzari, R. A. Knight, D. R. Green, C. Thompson and K. H. Vousden (2004). "p73 Induces apoptosis via PUMA transactivation and Bax mitochondrial translocation." J Biol Chem 279(9): 8076-8083.

Melino, G., V. De Laurenzi and K. H. Vousden (2002). "p73: Friend or foe in tumorigenesis." Nat Rev Cancer 2(8): 605-615.

Messmer, U. K. and B. Brune (1995). "Nitric oxide-induced apoptosis: p53-dependent and p53-independent signaling pathways." Biochem J 319(Pt 1): 299-305.

Meyer, B. E. and M. H. Malim (1994). "The HIV-1 Rev trans-activator shuttles between the nucleus and the cytoplasm." Genes Dev 8(13): 1538-1547.

Mi, Z., B. Hong, Z. K. Mirnics, Y. Y. Tyurina, V. E. Kagan, Y. Liang and N. F. Schor (2006). "Bcl-2-mediated potentiation of neocarzinostatin-induced apoptosis: requirement for caspase-3, sulfhydryl groups, and cleavable Bcl-2." Cancer Chemother Pharmacol 57(3): 357-367.

Michael, W. M. and G. Dreyfuss (1996). "Distinct domains in ribosomal protein L5 mediate 5 S rRNA binding and nucleolar localization." J Biol Chem 271(19): 11571-11574.

Midgley, C. A., B. Owens, C. V. Briscoe, D. B. Thomas, D. P. Lane and P. A. Hall (1995). "Coupling between gamma irradiation, p53 induction and the apoptotic response depends upon cell type in vivo." J Cell Sci 108(Pt 5): 1843-1848.

Miersch, S. and B. Mutus (2005). "Protein S-nitrosation: biochemistry and characterization of protein thiol-NO interactions as cellular signals." Clin Biochem 38(9): 777-791.

Mietz, J. A., T. Unger, J. M. Huibregtse and P. M. Howley (1992). "The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein." EMBO J 11(13): 5013-5020.

Mihara, M., S. Erster, A. Zaika, O. Petrenko, T. Chittenden, P. Pancoska and U. M. Moll (2003). "p53 has a direct apoptogenic role at the mitochondria." Mol Cell 11(3): 577-590.

Mihara, M. and U. M. Moll (2003). "Detection of mitochondrial localization of p53." Methods Mol Biol 234: 203-209.

Mikhailov, V., M. Mikhailova, K. Degenhardt, M. A. Venkatachalam, E. White and P. Saikumar (2003). "Association of Bax and Bak Homo-oligomers in Mitochondria. Bax REQUIREMENT FOR

Bak REORGANIZATION AND CYTOCHROME c RELEASE." J Biol Chem 278(7): 5367-5376.

Milne, D. M., L. E. Campbell, D. G. Campbell and D. W. Meek (1995). "p53 Is Phosphorylated *in vitro* and *in vivo* by an Ultraviolet Radiation-induced Protein Kinase Characteristic of the c-Jun Kinase, JNK1." J Biol Chem 270(10): 5511-5518.

Minn, A. J., P. Velez, S. L. Schendel, H. Liang, S. W. Muchmore, S. W. Fesik, M. Fill and C. B. Thompson (1997). "Bcl-x(L) forms an ion channel in synthetic lipid membranes." Nature 385(6614): 353-357.

Miyashita, T., S. Krajewski, M. Krajewska, H. G. Wang, H. K. Lin, D. A. Liebermann, B. Hoffman and J. C. Reed (1994). "Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and in vivo." Oncogene 9(6): 1799-1805.

Molitor, I. M., S. Knöbel, C. Dang, T. Spielmann, A. Alléra and G. M. König (2004). "Translation initiation factor eIF-5A from Plasmodium falciparum." Mol Biochem Parasitol 137(1): 65-74.

Mollinedo, F. and C. Gajate (2006). "Fas/CD95 death receptor and lipid rafts: new targets for apoptosis-directed cancer therapy." Drug Resist Updat 9(1-2): 51-73.

Moncada, S. and J. D. Erusalimsky (2002). "Does nitric oxide modulate mitochondrial energy generation and apoptosis?" Nat Rev Mol Cell Biol 3(3): 214-220.

Montagut, C., A. Rovira and J. Albanell (2006). "The proteasome: a novel target for anticancer therapy." Clin Transl Oncol 8(5): 313-317.

Moore, S., L. Alexander, M. Brownstein, L. Guan, S. Lobo, Y. Meng, M. Tanaguchi, Z. Wang, J. Yu, C. Prange, K. Schreiber, C. Shenmen, L. Wagner, M. Bala, S. Barbazuk, S. Barber, R. Babakaiff, J. Beland, E. Chun, L. Del Rio, S. Gibson, R. Hanson, R. Kirkpatrick, J. Liu, C. Matsuo, M. Mayo, R. R. Santos, J. Stott, M. Tsai, D. Wong, A. Siddiqui, R. Holt, S. J. Jones and M. A. Marra (2005). "GenBank accession number GI79153704."

Mootha, V. K., M. C. Wei, K. F. Buttle, L. Scorrano, V. Panoutsakopoulou, C. A. Mannella and S. J. Korsmeyer (2001). "A reversible component of mitochondrial respiratory dysfunction in apoptosis can be rescued by exogenous cytochrome c." EMBO J 20(4): 661-71.

Mori, H., K. Kawai, F. Ohbayashi, T. Kuniyasu, M. Yamazaki, T. Hamasaki and G. M. Williams (1984). "Genotoxicity of a variety of mycotoxins in the hepatocyte primary culture/DNA repair test using rat and mouse hepatocytes." Cancer Res 44(7): 2918-2923.

Mori, S., K. Murakami-Mori, S. Nakamura, A. Ashkenazi and B. Bonavida (1999). "Sensitization of AIDS-Kaposi's sarcoma cells to Apo-2 ligand-induced apoptosis by actinomycin." J Immunol. 162(9): 5616-5623.

Muchmore, S. W., M. Sattler, H. Liang, R. P. Meadows, J. E. Harlan, H. S. Yoon, D. Nettesheim, B. S. Chang, C. B. Thompson, S. L. Wong, S. L. Ng and S. W. Fesik (1996). "X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death." Nature 381(6580): 335-341.

Muller, H., A. P. Bracken, R. Vernell, M. C. Moroni, F. Christians, E. Grassilli, E. Prosperini, E. Vigo, J. D. Oliner and K. Helin (2001). "E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis." Genes Dev 15(3): 267-285.

Muller, M., S. Wilder, D. Bannasch, D. Israeli, K. Lehlbach, M. Li-Weber, S. L. Friedman, P. R. Galle, W. Stremmel, M. Oren and P. H. Krammer (1998). "p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs." J Exp Med 188(11): 2033-2045.

Mundle, S. D., X. Z. Gao, S. Khan, S. A. Gregory, H. D. Preisler and A. Raza (1995). "Two in situ labeling techniques reveal different patterns of DNA fragmentation during spontaneous apoptosis *in vivo* and induced apoptosis in vitro." Anticancer Res 15(5B): 1895-1904.

Munoz-Pinedo, C., A. Guio-Carrion, J. C. Goldstein, P. Fitzgerald, D. D. Newmeyer and D. R. Green (2006). "Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration." Proc Natl Acad Sci U S A 103(31): 11573-11578.

Murphy, K. M., U. N. Streips and R. B. Lock (1999). "Bax membrane insertion during Fas(CD95)-induced apoptosis precedes cytochrome c release and is inhibited by Bcl-2." Oncogene 18(44): 5991-5999.

Muzio, M., B. R. Stockwell, H. R. Stennicke, G. S. Salvesen and V. M. Dixit (1998). "An Induced Proximity Model for Caspase-8 Activation." J Biol Chem 273(5): 2926-2930.

Nagata, S. (1997). "Apoptosis by death factor." Cell 88(3): 355-365.

Nagata, S. (1999). "Fas ligand-induced apoptosis." Annu Rev Genet 33: 29-55.

Nagy, K., K. Szekely-Szuts, K. Izeradjene, L. Douglas, M. Tillman, H. Barti-Juhasz, M. Dominici, C. Spano, G. Luca Cervo, P. Conte, J. A. Houghton, R. Mihalik, L. Kopper and I. Peta (2006). "Proteasome inhibitors sensitize colon carcinoma cells to TRAIL-induced apoptosis via enhanced release of Smac/DIABLO from the mitochondria." Pathol Oncol Res 12(3): 133-142.

Nahle, Z., J. Polakoff, R. V. Davuluri, M. E. Mccurrach, M. D. Jacobson, M. Narita, M. Q. Zhang, Y. Lazebnik, D. Bar-Sagi and S. W. Lowe (2002). "Direct coupling of the cell cycle and cell death machinery by E2F." Nat Cell Biol 4(11): 859-864.

Nakagawa, T., H. Zhu, N. Morishima, E. Li, J. Xu, B. A. Yankner and J. Yuan (2000). "Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta." Nature 403(6765): 98-103.

Nakajima, W. and N. Tanaka (2007). "Synergistic induction of apoptosis by p53-inducible Bcl-2 family proteins Noxa and Puma." J Nippon Med Sch 74(2): 148-157.

Nakano, K. and K. H. Vousden (2001). "PUMA, a novel pro-apoptotic gene, is induced by p53." Mol Cell 7(3): 683-694.

Narita, M., S. Shimizu, T. Ito, T. Chittenden, R. J. Lutz, H. Matsuda and Y. Tsujimoto (1998). "Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria." Proc Natl Acad Sci U S A 95(25): 14681-14686.

Naujokat, C., O. Sezer, H. Zinke, A. Leclere, S. Hauptmann and K. Possinger (2000). "Proteasome inhibitors induced caspase-dependent apoptosis and accumulation of p21WAF1/Cip1 in human immature leukemic cells." Eur J Haematol 65(4): 221-236.

Nawrocki, S. T., J. S. Carew, K. Dunner Jr, L. H. Boise, P. J. Chiao, P. Huang, J. L. Abbruzzese and D. J. Mcconkey (2005). "Bortezomib inhibits PKR-like endoplasmic reticulum (ER) kinase and induces apoptosis via ER stress in human pancreatic cancer cells." Cancer Res 65(24): 11510-11519.

Newmeyer, D. D. and S. Ferguson-Miller (2003). "Mitochondria: releasing power for life and unleashing the machineries of death." Cell 112(4): 481-490.

Nicholson, D. W. (1999). "Caspase structure, proteolytic substrates, and function during apoptotic cell death." Cell Death Differ 6(11): 1028-1042.

Nicotera, P., M. Leist and E. Ferrando-May (1998). "Intracellular ATP, a switch in the decision between apoptosis and necrosis." Toxicol Lett 102-103: 139-42.

Nie, Z., B. N. Phenix, J. J. Lum, A. Alam, D. H. Lynch, B. Beckett, P. H. Krammer, P. P. Sekaly and A. D. Badley (2002). "HIV-1 protease processes procaspase 8 to cause mitochondrial release of cytochrome c, caspase cleavage and nuclear fragmentation." Cell Death Differ 9(11): 1172-1184.

Nijhawan, D., M. Fang, E. Traer, Q. Zhong, W. Gao, F. Du and X. Wang (2003). "Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation." Genes Dev 17(12): 1475-1486.

Nikrad, M., T. Johnson, H. Puthalalath, L. Coultas, J. Adams and A. S. Kraft (2005). "The proteasome inhibitor bortezomib sensitizes cells to killing by death receptor ligand TRAIL via BH3-only proteins Bik and Bim." Mol Cancer Ther 4:(3): 443-449.

Nishimura, K., K. Murozumi, A. Shirahata, M. H. Park, K. Kashiwagi and K. Igarashi (2005). "Independent roles of eIF5A and polyamines in cell proliferation." Biochem J. 385(Pt 3): 779-785.

Nishimura, K., Y. Ohki, T. Fukuchi-Shimogori, K. Sakata, K. Saiga, T. Beppu, A. Shirahata, K. Kashiwagi and K. Igarashi (2002). "Inhibition of cell growth through inactivation of eukaryotic translation initiation factor 5A (eIF5A) by deoxyspergualin." Biochem J. 363(Pt 3): 761-768.

Nishina, H., K. Nakagawa, N. Azuma and T. Katada (2003). "Activation mechanism and physiological roles of stress-activated protein kinase/c-Jun NH2-terminal kinase in mammalian cells." J Biol Regul Homeost Agents 17(4): 295-302.

Nishina, H., T. Wada and T. Katada (2004). "Physiological roles of SAPK/JNK signaling pathway." J Biochem (Tokyo). 136(2): 123-126.

231

Nutt, L. K., A. Pataer, J. Pahler, B. Fang, J. Roth, D. J. Mcconkey and S. G. Swisher (2002). "Bax and Bak Promote Apoptosis by Modulating Endoplasmic Reticular and Mitochondrial Ca2+ Stores." J Biol Chem 277(11): 9219-9225.

O'connell, J., M. W. Bennett, K. Nally, A. Houston, G. C. O'sullivan and F. Shanahan (2000). "Altered mechanisms of apoptosis in colon cancer: Fas resistance and counterattack in the tumor-immune conflict." Ann N Y Acad Sci 910(1): 178-195.

O'connor, L., A. Strasser, L. A. O'reilly, G. Hausmann, J. M. Adams, S. Cory and D. C. Huang (1998). "Bim: a novel member of the Bcl-2 family that promotes apoptosis." EMBO J 17(2): 384-395.

Oda, E., R. Ohki, H. Murasawa, J. Nemoto, T. Shibue, T. Yamashita, T. Tokino, T. Taniguchi and N. Tanaka (2000). "Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis." Science 288(5468): 1053-1058.

Odin, J. A., R. C. Huebert, L. Casciola-Rosen, N. F. Larusso and A. Rosen (2001). "Bcl-2-dependent oxidation of pyruvate dehydrogenase-E2, a primary biliary cirrhosis autoantigen, during apoptosis." J Clin Invest 108(2): 223-232.

Ohsato, T., N. Ishihara, T. Muta, S. Umeda, S. Ikeda, K. Mihara, N. Hamasaki and D. Kang (2002). "Mammalian mitochondrial endonuclease G. Digestion of R-loops and localization in intermembrane space." Eur J Biochem 269(23): 5765-5770.

Ohta, S. (2003). "A multi-functional organelle mitochondrion is involved in cell death, proliferation and disease." Curr Med Chem 10(23): 2485-2494.

Ohtani, K., J. Degregori and J. R. Nevins (1995). "Regulation of the cyclin E gene by transcription factor E2F1." Proc Natl Acad Sci U S A 92(26): 12146-12150.

Olivier, S., M. Fillet, M. Malaise, J. Piette, V. Bours, M. P. Merville and N. Franchimont (2005). "Sodium nitroprusside-induced osteoblast apoptosis is mediated by long chain ceramide and is decreased by raloxifene." Biochem Pharmacol 69(6): 891-901.

Oltvai, Z. N., C. L. Milliman and S. J. Korsmeyer (1993). "Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death." Cell 74(7): 609-619.

Opferman, J. T., A. Letai, C. Beard, M. D. Sorcinelli, C. C. Ong and S. J. Korsmeyer (2003). "Development and maintenance of B and T lymphocytes requires anti-apoptotic MCL-1." Nature 426(6967): 671-676.

Orlowski, R. Z. (1999). "The role of the ubiquitin-proteasome pathway in apoptosis." Cell Death Differ 6(4): 303-313.

Orru, S., L. Vitagliano, L. Esposito, L. Mazzarella, G. Marino and M. Ruoppolo (2000). "Effect of deamidation on folding of ribonuclease A." Protein Sci 9(12): 2577-2582.

Ossareh-Nazari, B., F. Bachelerie and C. Dargemont (1997). "Evidence for a role of CRM1 in signal-mediated nuclear protein export." Science 278(5335): 141-144.

Ossina, N. K., A. Cannas, V. C. Powers, P. A. Fitzpatrick, J. D. Knight, J. R. Gilbert, E. M. Shekhtman, L. D. Tomei, S. R. Umansky and M. C. Kiefer (1997). "Interferon-gamma modulates a p53-independent apoptotic pathway and apoptosis-related gene expression." J Biol Chem 272(26): 16351-16357.

Ott, M., J. D. Robertson, V. Gogvadze, B. Zhivotovsky and S. Orrenius (2002). "Cytochrome c release from mitochondria proceeds by a two-step process." Proc Natl Acad Sci U S A 99(3): 1259-1263.

Oya, N., F. Zolzer, F. Werner and C. Streffer (2003). "Effects of serum starvation on radiosensitivity, proliferation and apoptosis in four human tumor cell lines with different p53 status." Strahlenther Onkol 179(2): 99-106.

Oyadomari, S., K. Takeda, M. Takiguchi, T. Gotoh, M. Matsumoto, I. Wada, S. Akira, E. Araki and M. Mori (2001). "Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway." Proc Natl Acad Sci U S A 98(19): 10845-50.

Palmero, I., C. Pantoja and M. Serrano (1998). "p19ARF links the tumour suppressor p53 to Ras." Nature 395(6698): 125-6.

Pan, G., J. Ni, Y. F. Wei, G. Yu, R. Gentz and V. M. Dixit (1997). "An antagonist decoy receptor and a death domain-containing receptor for TRAIL." Science 277(5327): 815-818.

Papoff, G., P. Hausler, A. Eramo, M. G. Pagano, G. Di Leve, A. Signore and G. Ruberti (1999). "Identification and characterization of a ligand-independent oligomerization domain in the extracellular region of the CD95 death receptor." J Biol Chem 274(53): 38241-38250.

Park, J. H., L. Aravind, E. C. Wolff, J. Kaevel, Y. S. Kim and M. H. Park (2006). "Molecular cloning, expression, and structural prediction of deoxyhypusine hydroxylase: a HEAT-repeat-containing metalloenzyme." Proc Natl Acad Sci U S A. 103(1): 51-56.

Park, J. H., E. C. Wolff, J. E. Folk and M. H. Park (2003). "Reversal of the deoxyhypusine synthesis reaction. Generation of spermidine or homospermidine from deoxyhypusine by deoxyhypusine synthase." J Biol Chem 278(35): 32683-32691.

Park, M. H. (1987). "Regulation of biosynthesis of hypusine in Chinese hamster ovary cells. Evidence for eIF-4D precursor polypeptides." J Biol Chem 262(26): 12730-12734.

Park, M. H. (1989). "The essential role of hypusine in eukaryotic translation initiation factor 4D (eIF-4D). Purification of eIF-4D and its precursors and comparison of their activities." J Biol Chem 264(31): 18531-18535.

Park, M. H. (2006). "The post-translational synthesis of a polyamine-derived amino acid, hypusine, in the eukaryotic translation initiation factor 5A (eIF5A)." J Biochem (Tokyo). 139(2): 161-9.

Park, M. H., H. L. Cooper and J. E. Folk (1981). "Identification of hypusine, an unusual amino acid, in a protein from human lymphocytes and of spermidine as its biosynthetic precursor." Proc Natl Acad Sci U S A. 78(5): 2869-73.

Park, M. H., H. L. Cooper and J. E. Folk (1982). "The biosynthesis of protein-bound hypusine (N epsilon -(4-amino-2- hydroxybutyl)lysine). Lysine as the amino acid precursor and the intermediate role of deoxyhypusine (N epsilon -(4-aminobutyl)lysine)." J. Biol. Chem. 257(12): 7217-7222.

Park, M. H., Y. A. Joe and K. R. Kang (1998). "Deoxyhypusine synthase activity is essential for cell viability in the yeast Saccharomyces cerevisiae." J Biol Chem 273(3): 1677-1683.

Park, M. H., Y. B. Lee and Y. A. Joe (1997). "Hypusine is essential for eukaryotic cell proliferation." Biol Signals 6(3): 115-123.

Park, M. H., E. C. Wolff, A. Abbruzzese and J. E. Folk (1988). "Post-translational formation of hypusine: biogenesis of translation initiation factor eIF-4D." Adv Exp Med Biol 231: 633-640.

Park, M. H., E. C. Wolff and J. E. Folk (1993b). "Hypusine: its post-translational formation in eukaryotic initiation factor 5A and its potential role in cellular regulation." Biofactors 4(2): 95-104.

Park, M. H., E. C. Wolff and J. E. Folk (1993c). "Is hypusine essential for eukaryotic cell proliferation?" Trends Biochem Sci. 18(12): 475-479.

Park, M. H., E. C. Wolff, Y. B. Lee and J. E. Folk (1993a). "Antiproliferative effects of inhibitors of deoxyhypusine synthase. Inhibition of growth of Chinese hamster ovary cells by guanyl diamines." J Biol Chem 269(45): 27827-27832.

Park, M. H., E. C. Wolff, Y. B. Lee and J. E. Folk (1994). "Antiproliferative effects of inhibitors of deoxyhypusine synthase. Inhibition of growth of Chinese hamster ovary cells by guanyl diamines." J Biol Chem. 269(45): 27827-27832.

Park, M. H., E. C. Wolff, Z. Smit-Mcbride, J. W. Hershey and J. E. Folk (1991). "Comparison of the activities of variant forms of eIF-4D. The requirement for hypusine or deoxyhypusine." J Biol Chem 266(13): 7988-7994.

Park, M. T., J. A. Kang, J. A. Choi, C. M. Kang, T. H. Kim, S. Bae, S. Kang, S. Kim, W. I. Choi, C. K. Cho, H. Y. Chung, Y. S. Lee and S. J. Lee (2003). "Phytosphingosine induces apoptotic cell death via caspase 8 activation and Bax translocation in human cancer cells." Clin Cancer Res 9(2): 878-885.

Park, Y. W., J. D. Ji, J. S. Lee, D. W. Ryang and D. H. Yoo (2003). "Actinomycin D renders cultured synovial fibroblasts susceptible to tumour necrosis factor related apoptosis-inducing ligand (TRAIL)-induced apoptosis." Scand J Rheumatol 32(6): 356-363.

Parlato, S., A. M. Giammarioli, M. Logozzi, F. Lozupone, P. Matarrese, F. Luciani, M. Falchi, W. Malorni and S. Fais (2000). "CD95 (APO-1/Fas) linkage to the actin cytoskeleton through ezrin in human T lymphocytes: a novel regulatory mechanism of the CD95 apoptotic pathway." EMBO J 19(19): 5123-5134.

Parrish, J., L. Li, K. Klotz, D. Ledwich, X. Wang and D. Xue (2001). "Mitochondrial endonuclease G is important for apoptosis in C. elegans." Nature 412(6842): 90-94.

Pastorino, J. G., S. T. Chen, M. Tafani, J. W. Snyder and J. L. Farber (1998). "The over-expression of Bax produces cell death upon induction of the mitochondrial permeability transition." J Biol Chem 273(13): 7770-7775.

Patterson, H., S. Gill, C. Fisher, M. G. Law, H. Jayatilake, C. D. Fletcher, M. Thomas, R. Grimer, B. A. Gusterson and C. S. Cooper (1994). "Abnormalities of the p53 MDM2 and DCC genes in human leiomyosarcomas." Br J Cancer 69(6): 1052-1058.

Pay, A., E. Heberle-Bors and H. Hirt (1989). "Isolation and sequence determination of the plant homologue of the eukaryotic initiation factor 4D cDNA from alfalfa, Medicago sativa." Plant Mol Biol 17(4): 927-929.

Peat, T. S., J. Newman, G. S. Waldo, J. Berendzen and T. C. Terwilliger (1998). "Structure of translation initiation factor 5A from Pyrobaculum aerophilum at 1.75 A resolution." Structure 6(9): 1207-14.

Pegg, A. E. and P. P. Mccann (1982). "Polyamine metabolism and function." Am J Physiol 243(5): C212-221.

Pelengaris, S., B. Rudolph and T. Littlewood (2000). "Action of Myc *in vivo* - proliferation and apoptosis." Curr Opin Genet Dev 10(1): 100-105.

Perez-Galan, P., G. Roue, N. Villamor, E. Montserrat, E. Campo and D. Colomer (2006). "The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status." Blood 107(1): 257-264.

Perry, C., R. Sastry, I. M. Nasrallah and P. J. Stover (2005). "Mimosine attenuates serine hydroxymethyltransferase transcription by chelating zinc. Implications for inhibition of DNA replication." J Biol Chem 280(1): 396-400.

Peter, M. E. and P. H. Krammer (2003). "The CD95(APO-1/Fas) DISC and beyond." Cell Death Differ 10(1): 26-35.

Petit, P. X., H. Lecoeur, E. Zorn, C. Dauguet, B. Mignotte and M. L. Gougeon (1995). "Alterations in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis." J Cell Biol 130(1): 157-167.

Petit, P. X., S. A. Susin, N. Zamzami, B. Mignotte and G. Kroemer (1996). "Mitochondria and programmed cell death: back to the future." FEBS Lett 396(1): 7-13.

Phillips, A. C., M. K. Ernst, S. Bates, N. R. Rice and K. H. Vousden (1999). "E2F-1 potentiates cell death by blocking anti-apoptotic signaling pathways." Mol Cell 4(5): 771-781.

Phillips, A. C. and K. H. Vousden (2001). "E2F-1 induced apoptosis." Apoptosis 6(3): 173-182.

Pinsky, D. J., W. Aji, M. Szabolcs, E. S. Athan, Y. Liu, Y. M. Yang, R. P. Kline, K. E. Olson and P. J. Cannon (1999). "Nitric oxide triggers programmed cell death (apoptosis) of adult rat ventricular myocytes in culture." Am J Physiol 277(3 Pt 2): H1189-1199.

Piva, R., G. Belardo and M. G. Santoro (2006). "NF-kappaB: a stress-regulated switch for cell survival." Antioxid Redox Signal 8(3-4): 478-486.

Polevoda, B. and F. Sherman (2003). "N-terminal acetyltransferases and sequence requirements for N-terminal acetylation of eukaryotic proteins." J Mol Biol 325(4): 595-622.

Porter, C. W. and J. R. Sufrin (1986). "Interference with polyamine biosynthesis and/or function by analogs of polyamines or methionine as a potential anticancer chemotherapeutic strategy." Anticancer Res 6(4): 525-542.

Pruyne, D., A. Legesse-Miller, L. Gao, Y. Dong and A. Bretscher (2004). "Mechanisms of polarized growth and organelle segregation in yeast." Annu Rev Cell Dev Biol 20: 559-591.

Putcha, G. V., M. Deshmukh and E. M. Johnson Jr (1999). "BAX translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, BCL-2, and caspases." J Neurosci 19(17): 7476-7485.

Putcha, G. V., K. L. Moulder, J. P. Golden, P. Bouillet, J. A. Adams, A. Strasser and E. M. Johnson (2001). "Induction of BIM, a pro-apoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis." Neuron 29(3): 615-628.

Puthalakath, H., D. C. Huang, L. A. O'reilly, S. M. King and A. Strasser (1999). "The pro-apoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex." Mol Cell 3(3): 287-296.

Puthalakath, H. and A. Strasser (2002). "Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins." Cell Death Differ 9(5): 505-512.

Puthalakath, H., A. Villunger, L. A. O'reilly, J. G. Beaumont, L. Coultas, R. E. Cheney, D. C. Huang and A. Strasser (2001). "Bmf: a pro-apoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis." Science 293(5536): 1829-1832.

Pützer, B. M. (2007). "E2F1 death pathways as targets for cancer therapy." J Cell Mol Med 11(2): 239-251.

Pützer, B. M., T. Stiewe, K. Parssanedjad, S. Rega and H. Esche (2000). "E1A is sufficient by itself to induce apoptosis independent of p53 and other adenoviral gene products." Oncogene 7(2): 177-188.

Qin, X. Q., D. M. Livingston, W. G. Kaelin Jr and P. D. Adams (1994). "Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis." Proc Natl Acad Sci U S A 91(23): 10918-10922.

Rahman-Roblick, R., U. Johannes Roblick, U. Hellman, P. Conrotto, T. Liu, S. Becker, D. Hirschberg, H. Jornvall, G. Auer and K. G. Wiman (2007). "p53 targets identified by protein expression profiling." Proc Natl Acad Sci U S A 104(13): 5401-5406.

Ramadan, S., A. Terrinoni, M. V. Catani, A. E. Sayan, R. A. Knight, M. Mueller, P. H. Krammer, G. Melino and E. Candi (2005). "p73 induces apoptosis by different mechanisms." Biochem Biophys Res Commun 331(3): 713-717.

Ramakrishna Rao, D. N. and A. I. Cederbaum (1996). "Generation of reactive oxygen species by the redox cycling of nitroprusside." Biochim Biophys Acta 1289(2): 195-202.

Ramaswamy, S., K. N. Ross, E. S. Lander and T. R. Golub (2003). "A molecular signature of metastasis in primary solid tumors." Nat Genet 33(1): 49-54.

Rangarajan, E. S. and V. Shankar (2001). "Sugar non-specific endonucleases." FEMS Microbiol Rev 25(5): 583-613.

Rao, L., M. Debbas, P. Sabbatini, D. Hockenbery, S. Korsmeyer and E. White (1992). "The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins." Proc Natl Acad Sci U S A 89(16): 7742-7746.

Rao, R. V., S. Castro-Obregon, H. Frankowski, M. Schuler, V. Stoka, G. Del Rio, D. E. Bredesen and H. M. Ellerby (2002). "Coupling Endoplasmic Reticulum Stress to the Cell Death Program." J Biol Chem 277(24): 21836-21842.

Real, P. J., C. Sanz, O. Gutierrez, C. Pipaon, A. M. Zubiaga and J. L. Fernandez-Luna (2006). "Transcriptional activation of the pro-apoptotic bik gene by E2F proteins in cancer cells." FEBS Lett 580(25): 5905-5909.

Reed, J. C. (1994). "Bcl-2 and the regulation of programmed cell death." J Cell Biol 124(1-2): 1-6.

Rehemtulla, A., C. A. Hamilton, A. M. Chinnaiyan and V. M. Dixit (1997). "Ultraviolet radiation-induced apoptosis is mediated by activation of CD-95 (Fas/APO-1)." J Biol Chem 272(41): 25783-25786.

Richo, G. R. and G. E. Conner (1994). "Structural requirements of procathepsin D activation and maturation." J Biol Chem 269(20): 14806-14812.

Rinaudo, M. S., Y. A. Joe and M. H. Park (1993). "Cloning and sequencing of a chick embryo cDNA encoding the 20-kDa hypusine-containing protein, eIF-5A." Gene 137(2): 303-307.

Robertson, J. D., S. Orrenius and B. Zhivotovsky (2000). "Review: nuclear events in apoptosis." J Struct Biol 129(2-3): 346-358.

Robinson, A. B. and C. J. Rudd (1974). "Deamidation of glutaminyl and asparaginyl residues in peptides and proteins." Curr Top Cell Regul 8(0): 247-295.

Robinson, N. E. and A. B. Robinson (2001). "Molecular clocks." Proc Natl Acad Sci U S A 98(3): 944-949.

Robinson, N. E. and A. B. Robinson (2004). "Amide molecular clocks in drosophila proteins: potential regulators of aging and other processes." Mech Ageing Dev 125(4): 259-267.

Robles, A. I., N. A. Bemmels, A. B. Foraker and C. C. Harris (2001). "APAF-1 is a transcriptional target of p53 in DNA damage-induced apoptosis." Cancer Res 61(18): 6660-6664.

Rodrigues, N. R., A. Rowan, M. E. Smith, I. B. Kerr, W. F. Bodmer, J. V. Gannon and D. P. Lane (1990). "p53 mutations in colorectal cancer." Proc Natl Acad Sci U S A 87(19): 7555-75559.

Rodriguez, J. and Y. Lazebnik (1999). "Caspase-9 and APAF-1 form an active holoenzyme." Genes Dev 13(24): 3179-3184.

Roos, W. P. and B. Kaina (2006). "DNA damage-induced cell death by apoptosis." Trends Mol Med 12(9): 440-450.

Rosorius, O., B. Reichart, F. Kratzer, P. Heger, M. C. Dabauvalle and J. Hauber (1999). "Nuclear pore localization and nucleocytoplasmic transport of eIF-5A: evidence for direct interaction with the export receptor CRM1." J Cell Sci 112(Pt 14): 2369-2380.

Rosse, T., R. Olivier, L. Monney, M. Rager, S. Conus, I. Fellay, B. Jansen and C. Borner (1998). "Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c." Nature 391(6666): 496-499.

Rossi, M., A. E. Sayan, A. Terrinoni, G. Melino and R. A. Knight (200). "Mechanism of induction of apoptosis by p73 and its relevance to neuroblastoma biology." Ann N Y Acad Sci 1028: 143-149.

Rothe, M., M. G. Pan, W. J. Henzel, T. M. Ayres and D. V. Goeddel (1995). "The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins." Cell 81(3): 1243-1252.

Ruhl, M., M. Himmelspach, G. M. Bahr, F. Hammerschmid, H. Jaksche, B. Wolff, H. Aschauer, G. K. Farrington, H. Probst, D. Bevec and J. Hauber (1993). "Eukaryotic initiation factor 5A is a cellular target of the human immunodeficiency virus type 1 Rev activation domain mediating trans-activation." J Cell Biol 123(6 Pt 1): 1309-1320.

Ruiz-Vela, A., J. T. Opferman, E. H. Cheng and S. J. Korsmeyer (2005). "Pro-apoptotic BAX and BAK control multiple initiator caspases." EMBO Rep 6(4): 379-385.

Sachs, A. B. and G. Varani (2000). "Eukaryotic translation initiation: there are (at least) two sides to every story." Nat Struct Biol 7(5): 356-61.

Sala, A., N. C. Nicolaides, A. Engelhard, T. Bellon, F. C. Lawe, A. Arnold, X. Grana, A. Giordano and B. Calabretta (1994). "Correlation between E2F-1 requirement in the S phase and E2F-1 transactivation of cell cycle-related genes in human cells." Cancer Res 54(6): 1402-1406.

Sanchez-Prieto, R., V. J. Sanchez-Arevalo, J. M. Servitja and J. S. Gutkind (2002). "Regulation of p73 by c-Abl through the p38 MAP kinase pathway." Oncogene 21(6): 974-979.

Sandholzer, U., M. Centea-Intemann, A. A. Noegel and F. Lottspeich (1989). "cDNA and derived amino acid sequence of the hypusine containing protein from Dictyostelium discoideum." FEBS Lett 246(1-2): 94-100.

Sansome, C., A. Zaika, N. D. Marchenko and U. M. Moll (2001). "Hypoxia death stimulus induces translocation of p53 protein to mitochondria. Detection by immunofluorescence on whole cells." FEBS Lett 488(3): 110-115.

Sasaki, K., M. R. Abid and M. Miyazaki (1996). "Deoxyhypusine synthase gene is essential for cell viability in the yeast Saccharomyces cerevisiae." FEBS Lett 384(2): 151-154.

Sassone, J., A. Ciammola, C. Tiloca, M. Glionna, G. Meola, E. Mancinelli and V. Silani (2006). "Apoptosis induced by proteasome inhibition in human myoblast cultures." Eur J Histochem 50(2): 109-118.

Sattler, M., H. Liang, D. Nettesheim, R. P. Meadows, J. E. Harlan, M. Eberstadt, H. S. Yoon, S. B. Shuker, B. S. Chang, A. J. Minn, C. B. Thompson and S. W. Fesik (1997). "Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis." Science 275(5302): 983-986.

Sax, J. K. and W. S. El-Deiry (2003). "p53 downstream targets and chemosensitivity." Cell Death Differ 10(4): 413-417.

Sax, J. K., P. Fei, M. E. Murphy, E. Bernhard, S. J. Korsmeyer and W. S. El-Deiry (2002). "BID regulation by p53 contributes to chemosensitivity." Nat Cell Biol 4(11): 842-849.

Scaffidi, C., S. Fulda, A. Srinivasan, C. Friesen, F. Li, K. J. Tomaselli, K. M. Debatin, P. H. Krammer and M. E. Peter (1998). "Two CD95 (APO-1/Fas) signaling pathways." EMBO J 17(6): 1675-1687.

Schatz, O., M. Oft, C. Dascher, M. Schebesta, O. Rosorius, H. Jaksche, M. Dobrovnik, D. Bevec and J. Hauber (1998). "Interaction of HIV-1 Rev cofactor eukaryotic initiation factor 5A with ribosomal protein L5." Proc Natl Acad Sci U S A 95(4): 1607-1612.

Scheffner, M., K. Munger, J. C. Byrne and P. M. Howley (1991). "The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines." Proc Natl Acad Sci U S A 88(13): 5523-5527.

Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine and P. M. Howley (1990). "The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53." Cell 63(6): 1129-1136.

Schendel, S. L., R. Azimov, K. Pawlowski, A. Godzik, B. L. Kagan and J. C. Reed (1999). "Ion channel activity of the BH3 only Bcl-2 family member, BID." J Biol Chem 274(31): 21932-21936.

Schendel, S. L., Z. Xie, M. O. Montal, S. Matsuyama, M. Montal and J. C. Reed (1997). "Channel formation by anti-apoptotic protein Bcl-2." Proc Natl Acad Sci U S A 94(10): 5113-5118.

Schindler, P., D. Müller, W. Märki, H. Grossenbacher and W. J. Richter (1996). "Characterization of a beta-Asp33 isoform of recombinant hirudin sequence variant 1 by low-energy collision-induced dissociation." J Mass Spectrom 31(9): 967-974.

Schinzel, A., T. Kaufmann, M. Schuler, J. Martinalbo, D. Grubb and C. Borner (2004). "Conformational control of Bax localization and apoptotic activity by Pro168." J Cell Biol 164(7): 1021-1032.

Schnier, J., H. G. Schwelberger, Z. Smit-Mcbride, H. A. Kang and J. W. Hershey (1991). "Translation initiation factor 5A and its hypusine modification are essential for cell viability in the yeast Saccharomyces cerevisiae." Mol Cell Biol 11(6): 3105-3114.

Schnuchel, A., R. Wiltscheck, M. Czisch, M. Herrler, G. Willimsky, P. Graumann, M. A. Marahiel and T. A. Holak (1993). "Structure in solution of the major cold-shock protein from Bacillus subtilis." Nature 365(6433): 169-171.

Schrader, R., C. Young, D. Kozian, R. Hoffmann and F. Lottspeich (2006). "Temperature-sensitive eIF5A mutant accumulates transcripts targeted to the nonsense-mediated decay pathway." J Biol Chem 281(46): 35336-35346.

Schroder, H. (2006). "No nitric oxide for HO-1 from sodium nitroprusside." Mol Pharmacol 69(5): 1507-1509.

Schwartzman R. A. and J. A. Cidlowski (1993). "Apoptosis: the biochemistry and molecular biology of programmed cell death." Endocr Rev 14(2):133-51.

Schwarz, J. K., C. H. Bassing, I. Kovesdi, M. B. Datto, M. Blazing, S. George, X. F. Wang and J. R. Nevins (1995). "Expression of the E2F1 transcription factor overcomes type beta transforming growth factor-mediated growth suppression." Proc Natl Acad Sci U S A 92(2): 483-487.

Schwarz, M., M. A. Andrade-Navarro and A. Gross (2007). "Mitochondrial carriers and pores: Key regulators of the mitochondrial apoptotic program?" Apoptosis 12(5): 869-876.

Schwelberger, H. G., H. A. Kang and J. W. Hershey (1993). "Translation initiation factor eIF-5A expressed from either of two yeast genes or from human cDNA. Functional identity under aerobic and anaerobic conditions." J Biol Chem 268(19): 14018-14025.

Scoltock, A. B. and J. A. Cidlowski (2004). "Activation of intrinsic and extrinsic pathways in apoptotic signaling during UV-C-induced death of Jurkat cells: the role of caspase inhibition." Exp Cell Res 297(1): 212-223.

Scorrano, L. (2003). "Divide et impera: Ca2+ signals, mitochondrial fission and sensitization to apoptosis." Cell Death Differ 10(12): 1287-1289.

Scorrano, L. and S. J. Korsmeyer (2003). "Mechanisms of cytochrome c release by pro-apoptotic BCL-2 family members." Biochem Biophys Res Commun 304(3): 437-444.

Scorrano, L., S. A. Oakes, J. T. Opferman, E. H. Cheng, M. D. Sorcinelli, T. Pozzan and S. J. Korsmeyer (2003). "BAX and BAK Regulation of Endoplasmic Reticulum Ca2+: A Control Point for Apoptosis." Science 300(5616): 135 - 139.

Sedlak, T. W., Z. N. Oltvai, E. Yang, K. Wang, L. H. Boise, C. B. Thompson and S. J. Korsmeyer (1995). "Multiple Bcl-2 family members demonstrate selective dimerizations with Bax." Proc Natl Acad Sci U S A 92(17): 7834-7838.

Seelan, R. S., M. Irwin, P. Van Der Stoop P, C. Qian, W. G. Kaelin Jr and W. Liu (2002). "The human p73 promoter: characterization and identification of functional E2F binding sites." Neoplasia 4(3): 195-203.

Shao, R. G., T. Shimizu and Y. Pommier (1996). "Brefeldin A is a potent inducer of apoptosis in human cancer cells independently of p53." Exp Cell Res 227(2): 190-196.

Sheikh, M. S., M. J. Antinore, Y. Huang and A. J. Fornace Jr (1998). "Ultraviolet-irradiation-induced apoptosis is mediated via ligand independent activation of tumor necrosis factor receptor 1." Oncogene 17(20): 2555-2563.

Sheridan, J. P., S. A. Marsters, R. M. Pitti, A. Gurney, M. Skubatch, D. Baldwin, L. Ramakrishnan, C. L. Gray, K. Baker, W. I. Wood, A. D. Goddard, P. Godowski and A. Ashkenazi (1997). "Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors." Science 277(5327): 818-21.

Sherman, F., J. W. Stewart and S. Tsunasawa (1985). "Methionine or not methionine at the beginning of a protein." Bioessays 3(1): 27-31.

Shi, X. P., K. C. Yin, J. Ahern, L. J. Davis, A. M. Stern and L. Waxman (1996b). "Effects of N1-guanyl-1,7-diaminoheptane, an inhibitor of deoxyhypusine synthase, on the growth of tumorigenic cell lines in culture." Biochim Biophys Acta 1310(1): 119-126.

Shi, X. P., K. C. Yin and L. Waxman (1997). "Effects of inhibitors of RNA and protein synthesis on the subcellular distribution of the eukaryotic translation initiation factor, eIF-5A, and the HIV-1 Rev protein." Biol Signals 6(3): 143-149.

Shi, X. P., K. C. Yin, Z. A. Zimolo, A. M. Stern and L. Waxman (1996a). "The subcellular distribution of eukaryotic translation initiation factor, eIF-5A, in cultured cells." Exp Cell Res 225(2): 348-356.

Shi, Y. (2002). "Mechanisms of caspase activation and inhibition during apoptosis." Mol Cell 9(3): 459-470.

Shiba, T., H. Mizote, T. Kaneko, T. Nakajima and Y. Kakimoto (1971). "Hypusine, a new amino acid occurring in bovine brain. Isolation and structural determination." Biochim Biophys Acta 244(3): 523-531.

Shibasaki, F., E. Kondo, T. Akagi and F. Mckeon (1997). "Suppression of signaling through transcription factor NF-AT by interactions between calcineurin and Bcl-2." Nature 386(6626): 728-731.

Shimizu, S., Y. Eguchi, W. Kamiike, Y. Funahashi, A. Mignon, V. Lacronique, H. Matsuda and Y. Tsujimoto (1998). "Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux." Proc Natl Acad Sci U S A 95(4): 1455-1459.

Shimizu, S., A. Konishi, T. Kodama and Y. Tsujimoto (2000). "BH4 domain of anti-apoptotic Bcl-2 family members closes voltage-dependent anion channel and inhibits apoptotic mitochondrial changes and cell death." Proc Natl Acad Sci U S A 97(7): 3100-3105.

Shimizu, S., M. Narita and Y. Tsujimoto (1999). "Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC." Nature 399(6735): 483-487.

Shimodaira, H., A. Yoshioka-Yamashita, R. D. Kolodner and J. Y. Wang (2003). "Interaction of mismatch repair protein PMS2 and the p53-related transcription factor p73 in apoptosis response to cisplatin." Proc Natl Acad Sci U S A 100(5): 2420-2425.

Shinjyo, T., R. Kuribara, T. Inukai, H. Hosoi, T. Kinoshita, A. Miyajima, P. J. Houghton, A. T. Look, K. Ozawa and T. Inaba (2001). "Downregulation of Bim, a pro-apoptotic relative of Bcl-2, is a pivotal step in cytokine-initiated survival signaling in murine hematopoietic progenitor." Mol Cell Biol 21(3): 854-864.

Shinoura, N., M. Ohashi, Y. Yoshida, T. Kirino, A. Asai, M. Hashimoto and H. Hamada (2000). "Adenovirus-mediated over-expression of Fas induces apoptosis of gliomas." Cancer Gene Ther 7(2): 224-232.

Shiraishi, H., H. Okamoto, A. Yoshimura and H. Yoshida (2006). "ER stress-induced apoptosis and caspase-12 activation occurs downstream of mitochondrial apoptosis involving Apaf-1." J Cell Sci 119: 3958-3966.

Shore, G. C. and J. Viallet (2005). "Modulating the bcl-2 family of apoptosis suppressors for potential therapeutic benefit in cancer." Hematology Am Soc Hematol Educ Program: 226-230.

Shou, Y., L. Li, K. Prabhakaran, J. L. Borowitz and G. E. Isom (2003). "p38 Mitogen-activated protein kinase regulates Bax translocation in cyanide-induced apoptosis." Toxicol Sci 75(1): 99-107.

Si, K., K. Das and U. Maitra (1996). "Characterization of multiple mRNAs that encode mammalian translation initiation factor 5 (eIF-5)." J Biol Chem 271(28): 16934-16938.

Sidow, A. (1996). "Gen(om)e duplications in the evolution of early vertebrates." Curr Opin Genet Dev 6(6): 715-722.

Simbula, G., P. A. Glascott Jr, S. Akita, J. B. Hoek and J. L. Farber (1997). "Two mechanisms by which ATP depletion potentiates induction of the mitochondrial permeability transition." Am J Physiol 273(2 Pt 1): C479-488.

Singh, U. S., Q. Li and R. Cerione (1998). "Identification of the eukaryotic initiation factor 5A as a retinoic acid-stimulated cellular binding partner for tissue transglutaminase II." J Biol Chem 273(4): 1946-1950.

Sionov, R. V. and Y. Haupt (1999). "The cellular response to p53: the decision between life and death." Oncogene 18(45): 6145-6157.

Slee, E. A., M. T. Harte, R. M. Kluck, B. B. Wolf, C. A. Casiano, D. D. Newmeyer, H. G. Wang, J. C. Reed, D. W. Nicholson, E. S. Alnemri, D. R. Green and S. J. Martin (1999). "Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner." J cell biol 144(2): 281-292.

Smit-Mcbride, Z., T. E. Dever, J. W. Hershey and W. C. Merrick (1989a). "Sequence determination and cDNA cloning of eukaryotic initiation factor 4D, the hypusine-containing protein." J Biol Chem 264(3): 1578-1583.

Smit-Mcbride, Z., J. Schnier, R. J. Kaufman and J. W. Hershey (1989b). "Protein synthesis initiation factor eIF-4D. Functional comparison of native and unhypusinated forms of the protein." J Biol Chem 264(31): 18527-18530.

Sobell, H. M. (1985). "Actinomycin and DNA transcription." Proc Natl Acad Sci U S A 82(16): 5328-5331.

Sohn, D., K. Schulze-Osthoff and R. U. Janicke (2005). "Caspase-8 can be activated by interchain proteolysis without receptor-triggered dimerization during drug-induced apoptosis." J Biol Chem 280(7): 5267-5273.

Song, Q., S. P. Lees-Miller, S. Kumar, Z. Zhang, D. W. Chan, G. C. Smith, S. P. Jackson, E. S. Alnemri, G. Litwack, K. K. Khanna and M. F. Lavin (1996). "DNA-dependent protein kinase catalytic subunit: a target for an ICE-like protease in apoptosis." EMBO J 15(13): 3238-3246.

Song, S., G. A. Gulliver and P. F. Lambert (1998). "Human papillomavirus type 16 E6 and E7 oncogenes abrogate radiation-induced DNA damage responses *in vivo* through p53-dependent and p53-independent pathways." Proc Natl Acad Sci U S A 95(5): 2290-2295.

Spradling, A. C., D. Stern, A. Beaton, E. J. Rhem, T. Laverty, N. Mozden, S. Misra and G. M. Rubin (1999). "The Berkeley Drosophila Genome Project gene disruption project: Single P-element insertions mutating 25% of vital Drosophila genes." Genetics 153(1): 135-77.

Sprick, M. R., E. Rieser, H. Stahl, A. Grosse-Wilde, M. A. Weigand and H. Walczak (2002). "Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signaling complexes in a FADD-dependent manner but can not functionally substitute caspase-8." EMBO J 21(17): 4520-4530.

Srinivasula, S. M., M. Ahmad, T. Fernandes-Alnemri and E. S. Alnemri (1998). "Autoactivation of procaspase 9 by Apaf-1-mediated oligomerization." Mol Cell 1(7): 949-957.

Stade, K., C. S. Ford, C. Guthrie and K. Weis (1997). "Exportin 1 (Crm1p) is an essential nuclear export factor." Cell 90(6): 1041-1050.

Stahl, M., P. F. Dijkers, G. J. Kops, S. M. Lens, P. J. Coffer, B. M. Burgering and R. H. Medema (2002). "The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2." J Immunol 168(10): 5024-5031.

Steinkasserer, A., T. Jones, D. Sheer, K. Koettnitz, J. Hauber and D. Bevec (1995). "The eukaryotic cofactor for the human immunodeficiency virus type 1 (HIV-1) rev protein, eIF-5A, maps to chromosome 17p12-p13: three eIF-5A pseudogenes map to 10q23.3, 17q25, and 19q13.2." Genomics 25(3): 749-752.

Stephenson, R. C. and S. Clarke (1989). "Succinimide formation from aspartyl and asparaginyl peptides as a model for the spontaneous degradation of proteins." J Biol Chem 264(11): 6164-6170.

Stiewe, T. and B. M. Pützer (2000). "Role of the p53-homologue p73 in E2F1-induced apoptosis." Nat Genet 26(4): 464-469.

Stiewe, T. and B. M. Pützer (2001). "p73 in apoptosis." Apoptosis 6(6): 447-452.

Stoka, V., B. Turk, S. L. Schendel, T. H. Kim, T. Cirman, S. J. Snipas, L. M. Ellerby, D. Bredesen, H. Freeze, M. Abrahamson, D. Bromme, S. Krajewski, J. C. Reed, X. M. Yin, V. Turk and G. S.

Salvesen (2001). "Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route." J Biol Chem 276(5): 3149-3157.

Strack, P. R., M. W. Frey, C. J. Rizzo, B. Cordova, H. J. George, R. Meade, S. P. Ho, J. Corman, R. Tritch and B. D. Korant (1996). "Apoptosis mediated by HIV protease is preceded by cleavage of Bcl-2." Proc Natl Acad Sci U S A 93(18): 9571-9576.

Strasser, A., A. W. Harris, T. Jacks and S. Cory (1994). "DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2." Cell 79(2): 329-339.

Sugimoto, A. (2004). "High-throughput RNAi in Caenorhabditis elegans: genome-wide screens and functional genomics." Differentiation 72(2-3): 81-91.

Sumitani, K., R. Kamijo and M. Nagumo (1997). "Cytotoxic effect of sodium nitroprusside on cancer cells: involvement of apoptosis and suppression of c-myc and c-myb proto-oncogene expression." Anticancer Res 17(2A): 865-871.

Sunters, A., S. Fernandez De Mattos, M. Stahl, J. J. Brosens, G. Zoumpoulidou, C. A. Saunders, P. J. Coffer, R. H. Medema, R. C. Coombes and E. W. Lam (2003). "FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines." J Biol Chem 278(50): 49795-49805.

Susin, S. A., E. Daugas, L. Ravagnan, K. Samejima, N. Zamzami, M. Loeffler, P. Costantini, K. F. Ferri, T. Irinopoulou, M. C. Prevost, G. Brothers, T. W. Mak, J. Penninger, W. C. Earnshaw and G. Kroemer (2000). "Two Distinct Pathways Leading to Nuclear Apoptosis." J Exp Med 192(4): 571-580.

Susin, S. A., H. K. Lorenzo, N. Zamzami, I. Marzo, B. E. Snow, G. M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, N. Larochette, D. R. Goodlett, R. Aebersold, D. P. Siderovski, J. M. Penninger and G. Kroemer (1999). "Molecular characterization of mitochondrial apoptosis-inducing factor." Nature 397(6718): 441-446.

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

Suzuki, M., R. J. Youle and N. Tjandra (2000). "Structure of Bax: coregulation of dimer formation and intracellular localization." Cell 103(4): 645-654.

Suzuki, Y., Y. Imai, H. Nakayama, K. Takahashi, K. Takio and R. Takahashi (2001). "A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death." Mol Cell 8(3): 613-621.

Szabadkai, G. and R. Rizzuto (2004). "Participation of endoplasmic reticulum and mitochondrial calcium handling in apoptosis: more than just neighborhood?" FEBS Lett 567(1): 111-115.

Takahashi, R., Q. Deveraux, I. Tamm, K. Welsh, N. Assa-Munt, G. S. Salvesen and J. C. Reed (1998). "A single BIR domain of XIAP sufficient for inhibiting caspases." J Biol Chem 272(14): 7787-7790.

Takamatsu, M., T. Fujita and H. Hotta (2001). "Suppression of serum starvation-induced apoptosis by hepatitis C virus core protein." Kobe J Med Sci 47(2): 97-112.

Takeuchi, K., K. Nakamura, M. Fujimoto, S. Kaino, S. Kondoh and K. Okita (2002). "Heat stress-induced loss of eukaryotic initiation factor 5A (eIF-5A) in a human pancreatic cancer cell line, MIA PaCa-2, analyzed by two-dimensional gel electrophoresis." Electrophoresis 23(4): 662-669.

Talis, A. L., J. M. Huibregtse and P. M. Howley (1998). "The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells." J Biol Chem 273(11): 6439-6445.

Tan, T. T., K. Degenhardt, D. A. Nelson, B. Beaudoin, W. Nieves-Neira, P. Bouillet, A. Villunger, J. M. Adams and E. White (2005). "Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy." Cancer Cell 7(3): 227-238.

Tang, D., J. M. Lahti and V. J. Kidd (2000). "Caspase-8 Activation and Bid Cleavage Contribute to MCF7 Cellular Execution in a Caspase-3-dependent Manner during Staurosporine-mediated Apoptosis." J Biol Chem 275(13): 9303-9307.

Tao, Y. and K. Y. Chen (1995). "Purification of deoxyhypusine synthase from Neurospora crassa to homogeneity by substrate elution affinity chromatography." J Biol Chem 270(1): 383-386.

Taylor, C. A., M. Senchyna, J. Flanagan, E. M. Joyce, D. O. Cliche, A. N. Boone, S. Culp-Stewart and J. E. Thompson (2004). "Role of eIF5A in TNF-alpha-mediated apoptosis of lamina cribrosa cells." Invest Ophthalmol Vis Sci. 45(10): 3568-3576.

Taylor, C. A., Z. Sun, D. O. Cliche, H. Ming, B. Eshaque, S. Jin, M. T. Hopkins, B. Thai and J. E. Thompson (2007). "Eukaryotic translation initiation factor 5A induces apoptosis in colon cancer cells and associates with the nucleus in response to tumour necrosis factor alpha signaling." Exp Cell Res 313(3): 437-449.

Taylor, I. W. (1980). "A rapid single step staining technique for DNA analysis by flow microfluorimetry." J Histochem Cytochem 28(9): 1021-1024.

Te Poele, R. H. and S. P. Joel (1999). "Schedule-dependent cytotoxicity of SN-38 in p53 wild-type and mutant colon adenocarcinoma cell lines." Br J Cancer 81(8): 1285-1293.

Te Poele, R. H., A. L. Okorokov and S. P. Joel (1999). "RNA synthesis block by 5, 6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) triggers p53-dependent apoptosis in human colon carcinoma cells." Oncogene 18(42): 5765-5772.

Teodoro, J. G., G. C. Shore and P. E. Branton (1995). "Adenovirus E1A proteins induce apoptosis by both p53-dependent and p53-independent mechanisms." Oncogene 11(3467-3474).

Terradillos, O., S. Montessuit, D. C. Huang and J. C. Martinou (2002). "Direct addition of Biml to mitochondria does not lead to cytochrome c release." FEBS Lett 522(1-3): 29-34.

Terrasson, J., S. Allart, H. Martin, J. Lule, H. Haddada, D. Caput and C. Davrinche (2005). "p73-dependent apoptosis through death receptor: impairment by human cytomegalovirus infection." Cancer Res 65(7): 2787-2794.

Terrinoni, A., M. Ranalli, B. Cadot, A. Leta, G. Bagetta, K. H. Vousden and G. Melino (2004). "p73-alpha is capable of inducing scotin and ER stress." Oncogene 23(20): 3721-3725.

Thomas, A., H. Goumans, H. Amesz, R. Benne and H. O. Voorma (1979). "A comparison of the initiation factors of eukaryotic protein synthesis from ribosomes and from the postribosomal supernatant." Eur J Biochem 98(2): 329-337.

Thomas, M., G. Matlashewski, D. Pim and L. Banks (1996). "Induction of apoptosis by p53 is independent of its oligomeric state and can be abolished by HPV-18 E6 through ubiquitin mediated degradation." Oncogene 2(13): 265-273.

Thompson, C. B. (1995). "Apoptosis in the pathogenesis and treatment of disease." Science 267(5203): 1456-1462.

Thornberry, N. A. (1997). "The caspase family of cysteine proteases." Br Med Bull 53(3): 478-490.

Tikhomirov, O. and G. Carpenter (2005). "Bax activation and translocation to mitochondria mediate EGF-induced programmed cell death." J Cell Sci 118(Pt 24): 5681-5690.

Tishler, R. B., S. K. Calderwood, C. N. Coleman and B. D. Price (1993). "Increases in sequence specific DNA binding by p53 following treatment with chemotherapeutic and DNA damaging agents." Cancer Res 53(10 Suppl): 2212-2216.

Tiwari, M. M., K. J. Messer and P. R. Mayeux (2006). "Inducible Nitric oxide synthase and apoptosis in Murine Proximal Tubule Epithelial Cells." Toxicological Sciences 91(2): 493-500.

Tobias, J. W., T. E. Shrader, G. Rocap and A. Varshavsky (1991). "The N-end rule in bacteria." Science 254(5036): 1374-1377.

Tome, M. E., S. M. Fiser, C. M. Payne and E. W. Gerner (1997). "Excess putrescine accumulation inhibits the formation of modified eukaryotic initiation factor 5A (eIF-5A) and induces apoptosis." Biochem J. 328(Pt 3): 847-54.

Tome, M. E. and E. W. Gerner (1997). "Cellular eukaryotic initiation factor 5A content as a mediator of polyamine effects on growth and apoptosis." Biol Signals 6(3): 150-156.

Torrelio, B. M., M. A. Paz and P. M. Gallop (1987). "The formation and stability of the hypusine containing protein in Chinese hamster ovary cells." Biochem Biophys Res Commun 145(3): 1335-1341.

Torres, R., B. L. Firestein, H. Dong, J. Staudinger, E. N. Olson, R. L. Huganir, D. S. Bredt, N. W. Gale and G. D. Yancopoulos (1998). "PDZ proteins bind, cluster, and synaptically colocalize with Eph receptors and their ephrin ligands." Neuron 21(6): 1453-1463.

Trachsel, H. and T. Staehelin (1978). "Binding and release of eukaryotic initiation factor eIF-2 and GTP during protein synthesis initiation." Proc Natl Acad Sci U S A 75(1): 204-208.

Tsi, C. J., Y. Chao, C. W. Chen and W. W. Lin (2002). "Aurintricarboxylic acid protects against cell death caused by lipopolysaccharide in macrophages by decreasing inducible nitric-oxide synthase induction via IkappaB kinase, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinase inhibition." Mol Pharmacol 62(1): 90-101.

Tsujimoto, Y., N. Ikegaki and C. M. Croce (1987). "Characterization of the protein product of bcl-2, the gene involved in human follicular lymphoma." Oncogene 2(1): 3-7.

Tuschl, T. (2001). "RNA interference and small interfering RNAs." Chembiochem 2(4): 239-245.

Tyler-Cross, R. and V. Schirch (1991). "Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides." J Biol Chem 266(33): 22549-22556.

Ueda, K. and D. Ganem (1996). "Apoptosis is induced by N-myc expression in hepatocytes, a frequent event in hepadnavirus oncogenesis, and is blocked by insulin-like growth factor II." J Virol 70(3): 1375-1383.

Ueda, Y., M. Hijikata, S. Takagi, T. Chiba and K. Shimotohno (1999). "New p73 variants with altered C-terminal structures have varied transcriptional activities." Oncogene 18(35): 4993-4998.

Unbehaun, A., S. I. Borukhov, C. U. Hellen and T. V. Pestova (2004). "Release of initiation factors from 48S complexes during ribosomal subunit joining and the link between establishment of codon-anticodon base-pairing and hydrolysis of eIF2-bound GTP." Genes Dev 18(24): 3078-3093.

Valentini, S. R., J. M. Casolari, C. C. Oliveira, P. A. Silver and A. E. Mcbride (2002). "Genetic interactions of yeast eukaryotic translation initiation factor 5A (eIF5A) reveal connections to poly(A)-binding protein and protein kinase C signaling." Genetics 160(2): 393-405.

Valko, M., H. Morris and M. T. Cronin (2005). "Metals, toxicity and oxidative stress." Curr Med Chem 12(10): 1161-1208.

Van De Craen, M., W. Declercq, I. Van Den Brande, W. Fiers and P. Vandenabeele (1999). "The proteolytic procaspase activation network: an *in vitro* analysis." Cell Death Differ 6(11): 1117-1124.

Van Golen, K. L., S. Davies, Z. F. Wu, Y. Wang, C. D. Bucana, H. Root, S. Chandrasekharappa, M. Strawderman, S. P. Ethier and S. D. Merajver (1999). "A novel putative low-affinity insulin-like growth factor-binding protein, LIBC (lost in inflammatory breast cancer), and RhoC GTPase correlate with the inflammatory breast cancer phenotype." Clin Cancer Res 5(9): 2511-2519.

Van Gurp, M., N. Festjens, G. Van Loo, X. Saelens and P. Vandenabeele (2003). "Mitochondrial intermembrane proteins in cell death." Biochem Biophys Res Commun 304(3): 487-497.

Van Loo, G., P. Schotte, M. Van Gurp M, H. Demol, B. Hoorelbeke, K. Gevaert, I. Rodriguez, A. Ruiz-Carrillo, J. Vandekerckhove, W. Declercq, R. Beyaert and P. Vandenvan Looabeele (2001). "Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation." Cell Death Differ 8(12): 1136-42.

Vander Heiden, M. G., N. S. Chandel, E. K. Williamson, P. T. Schumacker and C. B. Thompson (1997). "Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria." Cell 91(5): 627-637.

Varfolomeev, E. E., M. P. Boldin, T. M. Goncharov and D. Wallach (1996). "A potential mechanism of "cross-talk" between the p55 tumor necrosis factor receptor and Fas/APO1: proteins binding to the death domains of the two receptors also bind to each other." J Exp Med 183: 1271-1275.

Varfolomeev, E. E., M. Schuchmann, V. Luria, N. Chiannilkulchai, J. S. Beckmann, I. L. Mett, D. Rebrikov, V. M. Brodianski, O. C. Kemper, O. Kollet, T. Lapidot, D. Soffer, T. Sobe, K. B. Avraham, T. Goncharov, H. Holtmann, P. Lonai and D. Wallach (1998). "Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally." Immunity 9(2): 267-276.

Vayssiere, J. L., P. X. Petit, Y. Risler and B. Mignotte (1994). "Commitment to apoptosis is associated with changes in mitochondrial biogenesis and activity in cell lines conditionally immortalized with simian virus 40." Proc Natl Acad Sci U S A 91(24): 11752-11756.

Veress, I., S. Haghighi, A. Pulkka and A. Pajunen (2000). "Changes in gene expression in response to polyamine depletion indicates selective stabilization of mRNAs." Biochem J. 346(Pt 1): 185-91.

Verhagen, A. M., P. G. Ekert, M. Pakusch, J. Silke, L. M. Connolly, G. E. Reid, R. L. Moritz, R. J. Simpson and D. L. Vaux (2000). "Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins." Cell 102(1): 43-53.

Vermes, I., C. Haanen, H. Steffens-Nakken and C. Reutelingsperger (1997). "A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V." J Immunol Methods 184(1): 39-51.

Vermeulen, K., Z. N. Berneman and D. R. Van Bockstaele (2003). "Cell cycle and apoptosis." Cell Prolif 36(3): 165-175.

Villunger, A., E. M. Michalak, L. Coultas, F. Mullauer, G. Bock, M. J. Ausserlechner, J. M. Adams and A. Strasser (2003). "p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa." Science 302(5647): 1036-1038.

Violette, S., L. Poulain, E. Dussaulx, D. Pepin, A. M. Faussat, J. Chambaz, J. M. Lacorte, C. Staedel and T. Lesuffleur (2002). "Resistance of colon cancer cells to long-term 5-fluorouracil exposure is correlated to the relative level of Bcl-2 and Bcl-X(L) in addition to Bax and p53 status." Int J Cancer 98(4): 498-504.

Viswanath, V., Y. Wu, R. Boonplueang, S. Chen, F. F. Stevenson, F. Yantiri, L. Yang, M. F. Beal and J. K. Andersen (2001). "Caspase-9 activation results in downstream caspase-8 activation and bid cleavage in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease." J Neurosci 21(24): 9519-9528.

Vita, M. and M. Henriksson (2006). "The Myc oncoprotein as a therapeutic target for human cancer." Semin Cancer Biol 16(4): 318-330.

Vlahakis, S. R. and A. D. Badley (2006). "Influence of proteasome inhibitors on apoptosis." Curr Opin Clin Nutr Metab Care 9(1): 42-47.

Vousden, K. H. (2006). "Outcomes of p53 activation--spoilt for choice." J Cell Sci 119(Pt 24): 5015-5020.

Vousden, K. H. and X. Lu (2002). "Live or let die: the cell's response to p53." Nat Rev Cancer 2(8): 594-604.

Wada, T. and J. M. Penninger (2004). "Mitogen-activated protein kinases in apoptosis regulation." Oncogene 23(16): 2838-2849.

Wagenknecht, B., M. Hermisson, K. Eitel and M. Weller (1999). "Proteasome inhibitors induce p53/p21-independent apoptosis in human glioma cells." Cell Physiol Biochem 9(3): 117-125.

Wakayama, M., Y. Katsuno, S. Hayashi, Y. Miyamoto, K. Sakai and M. Moriguchi (1995). "Cloning and sequencing of a gene encoding D-aminoacylase from Alcaligenes xylosoxydans subsp. xylosoxydans A-6 and expression of the gene in Escherichia coli." Biosci Biotechnol Biochem 59(11): 2115-2119.

Walczak, H., R. E. Miller, K. Ariail, B. Gliniak, T. S. Griffith, M. Kubin, W. Chin, J. Jones, A. Woodward, T. Le, C. Smith, P. Smolak, R. G. Goodwin, C. T. Rauch, J. C. Schuh and D. H. Lynch (1999). "Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo." Nat Med 5(2): 157-163.

Wallach, D., E. E. Varfolomeev, N. L. Malinin, Y. V. Goltsev, A. V. Kovalenko and M. P. Boldin (1999). "Tumor necrosis factor receptor and Fas signaling mechanisms." Annu Rev Immunol 17(331-367).

Wang, C. Y., M. W. Mayo, R. G. Korneluk, D. V. Goeddel and A. S. Baldwin Jr (1998). "NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation." Science 281(5383): 1680-1683.

Wang, H. G., U. R. Rapp and J. C. Reed (1996). "Bcl-2 targets the protein kinase Raf-1 to mitochondria." Cell 87(4): 629-638.

Wang, P., A. P. Gilmore and C. H. Streuli (2004). "Bim is an apoptosis sensor that responds to loss of survival signals delivered by epidermal growth factor but not those provided by integrins." J Biol Chem 279(40): 41280-41285.

Wang, T. W., L. Lu, D. Wang and J. E. Thompson (2001). "Isolation and characterization of senescence-induced cDNAs encoding deoxyhypusine synthase and eucaryotic translation initiation factor 5A from tomato." J Biol Chem 276(20): 17541-17549.

Wang, X., H. Sun and C. Li (2007). "Nitric oxide induces promyelocytic cell growth arrest and apoptosis through deactivation of Akt pathway." Leuk Res 31(5): 653-660.

Waterhouse, N. J., J. C. Goldstein, O. Von Ahsen, M. Schuler, D. D. Newmeyer and D. R. Green (2001). "Cytochrome c Maintains Mitochondrial Transmembrane Potential and ATP Generation after Outer Mitochondrial Membrane Permeabilization during the Apoptotic Process." J Cell Biol 153(2): 319-328.

Webb, B. L., E. Jimenez and G. S. Martin (2000). "v-Src generates a p53-independent apoptotic signal." Mol Cell Biol 20(24): 9271-9280.

Wei, M. C., T. Lindsten, V. K. Mootha, S. Weiler, A. Gross, M. Ashiya, V. B. Thompson and S. J. Korsmeyer (2000). "tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c." Genes Dev 14(16): 2060-2071.

Wei, M. C., W. X. Zong, E. H. Cheng, T. Lindsten, V. Panoutsakopoulou, A. J. Ross, K. A. Roth, G. R. Macgregor, C. B. Thompson and S. J. Korsmeyer (2001). "Pro-apoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death." Science 292(5517): 727-730.

Wei, Q. (2005). "Pitx2a Binds to Human Papillomavirus Type 18 E6 Protein and Inhibits E6-mediated P53 Degradation in HeLa Cells." J Biol Chem 280(45): 37790-37797.

Weir, B. A. and M. P. Yaffe (2004). "Mmd1p, a novel, conserved protein essential for normal mitochondrial morphology and distribution in the fission yeast Schizosaccharomyces pombe." Mol Biol Cell 15(4): 1656-1665.

Wen, W., J. L. Meinkoth, R. Y. Tsien and S. S. Taylor (1995). "Identification of a signal for rapid export of proteins from the nucleus." Cell 82: 463-473.

Wenzel, U., S. Kuntz, M. D. Brendel and H. Daniel (2000). "Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells." Cancer Res 60(14): 3823-3831.

Wesierska-Gadek, J., D. Schloffer, V. Kotala and M. Horky (2002). "Escape of p53 protein from E6-mediated degradation in HeLa cells after cisplatin therapy." Int J Cancer 101(2): 128-136.

Wesselborg, S., I. H. Engels, E. Rossmann, M. Los and K. Schulze-Osthoff (1999). "Anticancer drugs induce caspase-8/FLICE activation and apoptosis in the absence of CD95 receptor/ligand interaction." Blood 93(9): 3053-3063.

Weston, C. R., K. Balmanno, C. Chalmers, K. Hadfield, S. A. Molton, R. Ley, E. F. Wagner and S. J. Cook (2003). "Activation of ERK1/2 by deltaRaf-1:ER* represses Bim expression independently of the JNK or PI3K pathways." Oncogene 22(9): 1281-1293.

Widmann, C., S. Gibson and G. L. Johnson (1998). "Caspase-dependent cleavage of signaling proteins during apoptosis. A turn-off mechanism for anti-apoptotic signals." J Biol Chem 273(12): 7141-7147.

Wiley, S. R., K. Schooley, P. J. Smolak, W. S. Din, C. P. Huang, J. K. Nicholl, G. R. Sutherland, T. D. Smith, C. Rauch, C. A. Smith and R. G. Goodwin (1995). "Identification and characterization of a new member of the TNF family that induces apoptosis." Immunity 3(6): 673-682.

Willis, S. N., L. Chen, G. Dewson, A. Wei, E. Naik, J. I. Fletcher, J. M. Adams and D. C. Huang (2005). "Pro-apoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins." Genes Dev 19: 1294-1305.

Wilson, R., R. Ainscough, K. Anderson, C. Baynes, M. Berks, J. Bonfield, J. Burton, M. Connell, T. Copsey, J. Cooper, A. Coulson, M. Craxton, S. Dear, Z. Du, R. Durbin, A. Favello, A. Fraser, L. Fulton, A. Gardner, P. Green, T. Hawkins, L. Hillier, M. Jier, L. Johnston, M. Jones, J. Kershaw, J. Kirsten, N. Laisster, P. Latreille, J. Lightning, C. Lloyd, B. Mortimore, M. O'callaghan, J. Parsons, C. Percy, L. Rifken, A. Roopra, D. Saunders, R. Shownkeen, M. Sims, N. Smaldon, A. Smith, M. Smith, E. Sonnhammer, R. Staden, J. Sulston, J. Thierry-Mieg, K. Thomas, M. Vaudin, K. Vaughan, R. Waterston, A. Watson, L. Weinstock, J. Wilkinson-Sproat and P. Wohldman (1994). "2.2 Mb of contiguous nucleotide sequence from chromosome III of C. elegans." Nature 368(6466): 32-38.

Wilson-Annan, J., L. A. O'reilly, S. A. Crawford, G. Hausmann, J. G. Beaumont, L. P. Parma, L. Chen, M. Lackmann, T. Lithgow, M. G. Hinds, C. L. Day, J. M. Adams and D. C. Huang (2003). "Pro-apoptotic BH3-only proteins trigger membrane integration of prosurvival Bcl-w and neutralize its activity." J Cell Biol 162(5): 877-887.

Wingfield, P. T., R. J. Mattaliano, H. R. Macdonald, S. Craig, G. M. Clore, A. M. Gronenborn and U. Schmeissner (1987). "Recombinant-derived interleukin-1 alpha stabilized against specific deamidation." Protein Eng 1(5): 413-417.

Wink, D. A., K. S. Kasprzak, C. M. Maragos, R. K. Elespuru, M. Misra, T. M. Dunams, T. A. Cebula, W. H. Koch, W. W. Andrews and J. S. Allen (1991). "DNA deaminating ability and genotoxicity of nitric oxide and its progenitors." Science 254(5034): 1001-1003.

Wistow, G. (1990). "Cold shock and DNA binding." Nature 344(6269): 823-4.

Wohl, T., H. Klier, H. Ammer, F. Lottspeich and V. Magdolen (1993). "The HYP2 gene of Saccharomyces cerevisiae is essential for aerobic growth: characterization of different isoforms of the hypusine-containing protein Hyp2p and analysis of gene disruption mutants." Mol Gen Genet 241(3-4): 305-311.

Wohl, T., H. Klier and F. Lottspeich (1995). "Detection of nonfunctional over-expression gene products using two-dimensional gel electrophoresis with a narrowed pH range." Electrophoresis 16(5): 876-878.

Wolff, B., G. Cohen, J. Hauber, D. Meshcheryakova and C. Rabeck (1995). "Nucleocytoplasmic transport of the Rev protein of human immunodeficiency virus type 1 is dependent on the activation domain of the protein." Exp Cell Res 217(1): 31-41.

Wolff, E. C., T. G. Kinzy, W. C. Merrick and M. H. Park (1992). "Two isoforms of eIF-5A in chick embryo. Isolation, activity, and comparison of sequences of the hypusine-containing proteins." J Biol Chem 267(9): 6107-6113.

Wolff, E. C., Y. B. Lee, S. I. Chung, J. E. Folk and M. H. Park (1995). "Deoxyhypusine synthase from rat testis: purification and characterization." J Biol Chem 270(15): 8660-8666.

Wolff, E. C., M. H. Park and J. E. Folk (1990). "Cleavage of spermidine as the first step in deoxyhypusine synthesis. The role of NAD." J Biol Chem 265(9): 4793-4799.

Wolter, K. G., Y. T. Hsu, C. L. Smith, A. Nechushtan, X. G. Xi and R. J. Youle (1997). "Movement of Bax from the cytosol to mitochondria during apoptosis." J Cell Biol 139(5): 1281-1292.

Woods, I. G., C. Wilson, B. Friedlander, P. Chang, D. K. Reyes, R. Nix, P. D. Kelly, F. Chu, J. H. Postlethwait and W. S. Talbot (2005). "The zebrafish gene map defines ancestral vertebrate chromosomes." Genome Res 15(9): 1307-1314.

Wright, H. T. (1991a). "Sequence and structure determinants of the nonenzymatic deamidation of asparagine and glutamine residues in proteins." Protein Eng 4(3): 283-294.

Wright, H. T. (1991b). "Nonenzymatic deamidation of asparaginyl and glutaminyl residues in proteins." Crit Rev Biochem Mol Biol 26(1): 1-52.

Wright, K., G. Kolios, J. Westwick and S. G. Ward (1999). "Cytokine-induced apoptosis in epithelial HT-29 cells is independent of nitric oxide formation. Evidence for an interleukin-13-driven phosphatidylinositol 3-kinase-dependent survival mechanism." J Biol Chem 274(24): 17193-17201.

Wu, G. S., T. F. Burns, E. R. Mcdonald 3rd, W. Jiang, R. Meng, I. D. Krantz, G. Kao, D. D. Gan, J. Y. Zhou, R. Muschel, S. R. Hamilton, N. B. Spinner, S. Markowitz, G. Wu and W. S. El-Deiry (1997). "KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene." Nat Genet 17(2): 141-143.

Wu, G. S., T. F. Burns, Y. Zhan, E. S. Alnemri and W. S. El-Deiry (1999). "Molecular cloning and functional analysis of the mouse homologue of the KILLER/DR5 tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor." Cancer Res 59(12): 2770-2775.

Wu, H. M., K. H. Chi and W. W. Lin (2002). "Proteasome inhibitors stimulate activator protein-1 pathway via reactive oxygen species production." FEBS Lett 526(1-3): 101-105.

Xiang, J., D. T. Chao and S. J. Korsmeyer (1996). "BAX-induced cell death may not require interleukin 1-converting enzyme-like proteases." Proc Natl Acad Sci U S A 93: 14559-14563.

Xu, A. and K. Y. Chen (2001). "Hypusine is required for a sequence-specific interaction of eukaryotic initiation factor 5A with postsystematic evolution of ligands by exponential enrichment RNA." J Biol Chem 276(4): 2555-2561.

Xu, A., D. L. Jao and K. Y. Chen (2004). "Identification of mRNA that binds to eukaryotic initiation factor 5A by affinity co-purification and differential display." Biochem J. 384(Pt 3): 585-590.

Yamaguchi, H. and H. G. Wang (2002). "Bcl-XL protects BimEL-induced Bax conformational change and cytochrome C release independent of interacting with Bax or BimEL." J Biol Chem 277(44): 41604-41612.

Yamamoto, A. M., L. Eloy, J. F. Bach and H. J. Garchon (1998). "N-terminus cleavage of bcl-2 by a novel cellular non-ICE cysteine proteinase." Leukemia 12(9): 1467-1472.

Yamamoto, T. and R. J. Bing (2000). "Nitric oxide donors." Proc Soc Exp Biol Med 225(3): 200-206.

Yang, E. and S. J. Korsmeyer (1996). "Molecular thanatopsis: a discourse on the BCL2 family and cell death." Blood 88(2): 386-401.

Yang, E., J. Zha, J. Jockel, L. H. Boise, C. B. Thompson and S. J. Korsmeyer (1995). "Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death." Cell 80(2): 285-291.

Yang, J., X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. I. Peng, D. P. Jones and X. Wang (1997). "Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked." Science 275(5303): 1129-1132.

Yang, Y. and X. Yu (2003). "Regulation of apoptosis: the ubiquitous way." FASEB J 17(8): 790-799.

Yao, M., A. Ohsawa, S. Kikukawa, I. Tanaka and M. Kimura (2003). "Crystal structure of hyperthermophilic archaeal initiation factor 5A: a homologue of eukaryotic initiation factor 5A (eIF-5A)." J Biochem (Tokyo). 133(1): 75-81.

Yin, X. M., Z. N. Oltvai and S. J. Korsmeyer (1994). "BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax." Nature 369(6478): 321-323.

Yonehara, S. (2002). "Death receptor Fas and autoimmune disease: from the original generation to therapeutic application of agonistic anti-Fas monoclonal antibody." Cytokine Growth Factor Rev 13(4-5): 393-402.

You, H., K. Yamamoto and T. W. Mak (2006). "Regulation of transactivation-independent pro-apoptotic activity of p53 by FOXO3a." Proc Natl Acad Sci U S A 103(24): 9051-9056.

Yu, C., M. Rahmani, P. Dent and S. Grant (2004). "The hierarchical relationship between MAPK signaling and ROS generation in human leukemia cells undergoing apoptosis in response to the proteasome inhibitor Bortezomib." Exp Cell Res 295(2): 555-566.

Yu, J., L. Zhang, P. M. Hwang, K. W. Kinzler and B. Vogelstein (2001). "PUMA induces the rapid apoptosis of colorectal cancer cells." Mol Cell 7(3): 673-682.

Yuan, Z. M., H. Shioya, T. Ishiko, X. Sun, J. Gu, Y. Y. Huang, H. Lu, S. Kharbanda, R. Weichselbaum and D. Kufe (1999). "p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage." Nature 399(6738): 814-817.

Zaika, A., M. Irwin, C. Sansome and U. M. Moll (2001). "Oncogenes induce and activate endogenous p73 protein." J Biol Chem 276(14): 11310-11316.

Zalman, L. S., H. Nikaido and Y. Kagawa (1980). "Mitochondrial outer membrane contains a protein producing nonspecific diffusion channels." J Biol Chem 255(5): 1771-1774.

Zamzami, N., C. Brenner, I. Marzo, S. A. Susin and G. Kroemer (1998). "Subcellular and submitochondrial mode of action of Bcl-2-like oncoproteins." Oncogene 16(17): 2265-2282.

Zamzami, N., P. Marchetti, M. Castedo, T. Hirsch, S. A. Susin, B. Masse and G. Kroemer (1996). "Inhibitors of permeability transition interfere with the disruption of the mitochondrial transmembrane potential during apoptosis." FEBS Lett 384(1): 53-57.

Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, J. L. Vayssiere, P. X. Petit and G. Kroemer (1995). "Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo." J Exp Med 181(5): 1661-1672.

Zanelli, C. F., A. L. Maragno, A. P. Gregio, S. Komili, J. R. Pandolfi, C. A. Mestriner, W. R. Lustri and S. R. Valentini (2006). "eIF5A binds to translational machinery components and affects translation in yeast." Biochem Biophys Res Commun 348(4): 1358-66.

Zanelli, C. F. and S. R. Valentini (2005). "Pkc1 acts through Zds1 and Gic1 to suppress growth and cell polarity defects of a yeast eIF5A mutant." Genetics 171(4): 1571-81.

Zapp, M. L. and M. R. Green (1989). "Sequence-specific RNA binding by the HIV-1 Rev protein." Nature 342(6250): 714-6.

Zha, J., H. Harada, E. Yang, J. Jockel and S. J. Korsmeyer (1996). "Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L)." Cell 87(4): 619-628.

Zha, J., S. Weiler, K. J. Oh, M. C. Wei and S. J. Korsmeyer (2000). "Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis." Science 290(5497): 1761-1765.

Zhang, H. G., J. Wang, X. Yang, H. C. Hsu and J. D. Mountz (2004). "Regulation of apoptosis proteins in cancer cells by ubiquitin." Oncogene 23(11): 2009-2015.

Zhang, H. M., J. Yuan, P. Cheung, D. Chau, B. W. Wong, B. M. Mcmanus and D. Yang (2005). "Gamma interferon-inducible protein 10 induces HeLa cell apoptosis through a p53-dependent pathway initiated by suppression of human papillomavirus type 18 E6 and E7 expression." Mol Cell Biol 25(14).

Zhang, J., M. Dong, L. Li, Y. Fan, P. Pathre, J. Dong, D. Lou, J. M. Wells, D. Olivares-Villagómez, L. Van Kaer, X. Wang and M. Xu (2003). "Endonuclease G is required for early embryogenesis and normal apoptosis in mice." Proc Natl Acad Sci U S A 100(26): 15782-15787.

Zhang, J., X. Wang, K. E. Bove and M. Xu (1999). "DNA fragmentation factor 45-deficient cells are more resistant to apoptosis and exhibit different dying morphology than wild-type control cells." J Biol Chem 274(52): 37450-4.

Zhang, J. and M. Xu (2002). "Apoptotic DNA fragmentation and tissue homeostasis." Trends Cell Biol 12(2): 84-89.

Zhao, Y., J. Tan, L. Zhuang, X. Jiang, E. T. Liu and Q. Yu (2005). "Inhibitors of histone deacetylases target the Rb-E2F1 pathway for apoptosis induction through activation of pro-apoptotic protein Bim." Proc Natl Acad Sci U S A 102(44): 16090-16095.

Zhong, Q., W. Gao, F. Du and X. Wang (2005). "Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis." Cell 121(7): 1085-1095.

Zhu, J., J. Jiang, W. Zhou and X. Chen (1998). "The potential tumor suppressor p73 differentially regulates cellular p53 target genes." Cancer Res 58(22): 5061-5065.

Zhu, Y., B. J. Swanson, M. Wang, D. A. Hildeman, B. C. Schaefer, X. Liu, H. Suzuki, K. Mihara, J. Kappler and P. Marrack (2004). "Constitutive association of the pro-apoptotic protein Bim with Bcl-2-related proteins on mitochondria in T cells." Proc Natl Acad Sci U S A 101(20): 7681-7686.

Zhuang, S. and I. E. Kochevar (2003). "Ultraviolet A radiation induces rapid apoptosis of human leukemia cells by Fas ligand-independent activation of the Fas death pathways." Photochem Photobiol 78(1): 61-67.

Zimmermann, P., D. Tomatis, M. Rosas, J. Grootjans, I. Leenaerts, G. Degeest, G. Reekmans, C. Coomans and G. David (2001). "Characterization of Syntenin, a Syndecan-binding PDZ Protein, as a Component of Cell Adhesion Sites and Microfilaments." Mol Biol Cell 12(2): 339-350.

Zindy, F., C. M. Eischen, D. H. Randle, T. Kamijo, J. L. Cleveland, C. J. Sherr and M. F. Roussel (1998). "Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization." Genes Dev 12(15): 2424-2433.

Zolzer, F. (2000). "Radiation and/or hyperthermia sensitivity of human melanoma cells after several days of incubation in media lacking serum or certain serum components." Int J Radiat Oncol Biol Phys 46(2): 491-497.

Zomber, G., S. Reuveny, N. Garti, A. Shafferman and E. Elhanany (2005). "Effects of spontaneous deamidation on the cytotoxic activity of the Bacillus anthracis protective antigen." J Biol Chem 280(48): 39897-39906.

Zong, W. X., C. Li, G. Hatzivassiliou, T. Lindsten, Q. C. Yu, J. Yuan and C. B. Thompson (2002). "Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis." J Cell Biol 162(1): 59-69.

Zong, W. X., T. Lindsten, A. J. Ross, G. R. Macgregor and C. B. Thompson (2001). "BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak." Genes Dev 15(12): 1481-1486.

Zoratti, M. and I. Szabo (1995). "The mitochondrial permeability transition." Biochim Biophys Acta 1241(2): 139-176.

Zou, H., Y. Li, X. Liu and X. Wang (1999). "An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase 9." J Biol Chem 274(17): 11549-11556.

Zuk, D. and A. Jacobson (1998). "A single amino acid substitution in yeast eIF-5A results in mRNA stabilization." EMBO J 17(10): 2914-2925.