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Posttranscriptional Regulation of the Inhibitor of Apoptosis Protein HIAP2 During Cellular Stress

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POSTTRANSCRIPTIONAL REGULATION OF THE INHIBITOR OF APOPTOSIS PROTEIN HIAP2 DURING CELLULAR STRESS

By

Tong T. Zhao

Thesis Submitted to the School of Graduate and Post-doctoral Studies, University of Ottawa, in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Apoptosis is a physiological process characteristic of pluricellular organisms leading to self-destruction of the cell. One of the hallmarks of apoptosis is the activation of caspases that in turn cleave cellular substrates, leading to an orderly disassembly of the cell. A family of intrinsic cellular inhibitor of Apoptosis (IAP) proteins mediates competitive inhibition of caspase activity. I have discovered that UVC stress in HEK293 cells leads to downregulation of HIAP2 expression that is due to mRNA destabilization. I have identified that there are four putative AU-rich (ARE) elements located within the 3'untranslated region of HIAP2 mRNA that are sufficient to mediate HIAP2 mRNA instability during UVC stress. I hypothesized that specific ARE-binding protein (AUBP) could interact with the HIAP2 AREs which results in the modulation of HIAP2 expression. To this end, I have identified hnRNPA1 as one of the HIAP2 3'UTR ARE binding proteins. HnRNP A1 is primarily nuclear but becomes localized into cytoplasm after exposure of cells to UVC. In addition, hnRNP A1 destabilizes HIAP2 mRNA under normal growth conditions and during UVC stress. Furthermore, more than one ARE may contribute to the binding of hnRNP A1 to the HIAP2 3'UTR. Lastly, knockdown of hnRNP A1 attenuates the UVC – induced apoptosis suggesting that hnRNP A1 is an essential post-transcriptional modulator of HIAP2 expression.

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DEDICATION

To my parents, who have always been there for me

To Caramel, my cat, who has filled my heart with love

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LIST OF ABBREVIATIONS

ActD	Actinomycin D
AIF	Apoptosis-Inducing Factor
Apaf-1	Apoptotic protease activating Factor-1
ARE	AU Rich Element
ATP	Adenosine Tri-Phosphate
AUF1	ARE/poly-(U)-binding/degradation Factor 1
BAK	Bcl-2 homologous Antagonist/killer
BAX	Bcl-2 Associated protein X
Bcl-2	B-cell lymphoma 2
Diablo	Direct IAP Binding Protein with Low PI
BIR	Baculoviral IAP Repeat
BMP	Bone Morphogenetic Protein
BRUCE	BIR-containing Ubiquitin Conjugating Enzyme
BSA	Bovine Serum Albumin
CARD	Caspase Recruitment Domain
CKII	Casein Kinase II
CAT	Chloramphenicol Acetyl Transferase
CHX	Cyclohexmide
COX-2	Cyclooxygenase-2
DISC	Death-Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid disodium salt
ERK	Extracellular signal-regulated Kinase
FADD	Fas Associated Death Domain
GAPDH	Transcript Glyceraldehyde-3-Phosphate Dehydrogenase
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
GST	Glutathione S Transferase
HEK	Human Embryonic Kidney
HIAP	Human Inhibitor of Apoptosis Protein
hnRNP	Heterogeneous Nuclear Ribonucleoprotein
HRP	Horseradish Peroxidase
IAP	Inhibitor of Apoptosis
IRES	Internal Ribosomal Entry Site
JNK	c-Jun N-terminal Kinase
KSRP	KH-type Splicing Regulatory Protein
LB	Luria-Bertani medium
MAPK	Mitogen-Activated Protein Kinase
MAPKKK	Mitogen-Activated Protein Kinase Kinase Kinase
MK2	MAPK activated protein Kinase-2
mRNA	Messenger Ribonucleic Acid
mRNP	mRNA–protein complex proteins
NEO	Neomycin

NAIP	Neuronal Apoptosis Inhibitory Protein
NF-kB	Nuclear Factor kappa B
NPC	Nuclear Pore Complex
PAN	poly (A) nuclease
PBS	Phosphate Buffered Saline
PKA	Protein Kinase A
PKC	Protein Kinase C
PMSF	Phenylmethylsulfonyl Fluoride
PVDF	Polyvinylidene fluoride
RING	Really Interesting New Gene
RNP	Ribonucleoprotein
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
siRNA	Small interfering RNA
Smac	Second Mitochondrial Activator of Caspases
TAB1	TGF-β Activated Kinase-1 Binding Protein-1
TAK1	TGF-β Activated Kinase-1
TIA-1	T-cell Internal Antigen 1
TIAR	TIA-1 Related protein
TNF	Tumor Necrosis Factors
TRAF	TNF Receptor Associated Factors
TRADD	TNF Associated Death Domain
TTP	Tristetraprolin
UTR	Untranslated Region
UV	Ultraviolet
XIAP	X-linked IAP

CHAPTER 1. INTRODUCTION

Cancer and Programmed Cell Death

Cancer is a genetic disease. Several consequential mutations in coding regions of oncogenes and tumor suppressor genes which are responsible for regulating fundamental cellular processes like proliferation, differentiation, and apoptosis, are necessary for cancer development (Kim et al., 2006). Cancer cells have lost their balancing ability, resulting in an accumulation of cells with the potential to progress unimpeded through the cell growth cycle (Watson and Fitzpatrick, 2005).

Programmed cell death plays an important role in the maintenance of homeostasis (Lee and Baehrecke, 2000). Homeostatic control of cell proliferation and cell death is necessary to an organism's ability to affect the normal turnover of healthy cells and removal of damaged or abnormal cells (Watson and Fitzpatrick, 2005). Cell death can proceed by several distinct mechanisms, including apoptosis, necrosis, and autophagy. Apoptosis has been recognized as an important control mechanism and it is highly regulated and avoids eliciting an inflammatory response from cell death (Fuchs and Bode, 2006; Takasawa and Tanuma, 2003). Apoptosis is characterized by a series of cellular changes including mitochondrial damage, nuclear membrane breakdown, chromatin condensation, DNA fragmentation, blebbing of the plasma membrane, and fragmentation of cytoplasm into apoptotic bodies that are subsequently phagocytosed by surrounding cells (Lee and Baehrecke, 2000; Thiede and Rudel, 2004; Yousefi et al., 2006). In addition, there are changes in phosphatidylserine localization from the inner plasma membrane to the cell surface. Cells loose their phospholipid membrane symmetry and expose phosphatidylserine

at the cell surface while the plasma membrane remains intact during the early stages of apoptosis (Watson and Fitzpatrick, 2005).

Apoptosis is critical for the normal development and homeostasis of multicellular organisms. The apoptotic pathways are evolutionarily conserved from worm to human. (Budihardjo et al., 1999). Loss of apoptotic regulation results in a wide variety of diseases. For example, abnormally high rate of cell death is found in neurodegenerative diseases, AIDS and cardiovascular diseases, whereas insufficient cell death contributes to development and progression of cancers, as well as autoimmune disorders (Nachmias et al., 2004; Young et al., 1999).

Apoptotic Pathways

There are two major pathways for apoptosis that have been elucidated so far, the extrinsic and the intrinsic pathways (Figure 1). Two pathways act as sensors for death signals and can activate the cellular cell death program (Deveraux and Reed, 1999; Hawkins et al., 2001; Holcik and Sonenberg, 2005). The extrinsic pathway is a sensor for cytokines and extracellular signals (Budihardjo et al., 1999). Signal stimulation of death receptors (such as Fas/CD95, TNF receptor, or TRAIL receptor) of the tumor necrosis factors (TNF) receptor superfamily results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) (Richter and Duckett, 2000). FADD in turn binds pro-caspase 8, thereby forming the death-inducing signaling complex (DISC) (Richter and Duckett, 2000). As pro-caspase 8 concentrates at the DISC, it dimerizes and becomes activated and initiates apoptosis cascade by direct cleavage of downstream effector caspases 3 and 7 (LeBlanc, 2003).

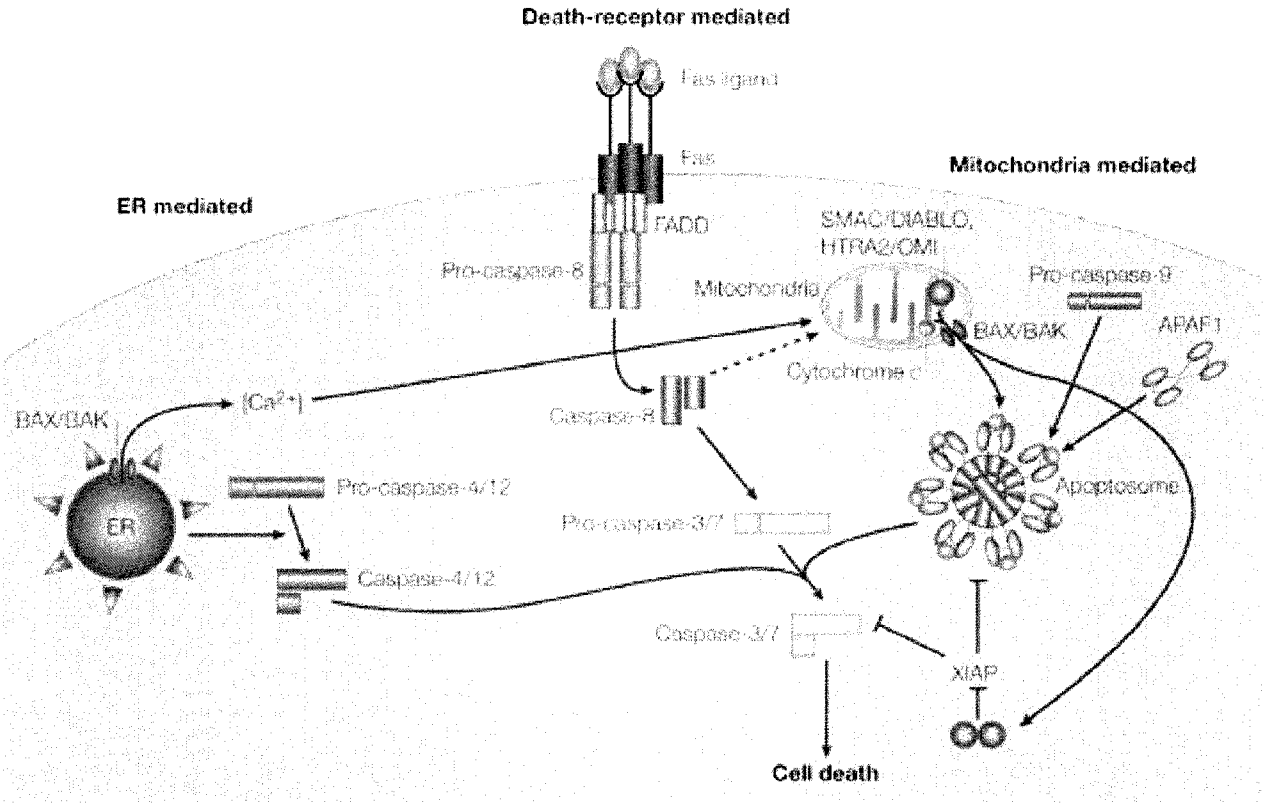

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Figure 1. IAPs are involved in the regulation of apoptosis

Both the death receptor (extrinsic) and mitochondria (intrinsic) pathways of apoptosis involve the activation of initiator caspases such as caspase 8 or 9 and effector caspases such as caspase 3 or 7. IAPs can inhibit both types of caspases at the junction of both pathways. IAPs are in turn controlled by several IAP-interacting proteins, thus creating a complex regulatory network. (Taken from Holcik and Sonenberg, 2005)

The intrinsic pathway is mediated by members of the BCL-2 family and is activated by intra-cellular stress, such as DNA damage, hypoxia or growth factor deprivation (de Graaf et al., 2004). The BCL-2 family possesses both anti-apoptotic and proapoptotic members (Nachmias et al., 2004). The anti-apoptotic members include BCL-2, BCL-X_L, MCL-1, A1 and BCL-W. Proapoptotics members are BAX, BAK (Fuchs and Bode, 2006) and BH3 domain-only proteins BID, BIM, NOXA and PUMAS (Fuchs and Bode, 2006). The intrinsic pathway for caspase activation is initiated by the release of cytochrome c from the mitochondria. Cytochrome c is normally sequestered between the inner and outer membranes of the mitochondria (LeBlanc, 2003). In response to a variety of proapoptotic stimuli, cytochrome c is released into the cytosol (Budihardjo et al., 1999). To allow cytochrome c release, BAX undergoes a conformational change, oligomerizes, and translocates to the mitochondria. This translocation to mitochondria can be triggered by BH3 domain only molecules. Bax can either form pores or induce the opening of preexisting pores in the mitochondria, resulting in the release of mitochondrial apoptogenic factors such as cytochrome c, apoptosis-inducing factor (AIF), Smac/Diablo, Omi/HtrA2, and endonuclease G (Schimmer, 2004). Smac/DIABLO and HtrA2/Omi are IAP antagonists (Wrzesien-Kus et al., 2004). They promote apoptosis by inhibiting IAP proteins that can inhibit the activation of apoptotic caspases (Li et al., 2004b). After cytochrome c is released from the mitochondria, it will associate with the apoptotic protein-activating factor-1 (Apaf-1), and procaspase-9 to form an apoptosome that results in an ATP-dependent activation of caspase-9. Active caspase-9 can then activate caspase-3 (LeBlanc, 2003; Schimmer, 2004).

The receptor (extrinsic) and the mitochondrial (intrinsic) pathway are interconnected at different levels, thus these two pathways are not isolated, and the activation of one usually involves the other (Scholzova et al., 2007). For example, caspase 8 in the extrinsic pathway can cleave Bid to generate tBid and this intermediate then translocates to the mitochondria and triggers the intrinsic pathway (Salvesen and Duckett, 2002). Both the intrinsic and extrinsic pathways converge at the activation of downstream effector caspases such as caspase 3. Active caspase 3 then cleaves critical intracellular proteins to induce the final stages of cell death (Schimmer, 2004).

Caspases

Apoptosis is typified by activation of a family of evolutionarily conserved intracellular cysteine proteases, known as caspases (Reed et al., 2004). Caspases can proteolytically cleave a broad spectrum of cellular targets, such as DNA-repair enzymes, lamin and Murine double minute 2 (Mdm2, a regulator of p53 stability), which ultimately leads to cell death (Ni et al., 2005). Caspases are synthesized as catalytically inactive zymogens that have two domains: a prodomain and a catalytic domain. The caspase zymogen is cleaved at a conserved aspartate residue, the prodomain is then separated from the catalytic domain, and becomes further processed to an active form (Eckelman et al., 2006; Thiede and Rudel, 2004). There are at least 14 mammalian caspases ranging in size from 32 to 55 kDa, all except caspase-11 and caspase-13 are found in humans (Richter and Duckett, 2000). This family of proteases is divided into initiator caspases, such as caspase-2, -8, -9 and -10 and the effector caspases, such as caspases-3, -6 and -7 (Eckelman et al., 2006; Thiede and Rudel, 2004). Caspases are functionally connected to each other, with initiator caspases cleaving and activating effector caspases in a hierarchical manner

(Richter and Duckett, 2000). Cancer cells negatively regulate caspases and thereby suppress apoptosis by three fundamental mechanisms: (i) preventing activation of caspase zymogen; (ii) neutralizing active caspases; and (iii) suppressing expression of genes encoding caspases or caspase-activating proteins (Debatin, 2004; Reed, 2006). One of the mechanisms that is used to neutralize caspase activity is facilitated by a family of proteins called the IAPs (Inhibitor of Apoptosis proteins) which are capable of directly binding caspases and inhibiting their proteolytic activity (Martin, 2002).

IAPs

Members of the inhibitor of apoptosis (IAP) protein family are key regulators of programmed cell death and their functions are tightly controled by several mechanisms including transcriptional, post-transcriptional and translational regulation (Lewis and Holcik, 2005; Watson and Fitzpatrick, 2005). The first IAP gene, *Cp-iap*, was discovered in the baculovirus Cydia pomonella granulovirus (Crook et al., 1993). The first human IAP discovered was Neuronal Apoptosis Inhibitory Protein, NAIP (Roy et al., 1995). To date, eight human IAP proteins (Figure 2) have been identified: XIAP, HIAP1/c-IAP2, HIAP2/c-IAP1, NAIP, Survivin, Livin, BRUCE, and Ts-IAP (Perrelet et al., 2000).

The IAP family of proteins serves as endogenous antagonists of the cell death proteases by interacting with and inhibiting the enzymatic activity of both initiator and effector caspases. Some of them can directly bind to active caspases and either suppress their protease activity or target them for destruction by ubiquitination and subsequent proteasome-mediated degradation (Reed, 2006). The functional unit in each IAP protein is the baculoviral IAP repeat (BIR), which contains approximately 80 amino acids folded

The IAP Family

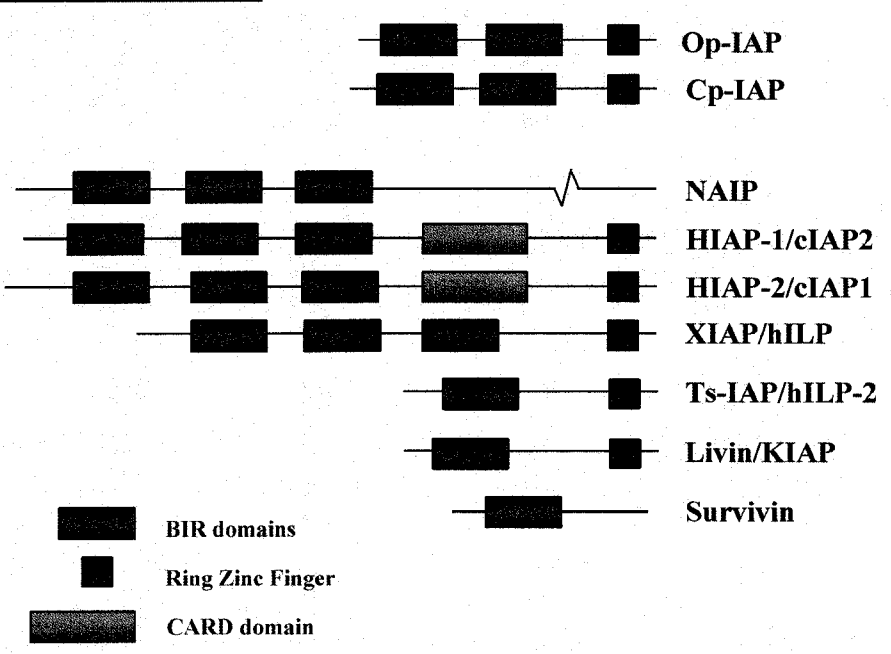


Figure 2. The mammalian IAP protein family.

Structural domains are indicated for each IAP. The abbreviations are as follows: BIR, baculovirus inhibitory repeat; CARD, caspase-recruitment domain; RING zinc finger motif, really interesting new gene. (Adapted from Liston et al, 2003)

around a zinc atom (Young et al., 1999). Most IAPs contain two or three copies of the BIR repeats, except for survivin which has only one repeat (Ni et al., 2005). The BIR regions of human XIAP, HIAP1 and HIAP2 were found to be necessary and sufficient for their caspase inhibitory and anti-apoptotic activities (Yang and Li, 2000). In addition to BIR, most IAPs, except for NAIP and survivin, also contain a RING (Really Interesting New Gene) finger zinc-binding domain C terminal to the BIR repeats (Ni et al., 2005). For example, XIAP, HIAP1, and HIAP2 all have a RING zinc-finger domain that allows these proteins to act as E3 ubiquitin ligases. So far, all RING-containing IAPs that have been tested possess autoubiquitylating activity, so they can control their own degradation through self-ubiquitination. In addition, IAP can control degradation of other IAPs. By doing this, they can act to lower the apoptotic threshold, thus allowing the cell to undergo apoptosis (Ni et al., 2005). IAPs can also promote the degradation of their target caspases. Furthermore, IAPs bind to and induce ubiquitination of endogenous IAP-antagonist proteins such as SMAC/DIABLO and serine protease Omi/HtrA2, both in insect and in mammalian cells (Reed et al., 2004). Furthermore, HIAP1 and HIAP2 also contain a caspase recruitment domain (CARD) (Budihardjo et al., 1999). Although the functions of CARD domain is not clear for the other IAPs, HIAP2 is capable of interacting with a pro-apoptotic kinase CARDIAk/RIP2/RICK through its CARD domain (Deveraux and Reed, 1999). In addition, both HIAP1 and HIAP2 interact with ARC, a CARD-domain-containing protein with anti-apoptotic activity (Deveraux and Reed, 1999; Huang, 2002; Miller, 1999).

IAPs can be grouped by the presence and number of BIR domain, RING finger and CARD domain (Liston et al., 2003). XIAP has three BIR domains and a RING finger. It binds and inhibits caspases 3, 7, and 9 with nanomolar affinity, but it does not bind or inhibit

caspase 8 (Schimmer, 2004). HIAP1 and HIAP2 are structurally related to XIAP with three BIR domains and a RING finger. HIAP1 and HIAP2 proteins are expressed in most human tissues, but HIAP2 expression is highest in the thymus, testis, and ovary, while HIAP1 expression is highest in the spleen and thymus (LeBlanc, 2003). HIAP1 and HIAP2 bind and inhibit caspase 3 and 7, but less strongly than XIAP (Liston et al., 2003; Schimmer, 2004). They do not inhibit caspases 1, 6, and 8. Livin and Ts-IAP have a RING finger and only one BIR domain, but their BIR domain is most homologous to the BIR3 domain of XIAP, HIAP1 and HIAP2. They both have been demonstrated to bind to caspase 9. NAIP has three BIR domains but no RING finger motif. It inhibits caspases 3, 7 and 9, but not caspases 1, 4, 5, or 8 (Davoodi et al., 2004; Warnakulasuriyarachchi et al., 2004). Survivin and Bruce contain only a single BIR domain and no RING finger. They both bind to and inhibit caspase 3 (Liston et al., 2003).

IAP involvement in signal transduction

Several studies indicate that the functions of the IAPs are not restricted to caspase inhibition, but may also include regulation of the cell cycle, signal transduction, and protein degradation (Liston et al., 2003). Some IAPs play a role in signal transduction from membrane-bound receptors (Deveraux and Reed, 1999). For example, HIAP1 and HIAP2 were identified originally as components of the TNF receptor 2 complex and are believed to influence the outcome of TNF signaling by both inhibiting caspases and activating the NF-kB pathway (Wilusz et al., 2001). XIAP can function as an adapter protein by recruiting TAB1 and TAK1 to BMP receptors, and this result in the activation of both the JNK and NF-kB survival pathways. In addition, XIAP can bind and activate JNK1 directly (Cheung et al., 2006; de Graaf et al., 2004; Huang, 2002; Liston et al., 1997).

Regulation of IAPs

Given the critical role the IAPs play in the regulation of apoptosis, their expression must be tightly controlled. This is achieved at different levels of gene expression. For example, HIAP1 is regulated transcriptionally by the NF-kB regulatory network (Chu et al., 1997). In contrast, the expression of XIAP is regulated translationally through an internal ribosome entry site (IRES) element (Holcik et al., 1999). Translation of a typical cellular mRNA is cap dependent, on the other hand, IRES translation does not require the presence of the m^7G cap-binding protein eIF4E. The IRES mechanism allows proteins to be continuously translated where cap-dependent translation is attenuated (Lewis and Holcik, 2005). Similarly, expression of HIAP2 is also controlled at the level of translation. There is a short regulatory upstream open reading frame (uORF) located in the 5'UTR of HIAP2 that exerts an inhibitory effect on the translation of HIAP2 (Warnakulasuriyarachchi et al., 2003). Furthermore, HIAP2 also contains an inducible IRES element. During endoplasmic reticulum (ER) stress, HIAP2 is translationally upregulated, to delay the onset of ER stress induced cell death (Warnakulasuriyarachchi et al., 2004). My work described in this thesis deals with the characterization of additional control mechanism that regulates HIAP2 expression by modulating HIAP2 mRNA stability.

3' UTR and AREs

There are multiple roles of the 3'untranslated region (3'UTR) of eukaryotic mRNA such as posttranscriptional regulation, mRNA localization, polyadenylation, mRNA stability and translation initiation (Glisovic et al., 2003). In the 3'UTR, specific *cis*-acting elements could interact with one or more trans-acting factors (Manjithaya and Dighe, 2004; Putland et

al., 2002). AU-rich elements (AREs) present in 3'UTRs that have been implicated in posttranscriptional regulatory processes, such as mRNA stability and translatability, are the most common example of such *cis* elements (Manjithaya and Dighe, 2004; Putland et al., 2002).

Several sequence elements can regulate the rate of turnover of a transcript. One of the best studied and most prevalent is the ARE (Jackman et al., 1994; King and Francomano, 2001). AREs usually promote the rapid cytoplasmic degradation of cytokine and proto-oncogene mRNAs (D'Orso and Frasch, 2001; Stoecklin et al., 2002). Accelerated decay of ARE-mRNAs is thought to proceed by rapid deadenylation, which, in turn, leads to rapid degradation of the body of the mRNA (Guhaniyogi and Brewer, 2001)

AREs have been grouped into three classes depending on the distribution and presence of the AUUUA pentanucleotide. Class I AREs (e.g. c-fos mRNA ARE) contain several AUUUA motifs interspersed within a less well-defined U-rich region. The AREs in the HIAP2 mRNA also falls into this category. Class II AREs (e.g. GM-CSF ARE) contain several overlapping AUUUA motifs. Class III AREs (e.g. c-jun ARE), although still AU-rich, contain no distinct AUUUA motif (Balmer et al., 2001; Brooks and Rigby, 2000; Park et al., 2000). Furthermore, it has been found that ARE containing the nonamer UUAUUUA (U/A) (U/A) is more indicative of rapid destabilization. Variations of the nonamer, such as extended pentamers (AUUUUA and AUUUUUA), are present in many complex AREs and have been identified as putative binding targets for various transacting factors (Balmer et al., 2001; Haeussler et al., 2000; Sureban et al., 2007). The combination of AU pentamers, nonamers, extended AU pentamers, and U-rich stretches determine the ultimate destabilizing ability of each particular ARE (Balmer et al., 2001; Wang et al., 1997). In

addition to the de-stabilization functions of the AREs, ARE-containing mRNAs are also known to be stabilized during various forms of cellular stress, including heat shock, UVC irradiation, hypoxia, and nutrient deprivation (Gallouzi et al., 2000; Jeyaraj et al., 2005). These observations indicate that AREs have both stabilizing and de-stabilizng effect on mRNAs which underscore the importance of AREs in the regulation of gene expression.

ARE binding proteins (AUBPs)

A number of RNA-binding proteins, including HuR, AUF1/hnRNP-D, Tristetraprolin, TIA-1, TIAR, and CUG-BP2/CELF2 have been shown to associate with AREs and to be responsible for post-transcriptional control of ARE containing mRNAs (Jing et al., 2005; Lee and Jeong, 2006; Lopez de Silanes et al., 2005; Paschoud et al., 2006; Prasanth et al., 2005; Pullmann et al., 2006; Vasudevan and Steitz, 2007; Zhang et al., 2005). ARE binding proteins can either destabilize (e.g. AUF-1, TTP) or stabilize (e.g. HuR) ARE-containing mRNAs (Capowski et al., 2001). For example, TTP is a mRNA destabilizing protein that acts at least in part by promoting deadenylation and by attracting the exosome to the transcript for rapid decay (Yu et al., 2003).

Usually, the effect on mRNA stability and protein expression are correlated. For instance, binding of Tristetraprolin to GM-CSF and TNF-α mRNAs decreases the stability of these mRNAs which causes the reduced expression of the encoded protein (Carballo et al., 2000; Lai et al., 1999). Similarly, binding of HuR to GM-CSF mRNA increased mRNA stability and correspondingly the amount of GM-CSF protein levels (Fan and Steitz, 1998; Raineri et al., 2004). However, this direct correlation is not always observed. HuR binding to TNF-α or Cyclooxygenase 2 (Cox-2) mRNAs which increase the stability of these

mRNAs also decrease their translation (Katsanou et al., 2005; Mukhopadhyay et al., 2003). Similar opposing effects have also been reported for CUG-BP2/CELF2 when bound to Cox-2 mRNA. These observations indicate that mRNA stability and translation are not always strictly correlated (Barreau et al., 2006).

Despite a large amount of knowledge about the AUBPs involved in regulating mRNA stability, exactly how mRNA affects the binding of different AUBPs and how the switch between destabilizing and stabilizing proteins is regulated remains poorly understood (Gringhuis et al., 2005). A number of factors can influence the association of an ARE-mRNA with one or several of RNA binding proteins (RBPs) (Dixon et al., 2003; Vasudevan and Steitz, 2007). These factors could be the affinity of the RBP for a particular RNA sequence, the subcellular localization, the posttranslational modification of the RBP or the specific cellular environment (normal condition vs stress) (Henics, 1999; Henics et al., 1997; Vasudevan and Steitz, 2007). Some ARE-mRNAs has been found to be targets of several regulatory RBPs (Lal et al., 2006; Lu and Schneider, 2004; Pioli et al., 2002). These AUBPs can compete for binding to the same RNA region in a mutually exclusive fashion. They can also bind cooperatively whereby binding of one RBP to one mRNA region facilitates the binding of another RBP to another mRNA region. They can also bind jointly on separate regions of the same mRNA without apparent impact of one upon the other (Lal et al., 2006). Interestingly, a single AUBP can bind to multiple AREs suggesting that these AREs may share characteristic features for AUBPs binding (Bevilacqua et al., 2007).

All AUBPs are believed to function in the cytoplasm but many also shuttle in and out of the nucleus as exemplified by the heterogeneous nuclear ribonucleoprotein family (Benjamin et al., 2006; Bonafe et al., 2005). These findings indicate that such RNA-binding

proteins may regulate mRNA stability by relocalizing between the nucleus and the cytoplasm (Kondo et al., 2006; Lapucci et al., 2002). Therefore, the degradation rate of a mRNA could be determined by the balance between stabilizing and destabilizing factors and their nuclear/cytoplasmic distribution (Barreau et al., 2005; Donnini et al., 2004; Sommer et al., 2005; Zhang et al., 2002).

Furthermore, although most of the AUBPs play a role in the regulation of mRNA stability, several of the AUBPs have also been shown to regulate translation of the mRNA (Colegrove-Otero et al., 2005; Duttagupta et al., 2005; Piecyk et al., 2000). For example, ARE-binding protein T-cell internal antigen 1 (TIA-1)/TIA-1-related protein (TIAR) can translational repress tumor necrosis factor alpha (TNF-α) mRNA (Duttagupta et al., 2005). At the same time, TIAR can modify the destabilizing effect of TTP on TNF-α mRNA by either affecting the subcelllular localization or the ARE-binding affinity of TTP. This brings the complexity of AUBPs to another level.

AREs, AUBP and decay

The first step in mammalian mRNA decay, including ARE-directed decay, appears to be shortening of the poly(A) tail. AUBP can recruit Poly(A) nuclease (PAN) deadenylase to the vicinity of the ARE-containing mRNAs to promote deadenylation (Chou et al., 2006; Kondo et al., 2006; Lal et al., 2004). In addition, AUBPs are involved in helping to recruit the exosome, a multiprotein complex of RNases involved in rapid 3'-to-5' exonucleolytic degradation of the mRNA (Quijada et al., 2002). Some ARE-binding proteins (including AUF1, TTP, and KSRP) were found associated with the exosome (Lu et al., 2006; Pan et al., 2005). The AUBPs may also recruit decapping enzymes and the 5′-to-3′ exonuclease to

promote mRNA decay via a 5′-to-3′ decay pathway. Finally, AUBPs escort their associated mRNAs to P-bodies which are believed to be the site of decapping and 5′–3′ mRNA decay (Amrani et al., 2006; Datta et al., 2005; Muhlemann, 2005; Newbury, 2006; Stoecklin et al., 2006; Stohr et al., 2006; Wilusz et al., 2001; Yamashita et al., 2005). Therefore, it has been suggested that the most important function of decay-promoting factors is the ability to recognize specific *cis* elements on the RNA (such as the ARE), and in turn increase the local concentration of RNA-degrading enzymes (such as recruiting the exosome) (Gherzi et al., 2004; Tran et al., 2004).

Signalling pathways that regulate mRNA stability

Several signalling pathways have been found to regulate mRNA stability (Eberhardt et al., 2007). These signalling pathways can modulate mRNP structure and mRNA stability by mediating phosphorylation of ARE-binding proteins. Phosphorylation can either alter the affinity of the protein for its substrate or results in the binding of other factors that change the function of the AUBP (Garneau et al., 2007; Stoecklin et al., 2004; Yasuda et al., 2004).

For example, it has been suggested that p38 MAP kinase pathway induces stabilization of the ARE-containing mRNAs (such as COX-2) through the p38 MAP kinase substrate MAPKAP kinase 2 (MK2) and ARE binding protein HuR (Akool el et al., 2003; Bollig et al., 2003; Espel, 2005; Sully et al., 2004).

RNPs and hnRNP A1

To function properly, eukaryotic messenger RNAs must contain information that specifies their nuclear export, subcellular localization, translation and stability (Patry et al., 2003; Patry et al., 2004). These functions are performed by specific RNA-binding proteins

such as heterogeneous nuclear ribonucleoproteins (hnRNP proteins) or mRNA–protein complex proteins (mRNP proteins) (Key et al., 1998; Lin and Yen, 2006; Pinol-Roma and Dreyfuss, 1992). Pre-mRNAs and mRNAs exist in cells in the functional forms such as the ribonucleoprotein complexes (RNPs). The protein components of the RNPs participate in pre-mRNA processing and are important determinants of mRNA export, localization, translation and stability (Feldherr et al., 2002; Stewart, 2007). The association of hnRNP proteins with pre-mRNAs initiates co-transcriptionally when pre-mRNAs are still nascent transcripts. Many RNPs remain bound to the resulting mRNAs all the way to ribosomes, and the RBPs thus shuttle back and forth between the nucleus and the cytoplasm (Kamma et al., 1999; Lichtenstein et al., 2001; Suzuki et al., 2005). The RNP is highly dynamic, the protein composition of hnRNP complexes is not temporally fixed, maturation and nuclear export of mRNA are accompanied by changes in the protein composition of hnRNP complexes (Kim et al., 2000a).

hnRNPs were originally defined as the proteins constituting the 40 S complexes isolated from nuclei (Kress et al., 2005; Lee et al., 2006). The hnRNP proteins range in mass from 30 to 120 kDa and associate with pre-mRNA as a complex. The protein composition of hnRNP complexes is best known in human cells with at least 20 major proteins identified (Iijima et al., 2006; Sandri-Goldin, 1998). They are designated from A1 (34 kDa) to U (120 kDa). Some hnRNP proteins are among the most abundant proteins in the nucleus. For example, the hnRNP proteins A1 and C1 are about ten times more abundant than the U1 snRNP (Kim et al., 2000a).

HnRNP A1 is known to participate in transcription, splicing, and mRNA export (Kim et al., 2000a). Under normal growth conditions, most hnRNP proteins are

concentrated in the nucleus. Some hnRNPs, such as hnRNPs A1 and K shuttle continuously between the nucleus and the cytoplasm, whereas others (e.g., hnRNP C1/C2 and hnRNP U) do not shuttle and are retained in the nucleus (Michael et al., 1997). hnRNP A1 is the most extensively characterized of the shuttling hnRNPs and requires continuous RNA polymerase II transcription for the return of hnRNP A1 to the nucleus, implying a link between transport and transcriptional activity. Transport of A1 is determined by the M9 domain, a 38 amino acid sequence located at the carboxy-terminus. The M9 signal functions as both nuclear export signal and as nuclear localization signal by mediating binding of its nuclear import receptor, transportin (Michael et al., 1997).

Studies of hnRNP-RNA interactions have demonstrated sequence-specific binding, as well as roles in pre-mRNA splicing and splice-site selection (Dreyfuss et al., 2002). The overall structure of the hnRNP protein is modular, for example, hnRNP A1 is composed of two RNP motifs RNA-binding domains (RBDs) as well as a third RNA-binding domain, the RGG (Arg–Gly–Gly) box (Dreyfuss et al., 2002). hnRNP A1 retains its ability to bind mRNA in the cytoplasm and also during its passage through the nuclear pore complex (NPC) (Mili et al., 2001). Others have found that cytoplasmic hnRNP A1 preferentially binds ARE relative to pre-mRNAs (Guil et al., 2006). This indicates that hnRNP A1 exhibits different RNA binding specificity in the cytoplasm relative to the nucleus, enabling distinct functional roles pertinent to RNA metabolism in each subcellular compartment (Hamilton et al., 1997).

Phosphorylation of hnRNP A1

hnRNP A1 is directly phosphorylated *in vitro* by PKA, casein kinase II (CKII) and PKC through its catalytic domain, but hnRNP A1 is not a direct target of p38 kinase (Perrotti and Calabretta, 2002). Others have found that phosphorylation of hnRNP A1

dramatically inhibits hnRNPA1 binding activity. For example, phosphorylation of hnRNP A1 by the Mnks decreases its binding to the TNFα 3′UTR both *in vitro* and in vivo (Buxade et al., 2005). Phosphorylation by PKC dramatically inhibits the binding of full length hnRNPA1 to RNA oligonucleotides. This indicates that residues phosphorylated by PKC are critically involved in the interaction of hnRNPA1 with RNA (Municio et al., 1995).

In addition to the altered binding affinity of hnRNP A1 for RNA, phosphorylation of hnRNP A1 can also regulate its subcellular localization. Others have shown that stress signals induced the cytoplasmic accumulation of hnRNP A1 with an increase in its phosphorylation through the $MKK_{3/6}$-p38 pathway (Allemand et al., 2005). In addition, the nucleocytoplasmic shuttling and RNA binding activities of hnRNP A1 are activated by the PKC, which directly phosphorylates hnRNP A1 on serine 199 (Iervolino et al., 2002). Therefore, both p38 and PKC kinase pathways appear to play a role during the stress-induced subcellular redistribution of hnRNP A1 (Allemand et al., 2005; Tan et al., 2006; van der Houven van Oordt et al., 2000). A recent interesting finding suggests that the stress-induced cytoplasmic accumulation of hnRNP A1 occurs in the cytoplasmic stress granules (SGs) with similar kinetics to that of TIA-1 (Guil et al., 2006). This is consistent with a model whereby hnRNP A1 exits the nucleus bound to mRNA, once in the cytoplasm and upon exposure to stress stimuli, mRNA-bound hnRNP A1 is phosphorylated by the Mnk1/2 protein kinases, causing the recruitment of hnRNP A1 and its associated mRNA to the SGs (Guil et al., 2006; van der Houven van Oordt et al., 2000). This is also consistent with another model whereby stress-induced phosphorylation of hnRNP A1 prevents its interaction with transportin, resulting in increased cytoplasmic hnRNP A1 which remains bound to poly(A) mRNAs that are then targeted to the SGs (Allemand et al., 2005).

UVC irradadiation

Ultraviolet (UVC) light can induce apoptosis (Debatin, 2004). The ultraviolet spectrum can be divided in to 3 sectors: UVA (320-400nm); UVB (280-320nm); and UVC (190-280nm) (Takasawa and Tanuma, 2003). UVC light is one of the DNA-damaging reagents and prolonged UVC exposure will result in inhibition of DNA synthesis and subsequent resumption of the cell cycle following apoptosis. UVC irradiation activates the caspase in the intrinsic pathway by cytochrome c release from the mitochondria (Schuler and Green, 2001; Schumacher et al., 2005).

During evolution, eukaryotes have developed structurally and functionally conserved complex systems to respond to UVC irradiation. One of the well-known systems is the mitogen-activated protein kinase, MAP kinase cascade (Bardeleben et al., 2003; Ouchi et al., 2005; Zhao et al., 2005). The MAPK pathways are composed of different members, including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 kinases (Christmann et al., 2006; Deng et al., 2002; Fernandez et al., 2003; Ferrer et al., 2001; Lagalwar et al., 2007; Liu et al., 2003; McKay et al., 2002; Nijhawan et al., 2003; Wu et al., 2002). MAPK cascade involves sequential activation of three distinct kinases through phosphorylation. First of all, an extracellular stimulus (e.g. small G proteins such as Ras, Rac and Rap1) activate MAPKKK, which then activates MAPKK, and MAPKK in turn activates MAPK by dually phosphorylating two residues, a threonine and a tyrosine (Larsen et al., 2002; Li et al., 2004a; Sauer et al., 2006; Song et al., 2005). The mammalian JNKs and p38 kinases are the best-studied MAPKs. They are known to be activated in response to a variety of stress conditions, including osmotic stress, heat shock, oxidative stress, UVC light and some DNA-damaging agents, and therefore they are collectively termed stress-

activated MAPKs (SAPKs) (Bergeron and Olivier, 2006; Bhatia et al., 2004; Cui et al., 2005; Demple, 2004; Faour et al., 2003). SAPK cascade is activated by UVC light and it has its own sensing and triggering mechanisms. SAPKs have recently been shown to be modulators of stability of several mRNAs through the 3'UTR. For example, SAPKs are involved in regulating mRNA stability via 3'-UTRs of IL-8, IL-6, c-Fos, GM-CSF mRNAs, cyclooxygenase 2 vascular endothelial growth factor, and TNF mRNAs (Fischer and Voynow, 2002; Geginat et al., 2000; Gorospe et al., 1998; Iliev et al., 2002; Kim et al., 2000b). In the case of 3'-UTR-dependent stability of IL-8 mRNA, there is evidence that the p38 MAP kinase pathway regulates ARE-based mRNA degradation via its downstream effector molecule MK2 (Force et al., 2004; Neininger et al., 2002; Winzen et al., 1999; Zhao et al., 2004).

In this work, I have investigated the mechanism of regulation of HIAP2 expression. I have identified that there are four AREs located within the 3'UTR of HIAP2 mRNA that are sufficient to mediate HIAP2 mRNA instability during UVC irradiation. In addition, I found that hnRNP A1 is one AUBP that binds to the AREs in the 3'UTR of HIAP2. Importantly, following UVC irradiation, hnRNP A1 relocalizes to the cytoplasm where it destabilizes HIAP2 mRNA. Taken together, my results suggest that hnRNP A1 is an essential post-transcriptional modulator of HIAP2 expression.

CHAPTER 2. Material and Methods

Cell Culture and Reagents

Human embryonic kidney cells (HEK293) were cultured in standard conditions in DMEM supplemented with 10% fetal calf serum, glutamate, and antibiotics (2.5 ml of penicillin and streptomycin each). Transient transfections were performed using LipofectAMINE PLUS according to the protocol provided by the manufacturer (Invitrogen, Carlsbad, CA). Briefly, cells were seeded at a density of 6 x 10^5 cells/well in six-well plates and were transfected 24 h later in serum-free DMEM with 0.5μg DNA. The transfection mixture was supplemented 3 h later with 2 ml DMEM containing 10% fetal calf serum, glutamate, and antibiotics. Cells were collected for analysis 24 h after transfection.

The expression plasmids pMC, pMC+HIAP2 3'UTR, pMC+TNF, and pMC+H2B were kindly provided by Mireille Cloutier.

RNA Interference

siRNA transfections were performed using Lipofectamine 2000 according to the protocol provided by the manufacturer (Invitrogen, Carlsbad, CA). Briefly, cells were seeded at a density of 3.0 x 10^5 cells/well in six-well plates and were transfected 24 h later in serum-free DMEM with a 10 nM final concentration of hnRNP A1 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA), a 5nM final concentration of HIAP2 siRNA (Dharmacon) or a nonsilencing control siRNA (Qiagen). Cells were collected for analysis 48 h after transfection. In the case of co-transfection, 0.5μg/well of pMC or H2B plasmid DNA was co-transfected at the same time as siRNA.

UVC Irradiation

Cells were seeded at a density of 6 X 10^5 cells/well in six-well plates. 24 hours later, cells were washed with phosphate buffered saline (PBS). Cells were irradiated at room temperature in 2 ml fresh medium with a 30-watt UVC light source (254 nm) with the cover removed. After irradiation, the cells were further incubated in culture medium for indicated times and harvested with a cell scraper. Both floating and attached cells were collected. PBS washed cells not exposed to UVC irradiation served as controls. The intensity of UVC light was measured prior to each experiment with a UVX radiometer (UVP Inc., Upland, CA). For the cycloheximide (CHX) experiments, cells were treated with UVC (150mW/cm^2) and 25μg/ml of CHX was added, cells were harvested at 0, 8, 24, 32, 48 hr point after addition of CHX. For the Actinomycin D experiments, cells were treated with UVC (150mW/cm^2), 10μg/ml of Act D was added and cells harvested at 0, 1, 2, 4, 8 hr time points. Cells untreated with UVC irradiation served as control. For cells transfected with an siRNA, they were treated with UVC (150mW/cm^2) 48 hours post siRNA transfection. Five hours later, cells were harvested as described above.

Western Blot Analysis

Cells were washed in 1 ml PBS and lysed in 150 μl RIPA buffer for 30 min at 4°C, followed by centrifugation at 12,000 x *g* for 10 min to pellet debris. Protein concentration was assayed by Bradford (Bio-Rad, Richmond, CA), and equal amounts of protein extract were separated by 10% SDS-PAGE. Proteins were electrophoretically transferred onto PVDF membrane. Samples were blocked overnight with Odyssey blocking buffer (LI-COR Biosciences) for HIAP2 or 1 hour for GAPDH and incubated with primary antibody (rabbit polyclonal anti-

HIAP2 1:1000 (abCAM), mouse-monoclonal anti-GAPDH 1:10000 (Advanced ImmunoChemical) for 1 hour. Blot was washed 3 X 10 min with Odyssey washing buffer and incubated with secondary antibody (goat anti-rabbit IgG Alexa Fluor 680 1:5000 for HIAP2 or goat anti-mouse IgG Alexa Fluor 680 1:10000 for GAPDH) for 1 hour. Blot was then washed once with wash buffer and two times with PBS. Antibody complexes were detected by direct infrared fluorescence by Odyssey infrared imaging system. Densitometry was performed using Odyssey software V1.1. For hnRNP A1, samples were blocked for 1 hr in 5% skim milk in PBS-Tween before adding the antibodies. This was followed by a 1 hr incubation in primary antibody (goat polyclonal anti-hnRNP A1 1:2000, Santa Cruz Biotechnology) diluted in PBS-T. Blots were washed 3 X 5 min in PBS-T then incubated for 1 hr in HRP-conjugated secondary antibody (horseradish peroxidase–conjugated anti-goat IgG 1:5000; Santa Cruz Biotechnology) diluted in PBS-T. Antibody complexes were detected using the ECL Plus systems (GE Healthcare) and visualized by autoradiography.

HIAP2 Protein Stability Analysis

Cells were treated with 25μg/ml cycloheximide (Sigma-Aldrich). After 45 min of cycloheximide treatment, cells were treated with or without UVC (150mW/cm^2) and protein extracts were harvested by lysis in 150 μl RIPA buffer (0-h time point). Protein extracts were subsequently harvested at 1-, 2-, 4-, and 8-h time points, protein concentration was assayed by Bradford assay (Bio-Rad), and equal amounts of protein extract were separated by 10% SDS-PAGE, transferred to PVDF membrane, and analyzed by Western blot using antibodies against HIAP2 and GAPDH as described above.

HIAP2 mRNA stability analysis

Cells were treated with or without UVC (150mW/cm^2) as described above. Actinomycin D (10μg/ml) was then added to each well. Cells were harvested by 200 μl Lysis buffer (Absolutely RNA miniprep) supplemented with 1.4 μl β-mercaptoethanol at 0 (immediately after adding the Actinomycin D), 1, 2, 4 and 8 hour time points. Cell lysate were then frozen in –20°C freezer for further analysis (see below).

RNA Isolation

Total RNA was isolated from cells using the Absolutely RNA miniprep kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). In brief, cell lysates were transferred to prefilter spin cup that was seated in a 2-ml receptacle tube and span in a microcentrifuge at maximum speed for 2 minutes. The spin cup was then removed and the filtrated solution was added an equal volume of 70% ethanol and vortexed. The mixture was then transferred to an RNA binding spin cup that is seated in a fresh 2-ml receptacle tube and span for 60 seconds. The spin cup was retained and the filtrate was discarded. 600 μl of 1 X low-salt wash buffer was used to wash the cup. The cup was then span untill dry for 2 minutes and incubated at 37°C for 15 minutes with DNAse added. The cup was washed with 600 μl of 1 X high-salt wash, 600 μl of 1 X low-salt wash and 300 μl of 1 x low-salt wash buffer. The spin cup was then transferred to a 1.5 ml microcentrifuge tube and 30μl of H_2O was added to elute the RNA. RNA concentration was determined by Nanodrop-1000 V3.3 spectrophotometer.

Quantitative RT-PCR Analysis

cDNA was generated using an oligo dT_{18} primer and the Bulk 1st-Strand Synthesis kit according to the protocol provided by the manufacturer (GE Healthcare). 1μg of RNA from each sample were added with water to the final volume of 8μl. The RNA solution was heated to 65°C for 10 minutes, then chilled on ice. Then 1μl of Not I-d(T)18 primer, 1μl of DTT solution, and 5μl of the bulk first-strand cDNA reaction mix were added to the RNA. The mixture was incubated at 37°C for 1 hour. The synthesized cDNA was used as the template for quantitative PCR using the QuantiTect SYBR Green PCR kit (Qiagen). 25μl of 2 X Quantitect SYBR green PCR master mix, 2 μl of forward primer, 2μl of reverse primer, 20μl of RNAse-free water and 1μl of template cDNA were added to each qRT-PCR reaction tube. The reaction was analyzed on an ABI Prism 7000 detection system (Columbia, MD) using the ABI Prism 7000 SDS software. Reaction cycles were set up as follows: 15 sec-95°C, (40 repeats of - 15 sec-94°C, 30sec -56°C, 30sec-72°C), 15sec-95°C, 20sec-60°C and 15sec-95°C. Quantitative PCR reactions were carried out to detect Ct values of NEO, CAT, HIAP2 and GAPDH genes. Forward and Reverse primer sequence for each gene are listed in Appendix I.

Data was analysised using Microsoft Excel and is presented as $2^{-CT\ (HIAP2-GAPDH)}$ and $2^{-CT\ (CAT-NEO)}$. In the Actinomycin D experiment, $2^{-CT\ (HIAP2-GAPDH)}$ normalized to 0hr CT values ($2^{-\Delta\Delta CT}$) calculation was used.

Plasmid DNA isolation

DNA was purified using compactprep plasmid purification kit from Qiagen. A single colony was picked from a freshly streaked selective plate and inoculated a starter culture of 2ml LB medium containing Ampicillin. The tube was incubated for 8h at 37°C with vigorous shaking. The starter culture was then diluted 1/500 into 100ml LB medium and grew for 12 hours with vigorous shaking. Bacterial cells were then harvest by centrifugation at 6000 x g for 15min at 4°C and resuspended in 5ml buffer P1. 5 ml of buffer P2 was then added and mixed thoroughly by inverting the sealed tube 4-6 times and incubated at room temperature for 3 min. 5 ml buffer S3 was added to the lysate and mixed immediately by vigorously inverting 4-6 times. The lysate was poured into the barrel of the QIAfilter cartridge, incubated at room temperature for 3 min. The lysate was then filtered into a new tube with 5ml of buffer BB and everything was added to the compact prep column. The vacuum source was switched on to draw the solution through the compactprep column and 0.7 ml buffer PE was used to wash the column. To elute the DNA, 100μl of water was added and the column was centrifuged for 1 min. DNA concentration was determined by Nanodrop-1000 V3.3 spectrophotometer.

Site directed Mutagenesis

Mutations of AREs were carried out using Quikchange II site-directed mutagenesis kit (Stratagene). 5μl of 10x reaction buffer, 50ng of dsDNA template, 125 ng of oligonucleotide primer #1, 125 ng of oligonucleotide primer #2, and 1μl of dNTP mix were put into the PCR reaction tube, with adding the water to the final volume of 50μl. Then 1μl of Pfu-ultra HF DNA polymerase was added. The PCR was set up to run 30 sec at 95°C,

16x (30 sec at 95°C, 1min at 55°C, 1min at 68°C). The reaction was then put on ice to cool before adding 1μl of Dpn I restriction enzyme. The reaction mixtures in a microcentrifuge was span down and incubated at 37°C for 1 hour to digest the parental plasmid. The newly synthesized DNA was transformed into XL1-blue supercompetent cells using heat pulse method. 0.5ml of LB medium was incubated with the transformation reaction at 37°C for 1 hour with shaking at 225-250 rpm. The transformation reaction was then plated on agar plates containing Ampicillin and incubated at 37°C for 16 hours.

Primer sequences for the mutants are listed in Appendix I.

Immunofluorescence Microscopy

Cells grown on coverslips were rinsed briefly in PBS and were then fixed in 4% paraformaldehyde for 30 min. The coverslips were then rinsed 3 X 5 min in PBS and the cells permeabilized in 0.2% Triton X-100 (Pierce) for 5 min. The coverslips were again rinsed 3 X 5 min in PBS. Immunofluorescence labeling was performed sequentially at room temperature. Fixed cells on coverslips were treated with 0.1% NaBH4 in PBS 3 x 4min. Cells were then rinsed 3 x 5 min with PBS. The coverslips were then inverted onto 100μl of primary antibody (goat polyclonal anti-hnRNP A1, Santa Cruz Biotechnology) and incubated for 1hr. The coverslips were then washed 3 X 5 min in PBS. Following the incubation of the secondary antibody (donkey anti goat IgG Alexa Fluor 488, Invitrogen), cells were washed 3 X 5 min in PBS again. Vectashield mounting medium containing DAPI was used (Vector, CA) and the edges of the cover slips were sealed with clear nail polish and let dry before visualizing. Immunolabeled samples were visualized using a Zeiss

Axiophot epifluorescence microscope equipped with a 100-W mercury arc lamp. Images were digitally recorded with an AxioCam CCD camera using Axio Vision v3.1 software.

RNA-Affinity Chromatography

RNA-affinity chromatography was kindly performed by Dr.Steve Lewis as described (Lewis et al., 2007).

GST-fusion purification

Overnight cultures of pGEX-hnRNP A1 or pGST were diluted 1:10 in fresh media and allowed to grow for 3 hr prior to induction. 0.1 mM IPTG was then added to the cultures and the cultures were induced at 37°C for 4 hrs. Bacteria were pelleted and resuspended in STE buffer (50 nM Tris-HCL, pH 8.0, 150 mM NaCl, 10 mM EDTA) containing protease inhibitors (10 uM Aprotinin, 100 uM Pepestatin A, 10 uM Leupeptin and 100 uM PMSF). The pellet was vortexed before adding 100μl of lysozyme (20 mg/ml stock) (Roche). The suspension was then incubated on ice for 15 min. Sarkosyl was added to 1.5% along with final concentrations of 0.1 mM DNase (Sigma) and 0.1mM $MgCl_2$. The suspension was incubated on ice for 45 min. Triton X-100 (Sigma) was added to 1% and then the suspension was centrifugated at 10,000g for 15 min at 4°C. The supernatant was collected. The glutathione sepharose beads (Amersham) were prepared before use to result in a 50% slurry that was added to the supernatant and incubated at room temperature with continuous rotation for 30min. Bead-protein complexes were collected and washed 4X in ice cold PBS. The bead complexes were then stored in 500 μl of PBS on ice until further use. GST-

hnRNP A1 or GST along on beads were run on 10% SDS-PAGE and stained with Coomassie Blue to confirm the presence and purity of the protein.

Preparation of the ^{32}P labeled RNA Probe

T7 promoter sequence was added to the H2B, mutant 1, mutant 2, mutant 3 or mutant 4 by PCR. 2.5μl of 10x buffer, 1.5μl $MgCl_2$, 100ng DNA, 100ng forward primer, 100ng reverse primer, 10mM dNTPs, and 0.5μl Taq polymerase were mixed in a PCR reaction tube and water was added to reach the final volume of 25μl. The reaction cycles were set up as 1 x 94°C for 3min, 30 x (94°C for 50sec, 56°C for 50sec, 72°C for 1min) and 1 x (94°C for 50sec, 56°C for 50sec, 72°C for 10min). Reaction tube was then incubated at 4°C.

Primer sequence to generate the probe T7-H2B or T7-mutants is listed in Appendix I.

PCR products were run on 1.6% agarose gel to confirm that they were the right size products. DNA was then extracted from an agarose gel using Ultraclean 15 DNA purification kit (Mo Bio Laboratories). Agarose gel band slice was weighted and 3 volumes of ultra salt were added. The mixture was incubated at 55°C to melt the gel and incubated with 5μl of resuspended ultra-bind for 5 minutes. Reaction tube was centrifuged for 10 seconds and supernatant was removed. The pellet was resuspended in 1ml of ultra wash, pelleted and resuspended again in 12 μl of water. DNA concentration was detected by Nanodrop-1000 V3.3 spectrophotometer.

In order to make the ^{32}P labeled RNA probe, MAXIscript *in vitro* transcription kit was used (Ambion). 500ng DNA template, 2μl 10x transcription buffer, 1μl 10mM ATP, 1μl 10mM CTP, 1μl 10mM GTP, 5μl of labeled ^{32}P UTP, and 2μl enzyme mix were added to the

reaction tube with the water to reach the final volume of 20μl. Reaction mixture was incubated at room temperature for 1hr. 1μl of DNase was then added to the reaction mixture and incubated at 37°C for 15 minutes. The generated ^{32}P labeled RNA samples were then run on a 5% Urea PAGE denaturing gel and visualized by autoradiography. Radioactive bands were then cut out from the gel and eluted overnight in 200μl elution buffer at 37°C. 2μl of each ^{32}P labeled probe was diluted in 400 μl of scintillation fluid and counted by 1450 LSC luminescence counter using Microbeta Trilux V4.7 software. All probes were diluted to 15,000 cpm/μl.

UV Cross-Linking of RNA–Protein Complexes

Binding buffer was prepared with 10mM Tris pH 7.4, 1.5mM $MgCl_2$, 150 mM Kcl, 0.5mM DTT, 0.1 mM PMSF and 0.1 mM Leupeptin. 7μl of recombinant protein was incubated with 1μl of each ^{32}P labeled RNA probe and 1μl of tRNA in binding buffer with the final volume of 11μl. Samples were incubated at room temperature for 30min. The samples were then transferred into a 96-well dish and irradiated on ice with a 254-nm UV light source at 400,000 uJ/cm^2. UV-irradiated RNA-protein complexes were treated with 2μl RNase A/T_1 each and incubated at room temperature for 10min. The complexes were then treated with 2μl heparin and incubated at room temperature for 10min. The RNA-protein complexes were released from the agarose beads by boiling in SDS loading buffer (100 mM Tris [pH 6.8], 2.5% SDS, 10% glycerol, 0.025% ß-mercaptoethanol, 0.1% bromophenol blue), resolved on an SDS-10% PAGE gel. The gel was fixed for 5 min in 5% MeOH, 10% CH_3COOH, it was then put into the drying solution for 5 min (7% CH_3COOH, 7% MeOH, 1% glycerol). The gel was dried at 80°C for 1hr and visualized by autoradiography.

Cell Viability Assay

Cells were plated on six-well plates, 24 hr later they were transfected with DNA or siRNA. 24 or 48 hours later, cells were treated with UVC irradiation of 0 mW/cm^2, 37 mW/cm^2, 74 mW/cm^2, 111 mW/cm^2 and 148 mW/cm^2. Cells were then put back into the incubator for another 24 hours before they were harvested. Cell viability was determined using the Vi-Cell cell viability analyzer (Beckman Coulter).

Statistical analysis

Data are expressed as mean +/- SD values. Statistical significance was assessed by two tailed *t* test or one-way ANOVA (comparison of more than two groups). If not otherwise stated, all experiments reported here represent at least three independent replications performed in triplicate.

CHAPTER 3. RESULTS

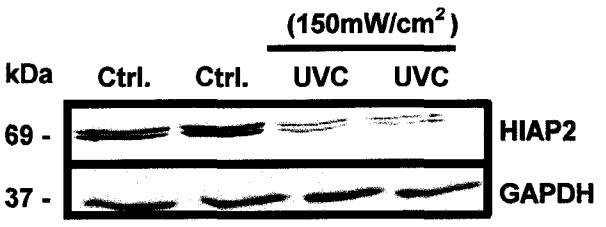
UVC irradiation reduces levels of HIAP2 protein

The first experiment was carried out to see the effect of UVC irradiation on HIAP2 expression. Cells were treated with a single dose of UVC irradiation (150mW/cm^2) and cell lysates were collected 24 h later for Western blot analysis. Densitometric analysis revealed that UVC irradiation induced more than a four fold decrease in HIAP2 protein levels (Figure 3A). HIAP2 protein was occasionally detected as a doublet, perhaps due to phosporylation of the protein. To test the effect of UVC irradiation on HIAP2 protein stability, cycloheximide, a translation elongation inhibitor was used. 25μg/μl of the inhibitor was added to the cell culture medium 3 minutes after radiation, and cells were harvested 0, 8, 24, 32 and 48 h later for Western analysis. Addition of cycloheximide did not alter the stability of HIAP2 protein in UVC-irradiated cells compared to untreated cells (Figure 3B), suggesting that changes in protein stability are not responsible for the radiation-induced down regulation of HIAP2.

UVC irradiation reduces HIAP2 mRNA Stability

Since the HIAP2 protein stability was not affected by UVC irradiation, it was reasonable to investigate if mRNA stability is involved in the radiation-induced down regulation of HIAP2. qRT-PCR was carried out to monitor the HIAP2 steady-state mRNA levels in cells treated with or without UVC irradiation. As shown in Figure 3C, there was a marked difference in the steady-state levels of HIAP2 mRNA observed in the irradiated cells compared to untreated control cells. Alterations in the rate of degradation of transcript and/or changes in the rate of transcription may be responsible for the changes in steady-state mRNA levels. Therefore the relative contribution of a post-transcriptional mechanism in the

A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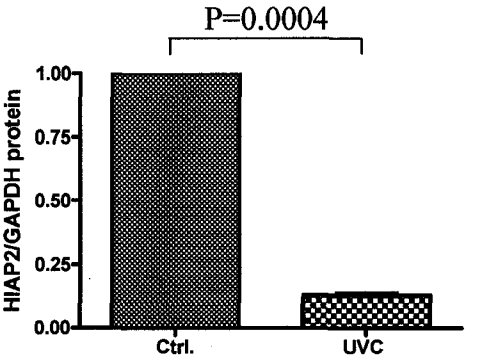
Ctrl.
Ctrl.
UVC
UVC
69 -
37 -
HIAP2
GAPDH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0.50
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0.00
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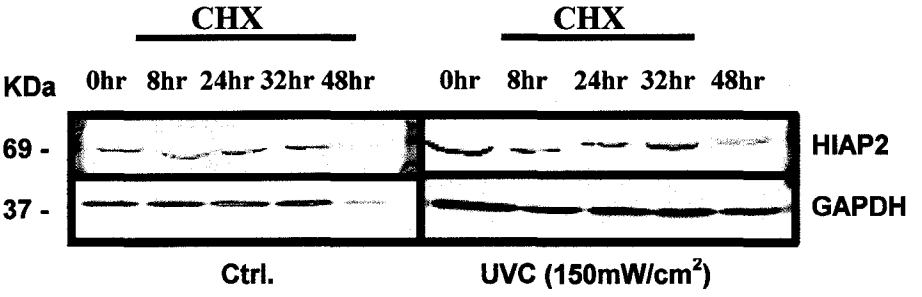
UVC

B


CHX
CHX
KDa
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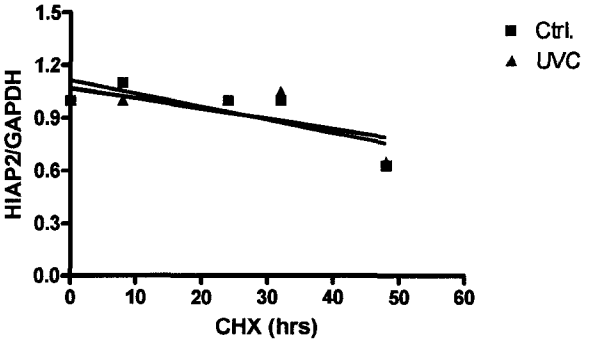
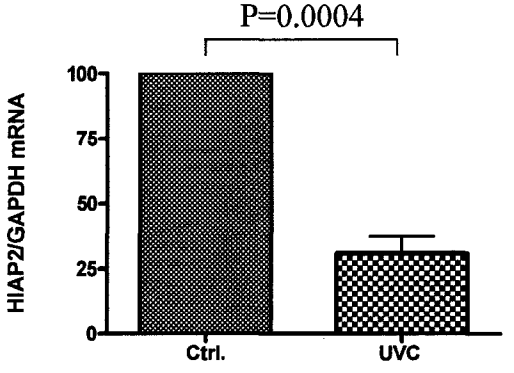
37 -
HIAP2
GAPDH
Ctrl.
UVC (150mW/cm²)


HIAP2/GAPDH
1.5
1.2
0.9
0.6
0.3
0.0
0
10
20
30
40
50
60
CHX (hrs)
Ctrl.
UVC

C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HIAP2/GAPDH mRNA
100
75
50
25
0
Ctrl.
UVC

D

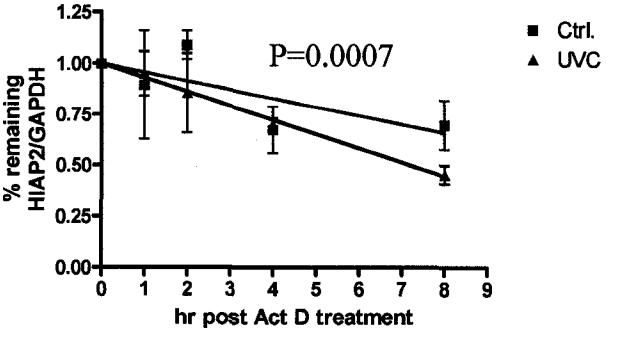
P=0.0007
% remaining HIAP2/GAPDH
1.25
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
8
9
hr post Act D treatment
Ctrl.
UVC

Figure 3. UVC stress in HEK293 cells leads to downregulation of HIAP2 expression which is due to mRNA destabilization.

(A) UVC irradiation represses endogenous HIAP2 expression. After exposure of HEK293 cells to 150mW/cm^2 UVC, the cells were incubated for 24hrs and the expression levels of HIAP2 and GAPDH were examined by Western blotting. Cells untreated with UVC irradiation served as the control. Representative blot of triplicate experiment is shown on the left. Quantification of the blot is shown on the right. (B) Stability of endogenous HIAP2 protein and GAPDH (control) in the presence or absence of UVC stress. Cells were treated with or without UVC irradiation and incubated with cycloheximide (25μg/ml) for indicated duration. Representative blot is shown on the top. Quantification of the blot is shown on the bottom (n=1). (C) Steady-state level of HIAP2/GAPDH mRNA in the presence or absence of UVC irradiation. Cells were treated with or without UVC irradiation dose and RNA samples prepared 24 hrs later. Results from RT-PCR quantification ($2^{-\Delta ct}$) after normalization to the control (GAPDH) in three experiments are shown. (D) Intracellular decay of HIAP2 mRNA in the presence of Actinomycin D. HEK293 cells were treated with or without UVC irradiation. *De novo* mRNA transcription was inhibited by the addition of Act D (10μg/ml) for the indicated times. Quantification of HIAP2 mRNA relative to GAPDH mRNA ($2^{-\Delta\Delta ct}$) of the remaining mRNA was performed by qRT-PCR. Values represent percentages of remaining mRNA relative to the mRNA level at time 0. Results are represented as the mean $\pm$ s.d. of three independent experiments.

irradiation-induced modulation of HIAP2 mRNA was determined using the transcription inhibitor Actinomycin D (ActD). HEK293 cells were irradiated with a single dose of UVC irradiation (150mW/cm^2). Three minutes later, these cells and untreated controls were incubated with 10μg/ml ActD to arrest transcription. Cells were harvested 0, 1, 2, 4, and 8 h after addition of ActD. The amount of HIAP2 mRNA was determined by qRT-PCR and normalized using the expression of GAPDH. The half life of mRNA (t1/2) was calculated from the slope of linear regression of the data (y=mx+b) and was determined to be ~ 8hr for the control cells and ~ 16hr for the UVC irradiated cells. UVC irradiation resulted in approximately 2-fold decrease in the half life of HIAP2 mRNA (Figure 3D). These findings suggest that the UVC irradiation-induced down-regulation of HIAP2 is due at least in part to decreased mRNA stability.

HIAP2 3'UTR Contains AU-rich Elements which are sufficient to cause HIAP2 mRNA Instability

3'UTR is often involved in the regulation of mRNA stability. Analysis of the 3'UTR of HIAP2 revealed that it contains multiple putative AU-rich elements (AREs), including at least three AUUUA motifs, which might regulate mRNA stability (Figure 4A). Thus, it was reasonable to propose that HIAP2 mRNA instability may be due to the presence of AREs in the 3'UTR. To test this hypothesis, the reporter plasmid pMC was constructed by inserting a synthetic polylinker into a modified pcDNA3 vector followed by insertion of the CAT (Chloramphenicol Acetyl Transferase) coding region. The Neomycin coding gene transcribed from an independent promoter is also present on the same plasmid, which serves as transfection efficiency control. To characterize the effect of the HIAP2 3'UTR on mRNA stability, the full length 3'UTR was cloned into the pMC downstream of the CAT coding

A

TAAAGAAAAATAGTCTATATTTTAACCTGCATAAAAAGGTCTT
TAAAATATTGTTGAACACTTGAAGCCATCTAAAGTAAAAAGGGAAT
TATGAGTTTTTCAATTAGTAACATTCATGTTCTAGTCTGCTTTGGTAC
T**(138)**AATAATCTTGTTTCTGAAAAGATGGTATCATATATTTAATCTT
AATCTGTTTATTTACAAGG(200)GAAGATTTATGTTTGGTGAACTATA
TTAGTATGTATGT**(238)**GTACCTAAGGGAGTAGTGTCACTGCTTGTTA
TGCATCATTTCAGGAGTTACTGGATTTGTTGTTCTTTCAGAAAGCTTT
GAATACTAAATTATAGTGTAGAAAAGAACTGGAAACCAGGAACTCT
GGAGTTCATCAGAGTTATGGTGCCGAATTGTCTTTGGTGCTTTTCAC
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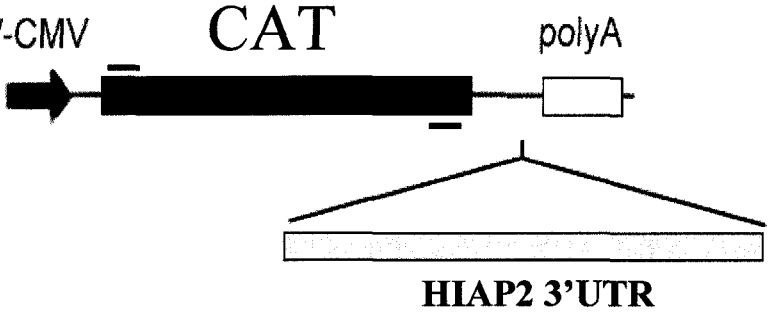
GAGAAACATCTCAATAAAGTGCTTT**(483)**

B



C

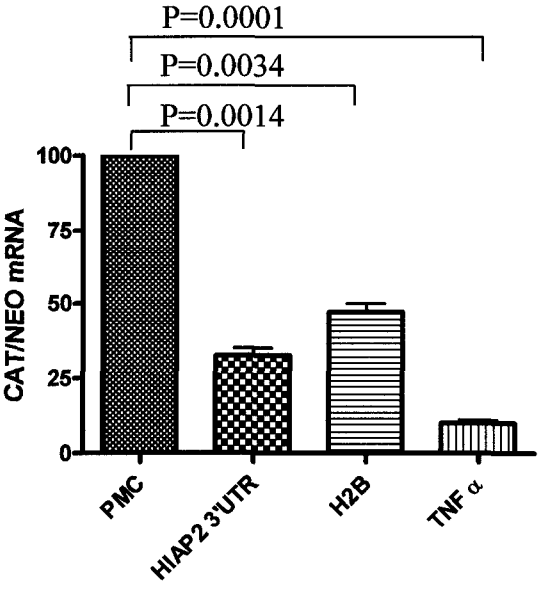


Figure 4. The AREs in the 3'UTR of HIAP2 destabilize HIAP2 mRNA.

(A) Sequence of the 3'UTR of HIAP2 mRNA. Four putative AREs are underlined. (B) Schematic representation of the reporter construct. CMV promoter controls the transcription of CAT gene. In the transcribed mRNA, the CAT coding sequence is followed by HIAP2 3'UTR. Neomycin gene is also on the same plasmid, under the control of an independent promoter. Measurement of expression from the NEO gene allows normalization between experiments. (C) The H2B fragment is sufficient to cause the destabilization of HIAP2 mRNA. Cells were transiently transfected with control plasmid pMC, plasmid containing the full length 3'UTR of HIAP2 (pMC+HIAP2), plasmid containing the H2B fragment (pMC-H2B) or the 3'UTR of TnFα (pMC-TNFα)that served as positive control. 24hrs after transfection, steady-state levels of CAT and NEO mRNAs were measured by qRT-PCR ($2^{-\Delta ct}$). Results from RT-PCR quantification after normalization to the control (NEO) signals in three independent experiments are shown as the mean $\pm$ s.d.

region (Figure 4B). In addition, a 100nt fragment containing the four AREs (H2B) of the HIAP2 3'UTR was also inserted downstream of the CAT coding region (plasmid pMC-H2B). It is known that TNFα 3'UTR contains an ARE that acts as an instability element, therefore it was used as positive control. Reporter plasmid pMC-TNFα was thus constructed by inserting the 3' UTR of TNFα downstream of CAT in the pMC construct. To determine relative stabilities of the resulting mRNAs, reporter constructs were transfected in equal amounts into HEK293 cells, and qRT-PCR was carried out to test the steady state mRNA levels of CAT produced from pMC, pMC+HIAP2 3'UTR, pMC+H2B and pMC+TNFα plasmids. Steady state levels of HIAP2 3'UTR reporter mRNA or the H2B fragment are all significantly less than the steady state mRNA level of the empty pMC construct. These data suggested that the 3'UTR of HIAP2 can confer instability of a reporter mRNA and the four AREs within the 100bp fragment are sufficient to recapitulate this effect (Figure 4C).

Specific trans-acting factors often bind to AREs of a given mRNA and thereby control its stability (Barreau et al., 2005). Since the ARE containing H2B fragment of HIAP2 3'UTR significantly decreased mRNA stability, it is reasonable to predict that the HIAP2 AREs might be associated with trans-acting factors that de-stabilize its mRNA. Subsequent experiments were therefore conducted to identify the HIAP2 ARE binding proteins (AUBPs). One such candidate is hnRNP A1, an RNA binding protein that is known to interact with a number of RNA regulatory sequences including AREs. Furthermore, it has been demonstrated that hnRNP A1 can relocalize from nucleus into cytoplasm during cellular stress (Iervolino et al., 2002) and in particular following UVC irradiation (van der Houven van Oordt et al., 2000). Therefore, I hypothesized that hnRNP A1 might be the trans-acting factor that modulates HIAP2 mRNA stability during UVC stress.

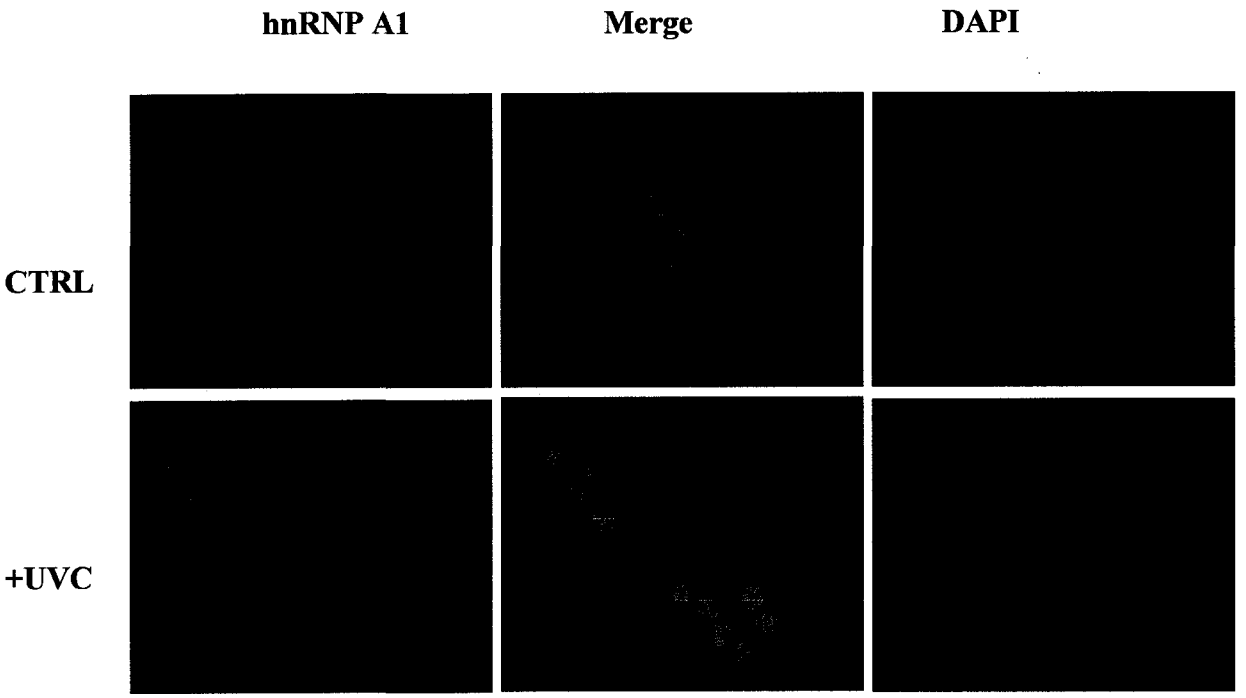
HnRNP A1 is primarily nuclear but becomes cytoplasmic after exposure to UVC.

First, I wished to confirm that UVC irradiation will cause relocalization of hnRNP A1 from the nucleus to the cytoplasm. To test the localization of hnRNP A1, HEK293 cells cultured on glass coverslips were left untreated or irradiated with UVC (150mW/cm^2) for 3 minutes. 5 hours later, cells were fixed and incubated with anti-hnRNP A1 monoclonal antibody to detect endogenous hnRNP A1 protein by immunofluorescence microscopy. DAPI was used to stain the nuclear DNA. As shown in Figure 5, hnRNP A1 in untreated cells was distributed primarily in nuclei. However, exposure of HEK293 cells to UVC irradiation induced a detectable accumulation of hnRNP A1 in the cytoplasm. These observations confirmed that hnRNPA1 accumulates in the cytoplasm following UVC irradiation of cells (Kim et al., 2000a).

hnRNP A1 interacts with the HIAP2 3'UTR and ARE elements of the 3'UTR

To test if hnRNP A1 is indeed HIAP2 AUBP that interacts with HIAP2 3'UTR, RNA-affinity chromatography was carried out. Whole-cell protein extract was prepared from HEK293 cells, which was incubated with either avidin-agarose beads alone or avidin-agarose beads conjugated to biotin-labeled HIAP2 3'UTR RNA. After protein binding, beads were washed extensively and pelleted, and proteins were eluted by boiling. Analysis of the eluates by SDS-PAGE and immunobloting with specific antibodies demonstrate that hnRNP A1, but not other known AUBPs such as TIAR or TIA-1 binds to the 3'UTR of HIAP2 (Figure 6A). Control reaction using avidin-agarose beads alone did not yield any proteins indicating that the binding of hnRNP A1 to the 3'UTR of HIAP2 is specific. To test if hnRNP A1 alone binds to the H2B fragment of the 3'UTR of HIAP2, *in vitro* binding assay was performed. Purified GST or GST-hnRNP A1 (2μl, 4μl, 7μl each) was incubated

A



B

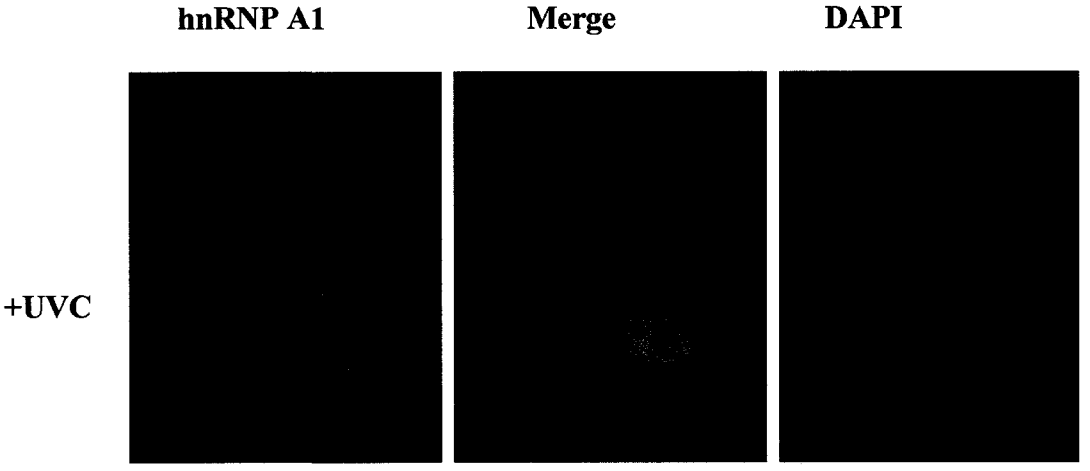
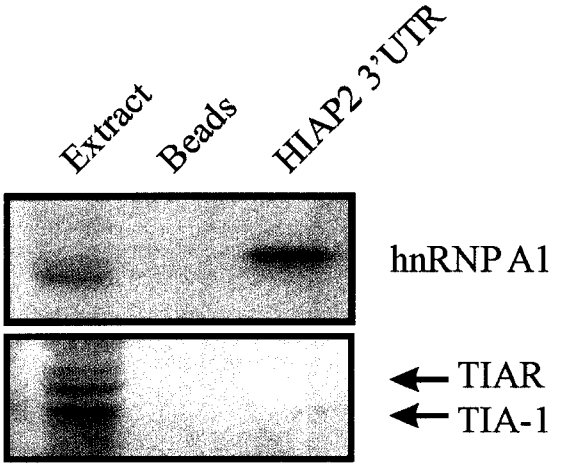


Figure 5. hnRNP A1 relocalizes to the cytoplasm during UVC stress.

(A) HEK293 cells cultured on glass coverslips were left untreated or were exposed to UVC irradiation (150mW/cm^2). 5 hours later, the cells were fixed and immunostained with anti-hnRNP A1 antibodies (green) to detect the endogenous proteins or Dapi (blue) to detect the nuclear DNA. Images were detected by Carl Zeiss Axioskop2 epifluorescent microscopy. (B) Magnified view of a single cell treated with UVC as in panel (A).

A


Extract
Beads
HIAP2 3'UTR
hnRNP A1
TIAR
TIA-1

B

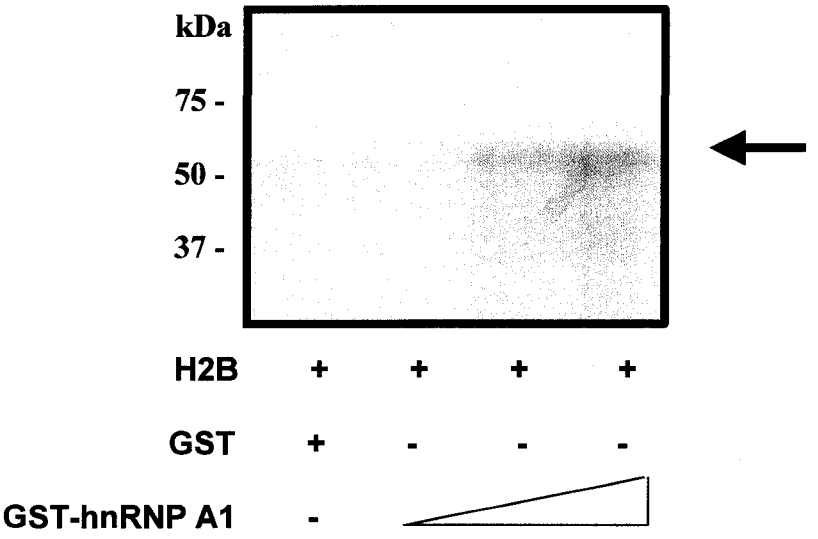

kDa
75 -
50 -
37 -
H2B + + + +
GST + - - -
GST-hnRNP A1 -

Figure 6. hnRNP A1 binds to 3'UTR of HIAP2.

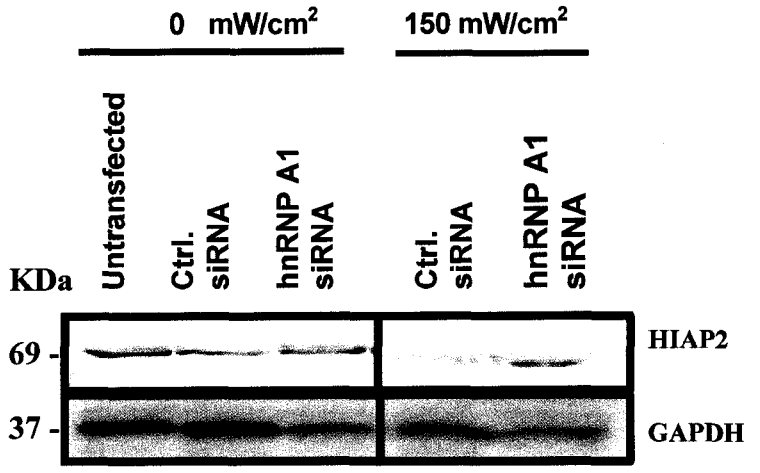
(A) RNA-affinity chromatography isolation of HIAP2 3'UTR-binding proteins. Precleared protein extracts from HEK293 cells were incubated with either agarose beads coated with HIAP2 3'UTR RNA, or agarose beads alone. After protein binding, beads were washed extensively and pelleted, and proteins were eluted by boiling and resolved by SDS-PAGE. Proteins were identified by Western blot using anti-hnRNP A1, anti-TIAR and anti-TIA-1 antibodies. (RNA-affinity chromatography was kindly performed by Dr. Stephen Lewis) (B) Purified GST or GST-hnRNP A1 (2μl, 4μl 7μl each) was incubated with ^{32}P-labeled H2B fragment of HIAP2 3'UTR and bound proteins were cross-linked to RNA by UVC-light irradiation. Free RNA was digested with RNaseA/T1. Following incubation, RNA-protein complexes were resolved on SDS-PAGE and visualized by autoradiography. The arrow indicates position of the RNA-hnRNP A1 complex. GST protein alone served as negative control.

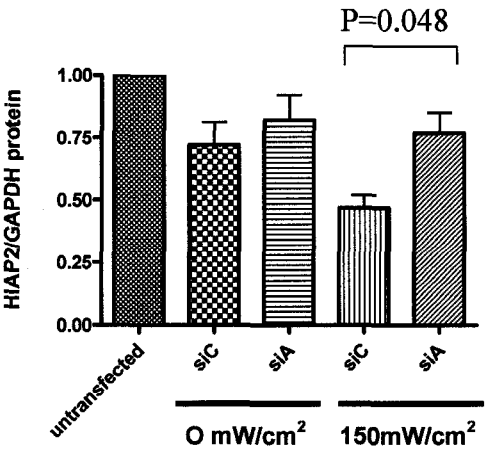
with ^{32}P-labeled H2B fragment of HIAP2 3'UTR and bound proteins were cross-linked to RNA by UVC-light irradiation. Free RNA was digested with RnaseA/T1. Following incubation, RNA-protein complexes were resolved on SDS-PAGE and visualized by autoradiography. Figure 6B shows that purified hnRNP A1 can bind to the H2B fragment in a concentration dependent manner.

HnRNP A1 destabilizes HIAP2 mRNA and the effect is enhanced during UVC stress

To evaluate the possibility that HIAP2 mRNA instability and hnRNP A1 binding to the HIAP2 ARE are two related events, siRNA experiments were carried out. RNA interference technology was used to knock down hnRNP A1 expression and determine its effect on HIAP2 levels. siRNA targeting hnRNP A1 was transfected into HEK293 cells, and forty-eight hours later cells were treated with or without UVC irradiation. Western blot analysis shows that there is at least 50% knowdown of hnRNP A1 after 48 hours transfection of siRNA (Figure 7). Figure 7A shows the expression of HIAP2 with the treatment of siRNA in the presence or absence of UVC irradiation. In the absence of UVC treatment, cells transfected with siRNA to hnRNP A1 showed slight increase of HIAP2 protein levels compared to the cells transfected with control siRNA. In contrast, following UVC irradiation the cells transfected with siRNA to hnRNP A1 showed notable increase in HIAP2 protein levels compared to the cells transfected with control siRNA. To confirm that the rescued HIAP2 protein expression was through the removal of hnRNP A1 destabilizing effect on the HIAP2 mRNA, the abundance of HIAP2 and GAPDH mRNAs were assessed by qRT-PCR. Again, cells transfected with siRNA to hnRNP A1 showed notable increase of HIAP2 steady state mRNA level compare to the cells transfected with control siRNA in the presence of UVC treatment (Figure 7B).

A


0 mW/cm2
150 mW/cm2
Untransfected
Ctrl. siRNA
hnRNP A1 siRNA
Ctrl. siRNA
hnRNP A1 siRNA
KDa
69 -
37 -
HIAP2
GAPDH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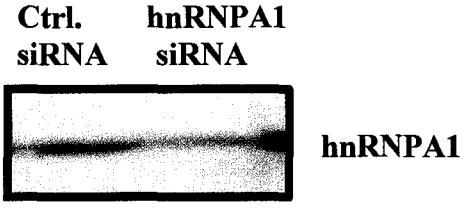
HIAP2/GAPDH protein
1.00
0.75
0.50
0.25
0.00
untransfected
siC
siA
siC
siA
O mW/cm2
150mW/cm2


Ctrl. siRNA
hnRNPA1 siRNA
hnRNPA1

B

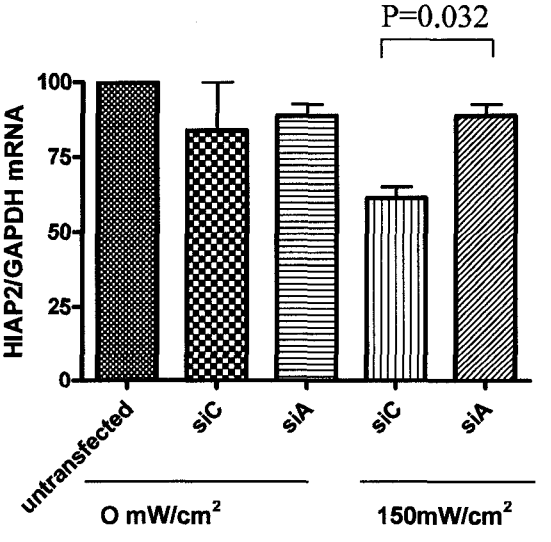

P=0.032
HIAP2/GAPDH mRNA
100
75
50
25
0
untransfected
siC
siA
siC
siA
O mW/cm2
150mW/cm2

Figure 7. Effect of siRNA-mediated knock down of hnRNP A1 levels on the expression and stability of HIAP2 mRNA.

(A) 48 hours after transfection of HEK293 cells with either an siRNA that suppressed hnRNP A1 or a control siRNA, cells were irradiated with or without UVC ($150mW/cm^2$). Five hours later, the levels of expression of HIAP2 and GAPDH were examined by Western blotting. Efficiency of knock-down was monitored by immunoblotting using anti-hnRNP A1 antibody. (B) Cells were treated the same way as in panel A. The abundance of HIAP2 and GAPDH mRNAs were assessed by qRT-PCR ($2^{-\Delta ct}$). Results are represented as the mean $\pm$ s.d. of three independent experiments.

Characterization of the AREs that destabilize HIAP2 mRNA

In order to identify which ARE is responsible for the binding of A1 and destabilizing HIAP2 mRNA, the AUUUA core elements were mutated individually using site directed mutagenesis. Sequences of the wild type and mutated H2B fragment of 3'UTR of HIAP2 are shown in Figure 8A. Potential ARE sites involved in the posttranscriptional regulation of HIAP2 mRNA are shown in red. The AREs were numberd 1- 4 to distinguish between the different ARE motifs within the HIAP2 3'UTR. To test if hnRNP A1 binds to a particular ARE, *in vitro* binding assay was performed with each mutant. Purified GST or GST-hnRNP A1 (7μl) was incubated with ^{32}P-labeled H2B fragment of HIAP2 3'UTR, mutant 1, mutant 2, mutant 3 and mutant 4. Bound proteins were cross-linked to RNA by UVC-light irradiation. Free RNA was digested with RnaseA/T1. Following incubation, RNA-protein complexes were resolved on SDS-PAGE and visualized by autoradiography. Figure 8B shows that hnRNP A1 binds to the H2B fragment as shown previously (Figure 6B). Mutations of ARE 1, ARE 3 or ARE 4 abolished the binding of hnRNP A1 suggesting that these sequences are involved in hnRNP A1 binding. In contrast, mutation of ARE 2 did not affect the binding of hnRNP A1 to the H2B fragment. qRT-PCR experiments were then performed to test the effect of ARE mutations on the stability of HIAP2 3'UTR reporter construct. CAT reporter construct containing the HIAP2 3'UTR segment H2B or the individual mutants were transfected into HEK293 cells. 24 hours later, steady-state levels of CAT mRNA were determined by real-time PCR analysis and normalized to the NEO mRNA levels. The results in Figure 8C indicate that mutation of ARE 1, 3 or 4 can rescue the stability of the HIAP2 reporter mRNA, although to a different extent. On the other hand, when ARE 2 is mutated, there is no change on the HIAP2 3'UTR reporter mRNA level

A

***H2B Fragment (bp 138-238)**

(138)-----AUAUAUUUAAU--------AUUUA-------AUUUA---------UAUAUUA------- (238)

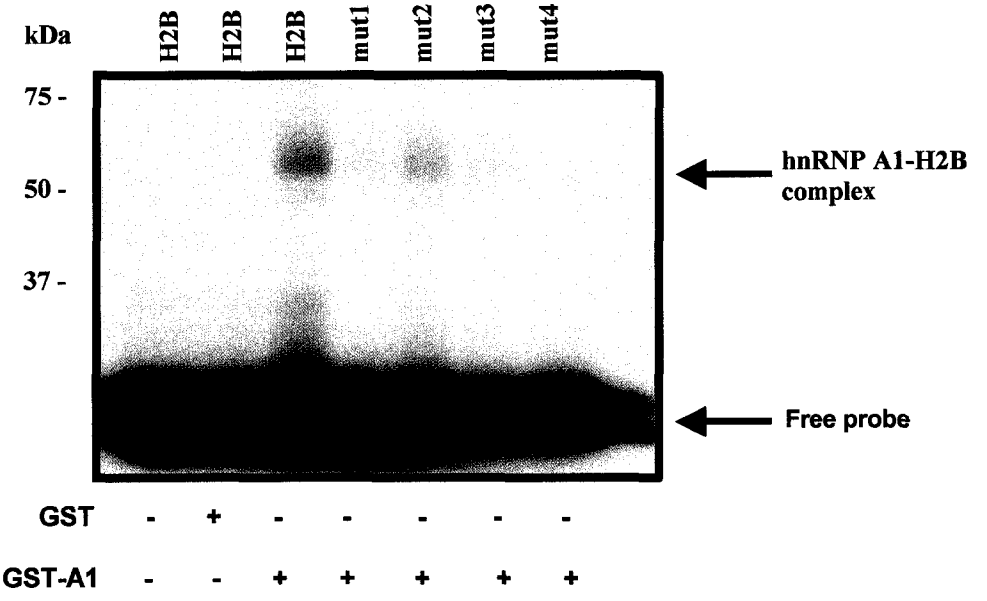
ARE 1 **ARE 2** **ARE 3** **ARE 4**

***H2B Fragment mutated**

(138)-------AUAUAGCAAU----------AGCA---------AGCA----------UAUAGCA-------(238)

mutant 1 **mutant 2** **mutant 3** **mutant 4**

B



C

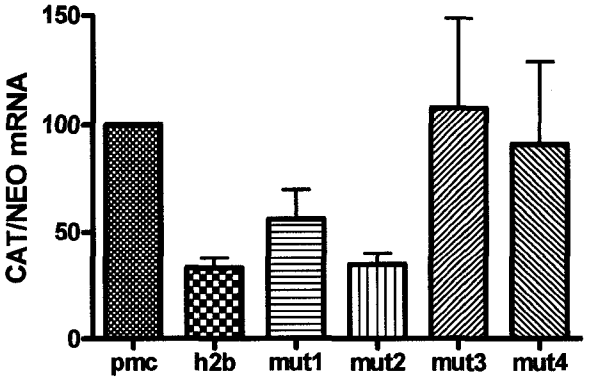


Figure 8. Influence of the mutations in the ARE in the 3'UTR of HIAP2 on HIAP2 mRNA stability.

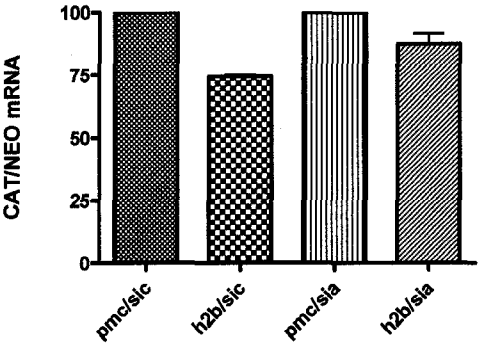
(A) Sequence of the wild type or mutated H2B fragment of 3'UTR of HIAP2. Potential ARE binding sites are shown in red. The AREs were numberd 1- 4 to distinguish between the different ARE motifs within the HIAP2 3'UTR. Mutations were generated by single site mutagenesis. (B) UVC-cross-linking assays identifies hnRNP A1 binding site on HIAP2 3'UTR. GST-hnRNP A1 (7μl) was incubated with ^{32}P-labeled H2B fragment, mutant 1, 2, 3, and 4. Bound proteins were cross-linked to RNA by UVC-light irradiation. Free RNA was digested with RnaseA/T1. Following incubation, RNA-protein complexes were resolved on SDS-PAGE and visualized by autoradiography. GST protein alone served as negative control. (C) CAT reporter construct with the H2B fragment (containing four putative AREs), the mutants (mutation of each AREs), or pMC were trasfected into HEK293 cells. 24 hours later, steady-state levels of CAT mRNA were determined by real-time PCR ($2^{-\Delta ct}$) analysis and normalized to the NEO mRNA levels. Results are represented as the mean $\pm$ s.d. of three independent experiments.

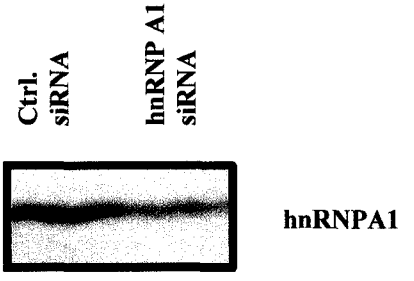
suggesting that this sequence does not contribute to the instability of HIAP2 mRNA. All of the above results suggest that ARE 1, 3 and 4 are all involved in the binding of hnRNP A1 to the H2B fragment thus affecting its mRNA stability.

siRNA-mediated knock down of hnRNP A1 levels stabilize HIAP2 3'UTR reporter mRNA and increase cell survival during UVC stress

I have shown that 3'UTR of HIAP2 is involved in the destabilization of its own mRNA and hnRNP A1 binds and destabilizes HIAP2 mRNA. I further wanted to confirm that this destabilizing effect is through the AREs in the 3'UTR of HIAP2. To evaluate the effects of siRNA mediated knock down of hnRNPA1 on the HIAP2 3'UTR reporter mRNA, HEK 293 cells were transiently transfected with control plasmid pMC and plasmid containing the H2B fragment. At the same time, cells were co-transfected with either an siRNA that suppressed hnRNP A1 protein levels or a control siRNA. 48 hours after co-transfection, cells were irradiated with (Figure 9A) or without (Figure 9B) UVC ($150mW/cm^2$). Five hours later, the abundance of CAT and NEO mRNAs was assessed by qRT-PCR. Western blot analysis revealed that there was at least 70% knock down of hnRNP A1. Knocking down hnRNP A1 caused a slightly elevated mRNA levels of HIAP2 3'UTR reporter mRNA compared to the cells that were treated with control siRNA. In contrast and consistent with the endogenous HIAP2 mRNA results (Figure 7B), following UVC irradiation, the cells transfected with siRNA to hnRNP A1 showed notable increase of HIAP2 3'UTR reporter steady state mRNA levels compared to the cells transfected with control siRNA (Figure 9B). This data suggests that hnRNP A1 destabilizes HIAP2 mRNA following UVC irradiation via the 3' UTR of HIAP2 .

A


CAT/NEO mRNA
100
75
50
25
0
pmc/siC
h2b/siC
pmc/siA
h2b/siA


Ctrl. siRNA
hnRNP A1 siRNA
hnRNPA1

B

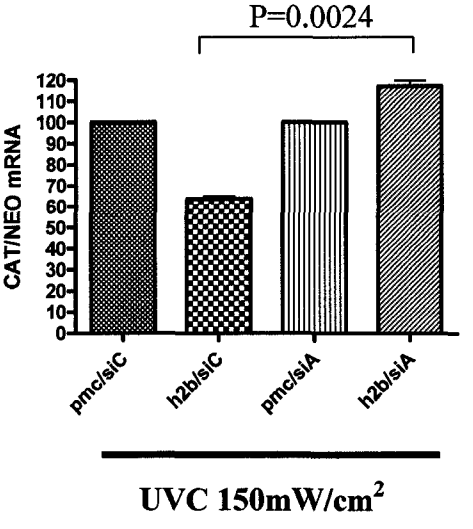
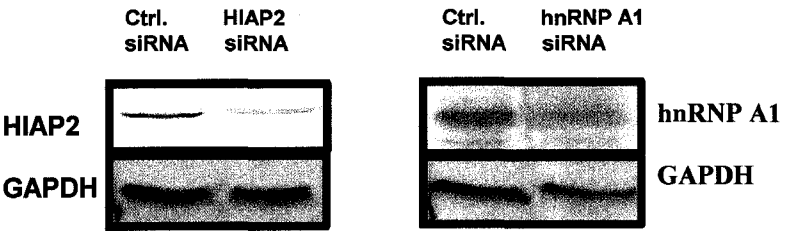

P=0.0024
CAT/NEO mRNA
120
110
100
90
80
70
60
50
40
30
20
10
0
pmc/siC
h2b/siC
pmc/siA
h2b/siA
UVC 150mW/cm2

Figure 9. Effect of siRNA-mediated knock down of hnRNP A1 levels on the stability of HIAP2 3'UTR reporter mRNA.

HEK293 cells were transiently transfected with control plasmid pMC or plasmid containing H2B fragment. At the same time, cells were co-transfected with either an siRNA that suppressed hnRNP A1 or a control siRNA. 48 hours after co-transfection, cells were irradiated with (A) or without (B) UVC (150mW/cm^2). Five hours later, the abundance of CAT and NEO mRNAs were assessed by qRT-PCR ($2^{-\Delta ct}$). Efficiency of knockdown was monitored by immunoblotting using anti-hnRNP A1 antibody. Results are represented as the mean $\pm$ s.d. of three independent experiments.

Finally, experiments were done to see the effect of knocking down HIAP2 or hnRNP A1 on cell viability following exposure to UVC. To investigate the effect of hnRNP A1 knock down on UVC-induced cell death, siRNA trasfected cells were exposed to increasing doses of UVC irradiation and cell viability was determined. Figure 10 shows that UVC irradiation was found to induce cell death in a concentration-dependent manner. As expected, cells transfected with HIAP2 siRNA were more sensitive to UVC induced cell death. In contrast, cells transfected with hnRNP A1 siRNA exhibited reduced sensitivity to UVC-induced cell death. All of the above results correspond well with the hypothesis that following exposure of cells to UVC irradiation, hnRNP A1 relocalizes to the cytoplasm and destabilizes HIAP2 mRNA. Therefore when hnRNP A1 levels are reduced, HIAP2 mRNA is stabilized resulting in increased levels of HIAP2 protein and attenuated cell death.


Ctrl. siRNA
HIAP2 siRNA
Ctrl. siRNA
hnRNP A1 siRNA
HIAP2
GAPDH
hnRNP A1
GAPDH

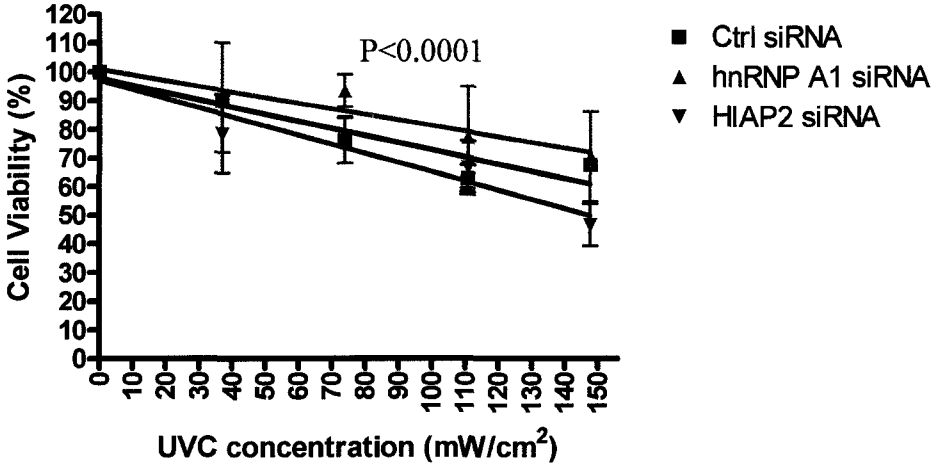

P<0.0001
Cell Viability (%)
UVC concentration (mW/cm2)
Ctrl siRNA
hnRNP A1 siRNA
HIAP2 siRNA

Figure 10. Effect of siRNA-mediated knock down of hnRNP A1 or HIAP2 on cell viability

HIAP2 siRNA, hnRNP A1 siRNA or control siRNA were transfected into HEK293 cells, 48 hours later, cells were irradiated with UVC for indicated doses. Analysis of cell viability was done 24 hours later using Vi-Cell cell viability analyzer. Results are represented as the mean $\pm$ s.d. of three independent experiments. Efficiency of knockdown was monitored by immunoblotting using anti-HIAP2 or anti-hnRNP A1 antibody.

CHAPTER 4. DISCUSSION

UVC light is a common type of environmental stress that causes DNA damage and induces apoptosis in mammalian cells through the mitochondrial branch of apoptotic pathway (Rehemtulla et al., 1997). UVC irradiation results in the modulation of expression of multiple genes that encode proteins involved in the repair of DNA damage, regulation of cell cycle progression, and regulation of apoptosis. The transcriptional regulation upon UVC irradiation has been extensively studied (Wu et al., 2002), but gene expression by cellular stress involves at least three types of coordinate regulation: regulation of transcription, regulation of mRNA stability, and regulation of mRNA translation (Fernandez et al., 2003). My thesis focused on how UVC irradiation alters gene expression of an important inhibitor of apoptosis, HIAP2. An important finding is that UVC irradiation reduces HIAP2 protein expression (Figure 3A). Assessment of protein stability was carried out and showed that the reduced HIAP2 protein expression was not due to changes in protein stability (Figure 3B). Instead, examination of RNA revealed that steady state levels of HIAP2 mRNA are substantially lower in UVC treated cells compared to normal cells (Figure 3C). Further experiments demonstrated that UVC irradiation resulted in approximately 2-fold decrease in the half life of HIAP2 mRNA (Figure 3D). A possible explanation for these observations could be that UVC irradiation can modulate the mRNA stability of HIAP2 gene. It has become evident in recent year that stress can alter the mRNA half-life of many genes coding for cytokines and stress response proteins. Although the mechanisms regulating mRNA stability of different genes have unique features, in general, sequences located in the 3'UTR and their interactions with specific proteins regulate mRNA stability.

To reveal if HIAP2 3'UTR could regulate its own mRNA stability, HIAP2 3'UTR reporter construct steady state mRNA levels were examined. Indeed, the steady-state mRNA levels were found to change markedly by insertion of either HIAP2 3'UTR or a 100nt H2B fragment into the pMC reporter control (Figure 4C). These results indicate that the stability of the HIAP2 message is controlled by a 3' untranslated region (UTR). In addition, sequence analysis was carried out. It was found that HIAP2 3'UTR contains four AREs that are located within the 100bp segment of the 3' UTR (termed H2B, Figure 4A). Mutational analysis of these elements disclosed that the AREs are involved in the modulation of HIAP2 mRNA stability (Figure 8C). Thus, the post-transcriptional regulation mechanism described here represents a novel mechanism of HIAP2 gene regulation under stress conditions.

One of the important findings presented in this work is the identification of hnRNP A1 as a novel HIAP2 ARE binding protein that modulates HIAP2 mRNA stability. HnRNP A1 is an ubiquitously expressed, predominantly nucleus-located member of the hnRNP family of RNA-binding proteins. It has been shown that hnRNP A1 can translocate to the cytoplasm and can bind to ARE-containing mRNAs (Hamilton et al., 1997). Consistent with this mechanism, I demonstrate that upon treatment of cells with UVC irradiation, the cytoplasmic content of hnRNP A1 is markedly increased in HEK293 cells (Figure 5).

In mammalian cells, hnRNP A1 undergoes several modifications such as methylation, sumoylation, ubiquitination, and phosphorylation (Hamilton et al., 1997; Iervolino et al., 2002). Endogenous hnRNP A1 is only very weakly phosphorylated in cells grown under normal conditions. However, various stress treatments of cells increase phosphorylation of hnRNP A1 and induce its cytoplasmic accumulation (Hamilton et al.,

1997; Kim et al., 2000a). Therefore specific phosphorylation could play a role in the regulation of the hnRNP A1 nucleocytoplasmic distribution. In fact, it has been shown that PKC directly associates with hnRNP A1 through its catalytic domain and phosphorylates it. Phosphorylation of Ser 199 by PKC is sufficient to regulate hnRNP A1 localization. Furthermore, the subcellular localization of hnRNP A1 can be modulated by the MKK3/6-p38 pathway in response to stress such as osmotic shock and irradiation with UVC light (Bonnal et al., 2005; Municio et al., 1995). Indeed, I have observed that hnRNP A1 relocalized to the cytoplasm of HEK293 cells following UVC irradiation. It has been suggested that phosphorylation of hnRNP A1 would cause hnRNP A1 to accumulate in the cytoplasm by inhibiting its association with transportin, which is responsible for transporting hnRNP A1 into the nucleus.

A number of members of the hnRNP family of proteins, including HuR and AUF1, have been shown to interact with AREs and affect the stability of ARE-containing transcripts. RNA affinity chromatography assay demonstrated that hnRNP A1 could bind *in vitro* to the 3' UTR sequence of the HIAP2 mRNA (Figure 6A). This is in distinct contrast to TIAR or TIA-1, which are also RNA binding proteins, but were not found to interact with HIAP2 3'UTR. Furthermore, the direct interaction of hnRNP A1 with HIAP2 3'UTR was demonstrated by the *in vitro* cross-linking assay (Figure 6B). I found that hnRNP A1 functions as HIAP2 mRNA-destabilizing factor. Indeed, knocking down hnRNP A1 by siRNA transfection increased HIAP2 protein expression through increasing steady state HIAP2 mRNA levels in both normal and UVC treated cells (Figure 7A and B).

Further research was done to identify the specific hnRNP A1-binding sites in the HIAP2 3'UTR mRNA. The results suggest that more than one ARE (ARE 1, 3 and 4)

contribute simultaneously to the binding of hnRNPA1 (Figure 8B). I propose that in addition to the primary sequences of the AREs, the secondary RNA structure of the ARE containing fragment may also play a role in the binding. This is supported by the previous findings which suggest that 3'UTR's secondary structure plays an important role for the binding of hnRNP A1 to CYP2A5 mRNA (Glisovic et al., 2003). Furthermore, the 3' UTR of G-CSF contains several copies of the ARE as well as a structurally and functionally distinct element that has been called the stem-loop destabilizing element (SLDE) (Putland et al., 2002). In the case of HIAP2, ARE 1, 3 and 4 act as cis-acting mRNA destabilizing elements which could form a SLDE or SLDE-like among themselves. Therefore mutation of either one of the AREs could disturb the secondary structure formed by them which could result in reduced binding of hnRNP A1. Further experiments could be carried out to test the binding affinity of hnRNP A1 for each AREs to dissect the key components that contribute to the binding of hnRNP A1 to the HIAP2 3' UTR.

To confirm that hnRNP A1 binding is responsible for HIAP2 mRNA instability, steady state mRNA level of each mutated AREs was assessed. There are four AREs between nucleotides 138 and 238 in the HIAP2 3'UTR, and within this region, sequences in ARE 1, 3 and 4 are necessary for destabilization of HIAP2 mRNA (Figure 8C). This is consistent with the hnRNP A1 binding results shown in Figure 8B. Those mutations that abolished the binding of hnRNP A1 also attenuated the mRNA instability (Figure 8B and C). In contrast, mutation of ARE 2 did not have any effect on the binding of hnRNP A1 and did not affect the steady state levels of the reporter construct mRNA. This indicates that the mutated segment still harbors the binding site for hnRNP A1 and that hnRNP A1 is required as a trans-acting factor for destabilizing activity of the HIAP2 ARE.

A growing number of ARE containing mRNAs has been found to be targets of several regulatory RNA binding proteins (RBPs). These RBPs can compete for binding to the same RNA region (in a mutually exclusive fashion), or bind cooperatively (whereby binding of one RBP to one mRNA region facilitates the binding of another RBP to another mRNA region), or bind jointly on separate regions of the same mRNA without apparent impact of one upon the other. In contrast, single RBP is sufficient to control the turnover of the HIAP2 mRNA since hnRNP A1 itself is sufficient for the binding to and the instability of HIAP2 mRNA.

From my previous results and the known shuttling properties of hnRNP A1, I hypothesized that following UVC irradiation, hnRNP A1 would redistribute to the cytoplasm and destabilize HIAP2 mRNA. The small amount of hnRNP A1 that is present in the cytoplasm under normal conditions can partially destabilize HIAP2 mRNA. This effect increases significantly following UVC irradiation that leads to increased amount of hnRNP A1 in the cytoplasm that is now available to bind to and destabilize HIAP2 mRNA. The siRNA transfection experiments indicate that under normal growth conditions, knock down of hnRNP A1 can enhance the steady state levels of HIAP2 3'UTR reporter mRNA (Figure 9B). This corresponds well with my hypothesis that there is a small amount of hnRNP A1 present in the cytoplasm under normal conditions. In fact, this was confirmed by microscopy data that shows low levels of hnRNP A1 in the cytoplasm of normal cells (Figure 5). In contrast, following UVC irradiation, hnRNP A1 accumulates in the cytoplasm. Therefore, when hnRNPA1 is knocked down under UVC irradiation conditions, the rescued levels of HIAP2 3'UTR reporter mRNA is higher compared to the rescued level in normal cells (Figure 9B).

UVC irradiation causes hnRNP A1 to accumulate in the cytoplasm by disturbing its normal shuttling mechanism. Many stress stimuli, such as osmotic shock or UVC irradiation result in the accumulation of hnRNP A1 in the cytoplasm, suggesting that translocation of hnRNP A1 could be a mechanism by which cells respond to various types of cellular stress in order to regulate a specific cohort of mRNAs under these conditions. There are still many questions to be elucidated. For example, if hnRNP A1 is an important cytoplasmic AUBP, do its cytoplasmic and nuclear forms differ in their RNA binding specificity? In addition, what modulates its ability to interact with ARE? It would also be of interest to investigate if additional stresses (i.e. osmotic shock, Arsenite treatment) of a different nature that are known to result in the cytoplasmic accumulation of hnRNP A1 also affect HIAP2 mRNA stability. This would suggests that changes in HIAP2 mRNA stability are not limited to UVC treatment but also occur with other stresses and may be a common stress response mediated by hnRNP A1.

UVC irradiation is known to activate MAPK pathway cascade and these pathways are involved in alteration of mRNA stability (Eberhardt et al., 2007). Stress-activated MAPKs (SAPKs) include the mammalian JNKs and p38 kinases. They are known to be activated in response to a variety of stress conditions, including osmotic stress, heat shock, oxidative stress, UVC light and some DNA-damaging agents (Kim et al., 2000b). Microarray experiments that globally tested mRNA stability found that 10% of transcripts were associated with p38 MAPK-dependent regulation (Cui et al., 2005; Demple, 2004). Accumulating data have highlighted that p38 MAPK regulates stability of many mRNA (for instance: cyclooxygenase-2, IL2, IL6, IL8, c-fos, Mmp2 or GM-CSF) through AU-rich regions of corresponding genes (Fischer and Voynow, 2002; Geginat et al., 2000; Hoffmann

et al., 2002; Iliev et al., 2002; Li et al., 2004a; Mandal and Hamilton, 2007; Zhao et al., 2004). It is known that activation of the MAPK family members results in a multitude of cytoplasmic and nuclear changes, which contribute to alterations in gene expression (Jang et al., 2000). MAP kinases serve to regulate the activity of critical transcription factors. In addition, the MAP kinase-activated protein kinase-2 (MK-2, downstream of p38 MAPK) is the only kinase to date that has been shown to be involved in regulating stability of specific mRNAs through direct phosphorylation of proteins bound to the 3'UTR. There exist additional examples of stress signaling pathways that phosphorylate AUBPs that in turn change the mRNA stability of the target gene through association with AREs. For example, ARE is involved in the destabilization of TNFα mRNA by the RNA binding protein TTP, which is regulated by the p38 pathway (Brook et al., 2000). Oxidative stress-induced γ-GCSh expression is mediated by the mRNA-stabilizing protein HuR, which interacts with an AU-rich sequence in the 3'-untranslated region (UTR) of γ-GCSh mRNA and the binding of HuR is regulated by the p38 (MAPK) signal transduction pathway (Larsen et al., 2002; Song et al., 2005). Furthermore, RhIL-17 mitigates COX-2 mRNA decay normally mediated by the AREs in the 3'UTR of COX-2 mRNA and involves the MKK3/6/SAPK2/p38 cascade (Faour et al., 2003). Therefore it is possible that SAPKs are involved in the regulation of HIAP2 mRNA stability and future experiments could be carried out to detect if there are changes in the binding affinity of hnRNP A1 to HIAP2 mRNA.

How does hnRNP A1 destabilize HIAP2 mRNA? Shortening of the poly(A) tail is the initial step to trigger mRNA for decay. There are two major mRNA degradation pathways in eukaryotic cells. In one pathway, the deadenylated mRNA is degraded by a cytoplasmic protein complex, the exosome. The exosome consists of a number of

exonucleases with 3'-5' activity (Eulalio et al., 2007; Garneau et al., 2007; Nonhoff et al., 2007). In the other pathway, shortening of the poly (A) tail leads to the removal of the cap structure of the mRNA by the decapping proteins DCP1 and DCP2 and subsequent 5'-3' degradation of the mRNA through the exoribonuclease XRN1 (Eulalio et al., 2007; Garneau et al., 2007; Nonhoff et al., 2007). The use of an *in vitro* RNA decay system provided evidence that 3′-to-5′ degradation is mediated by the exosome, which represents the major decay pathway for ARE-containing mRNAs in mammalian cells (Garneau et al., 2007). The exosome is likely to be recruited to unstable mRNAs *via* the AREs. It was found that several AUBPs, such as AUF1, TTP and KSRP physically associate with the exosome and that the isolated exosome preferentially degrades ARE-containing RNAs (Lal et al., 2004). These observations suggest that the exosome is recruited to inherently unstable mRNA substrates through its interaction with destabilizing AUBPs, and that this recruitment provides the basic mechanism responsible for rapid and preferential decay of ARE-containing mRNAs (Gherzi et al., 2004; Teixeira et al., 2005). Therefore it is possible that hnRNP A1 also adopts this mechanism and further experiments will be needed to obtain evidence of hnRNP A1-bound HIAP2 mRNAs associated with the exosome.

Is there a specific role for hnRNP A1 or, more generally, for hnRNPs in the stress response? In mammalian cells, different types of stress, including UVC irradiation, heat shock and oxidative stress inhibit translation of bulk mRNA, which aggregates in cytoplasmic structures known as stress granules (SGs). Because mRNAs in SGs, but not in processing bodies (P bodies), are associated with translation-initiation factors, it is assumed that mRNAs that have been released from polysomes in stressed cells are first directed to SGs (Eulalio et al., 2007; Gilks et al., 2004). In addition, during the recovery period, stress

granules may serve as a sorting domain for mRNAs. A subset of mRNAs may be recycled and returned to polysomes to restart protein synthesis. In contrast, other mRNAs may be transferred to adjacent P-bodies for destruction (Kedersha and Anderson, 2002; Kedersha et al., 2005; Yang and Bloch, 2007). The mechanism by which mRNAs are sorted, for either reuse or destruction, is unknown. Several lines of evidence in both *S. cerevisiae* and human cells suggest that processing bodies may be directly involved in mRNA degradation. (Fillman and Lykke-Andersen, 2005). Previous findings identified hnRNP A1 as a novel component of the SGs. In addition, stress-induced phosphorylation of hnRNP A1 prevents its interaction with transportin, resulting in increased cytoplasmic hnRNP A1 which remains bound to poly(A) mRNAs that are targeted to the SGs (Guil et al., 2006). Therefore it is possible that following UVC irradiation hnRNP A1 relocalizes into the cytoplasm where it associates with HIAP2 mRNA and targets HIAP2 mRNA to P bodies for degradation.

Finally, experiments were performed to see the effect of knocking down HIAP2 or hnRNP A1 on cell viability. Overexpression of several IAPs including XIAP, c-IAP1, c-IAP2, NAIP, or Survivin has been shown to suppress cell death induced by a variety of stimuli including TNFα, Fas, menadione, staurosporin, etoposide (VP16), Taxol, and growth factor withdrawal (Liston et al., 1996; Richter and Duckett, 2000; Salvesen and Duckett, 2002). I anticipated that knock down of HIAP2 would have the opposite effect on viability of cells exposed to UVC irradiation. Indeed, cells were more sensitive to UVC irradiation induced death when HIAP2 was knocked down, but the effect was modest when compared to the control siRNA transfected cells (Figure 10). It should be noted that mammalian IAPs have been reported to form homodimers and heterodimers (Rajalingam et al., 2006) and to cross-regulate each other, suggesting that knocking down HIAP2 may also affect the levels

of other IAPs. In fact, HIAP1 levels were observed to increase when HIAP2 was knocked down in HeLa cells (Conze et al., 2005). This suggests that simply reducing the levels of HIAP2 by siRNA may not be sufficient to dramatically affect the cell survival following UVC irradiation.

In addition, the activity of the XIAP or HIAP2 IRES has been shown to be induced by transient cellular stresses. The activity of the XIAP IRES is induced by anoxia, serum deprivation and low –dose γ-irradtioan. The ativity of the HIAP2 IRES can be stimulated by ER stress, etoposide or sodium arsenate treatment. The induction of their IRES activites by the stress results in an increase in protein levels, thereby increasing the amount of XIAP or HIAP2 to block the cell death under such stress conditions (Lewis and Holcik, 2005). It was also shown that both XIAP and HIAP2 may be translationally upregualted during Fas- or UVC-induced apoptosis by the FAST protein (Li et al., 2004b). Furthermore, pro-apoptotic protein Apaf-1 plays a central role in the mitochondrial apoptotic pathway, and its IRES-mediated translation is enhanced by UVC irradiation (Ungureanu et al., 2006). Therefore, following UVC irradiation, even if HIAP2 levels are reduced by siRNA treatment, the levels of other pro- or anti-apoptotic protein levels may vary and contribute to the overall cellular survival .

For the hnRNP A1 siRNA transfected experiments, I expected that cells would become more resistant to UVC irradiation due to the more stable HIAP2 mRNA and thus increase levels of HIAP2 protein. Although increased resistance to UVC irradiation was observed in cells with reduced levels of hnRNP A1, this effect was rather modest. It should be noted, however, that hnRNP A1 will likely affect other pro- or anti-apoptotic factors. For example, hnRNP A1 is a negative regulator of IRES-mediated XIAP translation. It has been

shown that osmotic stress causes the redistribution of hnRNP A1 to the cytoplasm where it can bind to the XIAP IRES to reduce XIAP translation (Lewis et al., 2007). In this case, hnRNP A1 has a pro-apoptotic role by decreasing XIAP level. In addition, hnRNP A1 was suggested to have anti-apoptotic function in part through the inhibition of the Apaf-1 IRES activity (Lewis and Holcik, unpublished data). Therefore, when hnRNP A1 is knocked down, both XIAP and Apaf-1 levels would be expected to increase and their relative abundance, together with levels of HIAP2 will contribute to the overall survival of cells following UVC irradiation. Thus, hnRNP A1 can have both apoptotic and anti-apoptotic effect. Further investigations will provide a clearer understanding of such complex system.

Conclusion

I have identified a novel regulatory mechanism that controls the expression of inhibitor of apoptosis protein HIAP2. Following UVC irradiation, RNA-binding protein hnRNP A1 relocalizes to cytoplasm, where it associates with HIAP2 mRNA *via* specific ARE sequences in the 3' UTR of HIAP2 and targets HIAP2 mRNA for degradation. The results presented here provide evidence for hnRNP A1 being an important player in the post-transcriptional regulation of HIAP2 expression. HIAP2 has been linked with several common malignancies including lung, ovarian, esophageal, and liver carcinoma. This suggests that overall contribution of HIAP2 to human cancers may be substantial, and the regulation of the expression of this gene should be closely examined.

Appendix I

Primer sequences for qRT-PCR

NEO (forward: 5'-tgaatgaactgcaggacgag; reverse: 5'-caatagcagccagtcccttc)

CAT (forward: 5'-gcgtgttacggtgaaaacct; reverse: 5'-gggcgaagaacttgtccata)

HIAP2 (forward: 5'-tctggagatgatccatgggtaga; reverse: 5'tggcctttcattcgtatcaaga-)

GAPDH (forward: 5'-acagtcagccgcatcttctt; reverse: 5'-acgaccaaatccgttgactc)

Primer sequences to generate mutant ARE

Mutant 1

Forward primer: 5'-ggtatcatatagcaatcttaatctg-3'

Reverse primer: 5'-cagattaagattgctatatgatacc-3'

Mutant 2

Forward primer: 5'-cttaatctgtttagcacaagggaag-3'

Reverse primer: 5'-cttcccttgtgctaaacagattaag-3'

Mutant 3

Forward primer: 5'-caagggaagagcatgtttggtgaac-3'

Reverse primer: 5'-gttcaccaaacatgctcttcccttg-3'

Mutant 4

Forward primer: 5'-ggtgaactatagcagtatgtatgtg-3'

Reverse primer: 5'-acatacatactgctatagttcacc-3'

Primer sequences to generate T7-H2B or T7-mutants (T7 sequence is underlined)

Forward primer: 5'-aatacgactcactatagggcgagttctagtctgctttggt-3'

Reverse primer: 5'-tctagacagtgacactactcccttagg-3'

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