

**Regulation of cIAP1 mRNA Stability Through Its 3' UTR by the RNA-Binding Protein HuR**

**By:**

**Peng Liu**

**Thesis submitted to the Faculty of Graduate and Postdoctoral Studies (FGPS), University of Ottawa in partial fulfillment of the requirements for degree of Master of Science**

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

## **Abstract**

The RNA-binding protein HuR is involved in numerous aspects of the RNA life-cycle. It is known for its ability to stabilize AU-Rich Element (ARE)-containing transcripts in the cytoplasm. The transcript of cIAP1, an important protein involved both in the regulation of apoptosis and NF- $\kappa$ B signaling, contains four such AREs, raising the question of whether HuR can modulate the stability of cIAP1 mRNA. First, using C2C12 cells, we observed a positive correlation between cIAP1 mRNA levels and HuR cytoplasmic localization. We then show that knockdown of HuR in U2OS cells results in a decrease in steady-state cIAP1 mRNA levels through destabilization of the cIAP1 mRNA. Furthermore, we are able to show *in vitro* that HuR binds directly to the second of the four AREs in the 3' UTR. The direct link between the binding of HuR to the second ARE and its effect on cIAP1 mRNA stability remains to be shown.

## **Acknowledgements**

I would like to thank all of the members of the Holcik lab, both past and present, for their help and guidance in both this project and my development as a Masters student. In particular, I am grateful to my supervisor, Dr. Martin Holcik, who provided me this amazing opportunity. His positive attitude, patience, and direction made for a great environment to work in and helped immensely in elucidating confounding issues. Thanks also to all of the members of the Apoptosis Research Centre in general for making my experience at the research institute fun and enjoyable. I'd also like to thank the members of my Thesis Advisory Committee, Drs. Martin Pelchat and Bruce McKay, who provided helpful suggestions to guide my research. I would like to acknowledge the Natural Sciences and Engineering Research Council for funding this work. Finally, I'd like to thank my parents and my girlfriend, Joanne, for their unconditional support in whatever I chose to pursue.

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## 1. List of Abbreviations

ActD	Actinomycin D
ARE	AU-rich element
ATP	Adenosine trisphosphate
AUF	AU-rich element RNA-binding protein
$\beta$ -Gal	$\beta$ -galactosidase
BIR	Baculovirus inhibitor of apoptosis protein repeat
BIRC	Baculoviral IAP repeat-containing protein
bp	Base pair
BSA	Bovine serum albumin
C2C12	Immortalized mouse myoblast cell line
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary DNA
cIAP	Cellular inhibitor of apoptosis protein
cpm	Counts per minute
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ELAV	Embryonic lethal abnormal vision
ELAVL	ELAV-like
ELISA	Enzyme-linked immuno sorbent assay
FBS	Fetal bovine serum
GST	Glutathione S-transferase
HIAP	Human inhibitor of apoptosis protein (alternate name for cIAP, but different numbering)
hnRNP	Heterogenous nuclear ribonucleoprotein
HNS	HuR nucleocytoplasmic shuttling sequence
Hu	Human antigen
HuR-CP	HuR cleavage product
IAP	Inhibitor of apoptosis protein
IRE	Iron-responsive element
IRES	Internal ribosome entry site
miRNA	MicroRNA
mRNA	Messenger RNA
mut. X	H2B with the x <sup>th</sup> ARE mutated
n.s.	Not significant (p-value > 0.05)
NES	Nuclear export signal
nt	Nucleotides
ONPG	o-nitrophenyl- $\beta$ -D-galactoside
NLS	Nuclear localization signal
PAGE	Polyacrylamide gel electrophoresis

PAR-CLIP	Photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative PCR (also known as real-time PCR)
RING	Really Interesting New Gene
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RPM	Revolutions per minute
RRM	RNA recognition motif
qRT-PCR	Quantitative reverse transcription PCR
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
siC	Non-targeting negative control siRNA purchased from Qiagen
siHuR	siRNA targeting the coding region of HuR
siRNA	Small interfering RNA
U2OS	Immortalized human osteosarcoma cell line
UTP	Uridine triphosphate
UTR	Untranslated region
x% PBST	Phosphate buffered saline supplemented with x% Tween 20
XIAP	X-linked inhibitor of apoptosis protein

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### **3. Introduction**

#### **3.1 Prologue**

Cells possess many mechanisms by which they can regulate their behaviour in response to any stimuli, but it is generally accepted that most of this regulation ultimately comes down to controlling protein levels and/or function. Protein levels and/or function can be regulated at multiple steps starting from the transcription of the encoding mRNA (messenger RNA). Following transcription, the mRNA can be further regulated by alternative splicing, during export into the cytoplasm, by altering its stability, and during translation of the message into the encoded protein. Even after translation, the protein itself can be regulated by post-translational modifications (i.e.: phosphorylation, ubiquitylation) to affect its stability and/or functionality. All of these events are themselves regulated, by proteins or other factors (i.e.: miRNAs [microRNAs]), leading to a complex network of interactions that ultimately governs how a cell behaves. In particular, I will focus on the regulation of mRNA stability.

Although traditionally research related to gene expression has focused on regulation at the transcriptional level, more recently, there has been increased interest in post-transcriptional regulation. For a living cell, regulating gene expression post-transcriptionally provides many benefits compared to transcriptional regulation alone. One of the most important of these benefits is the increased rapidness of the response that arises from post-transcriptional regulation. In general, the further downstream the regulation occurs, the quicker the response. Accordingly, this form of regulation may be favoured in responding to urgent, time-sensitive, or rapidly-changing stimuli (i.e.: apoptotic decisions), increasing the need to understand these additional levels of regulation.

### 3.2 mRNA stability

The central dogma of molecular biology states that information is passed along from DNA to mRNA by transcription and then from mRNA to protein by translation (Crick, 1970). This pathway only tells half of the story, however, and only allows for active upregulation of gene products, forcing the cell to rely on slow or non-specific, passive mechanisms to degrade and downregulate these mRNA and protein products. As cells require the ability to rapidly regulate protein levels bidirectionally, they thus also possess active means to degrade these products, allowing them to selectively degrade mRNAs and proteins that are no longer needed or defective. Although mRNA decay occurs in both the nucleus and cytoplasm (Houseley and Tollervey, 2009), we will focus on cytoplasmic decay because of its predominance and its ability to directly regulate the stability, and as a result, the quantity, of translation-competent transcripts.

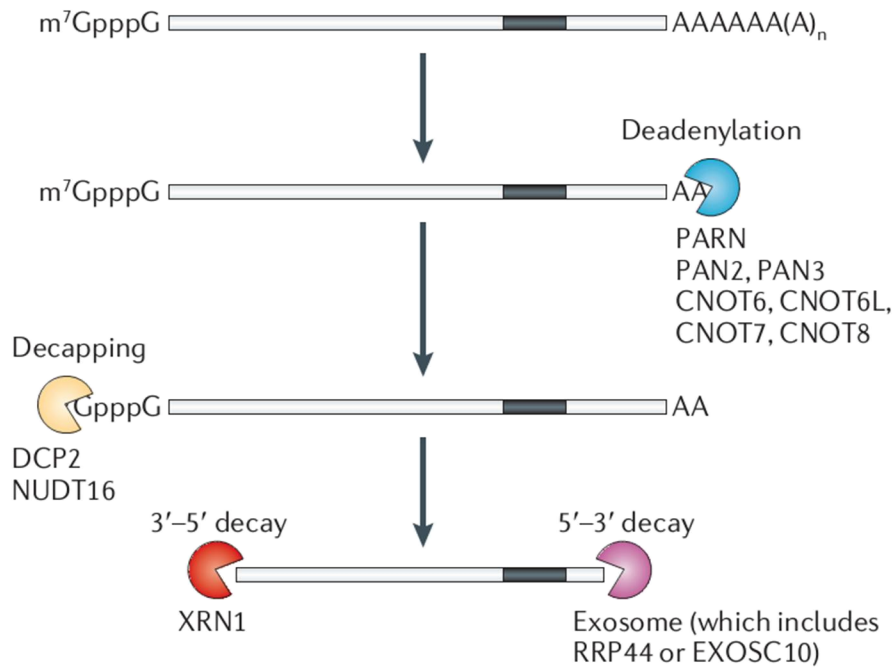
mRNA is protected from non-specific degradation by exonucleases from the 5' end by the 5' m<sup>7</sup>G cap structure and from the 3' end by the 3' poly(A) tail, although some exceptions apply (Fig. 1). In eukaryotic cells, deadenylation of the 3' poly(A) tail by the Ccr4-NOT complex, the Pan2-Pan3 complex, or PARN generally initiates and rate-limits mRNA degradation (Chen and Shyu, 2011). Once deadenylated, the mRNA can undergo 3' to 5' degradation by the exosome complex, with RRP44 as the active exonuclease, or decapped by either Dcp2 or Nudt16, further exposing the 5' end (Coller and Parker, 2004). The exposed 5' end can then be degraded in a 5' to 3' manner by Xrn1 (Li and Kiledjian, 2010). In contrast to this exonucleolytic initiation of degradation, some mRNAs are cleaved endonucleolytically, leaving two fragments, one with its 3' end exposed and the other with

its 5' end exposed, that are then further processed exonucleolytically by the aforementioned enzymes (Schoenberg, 2011).

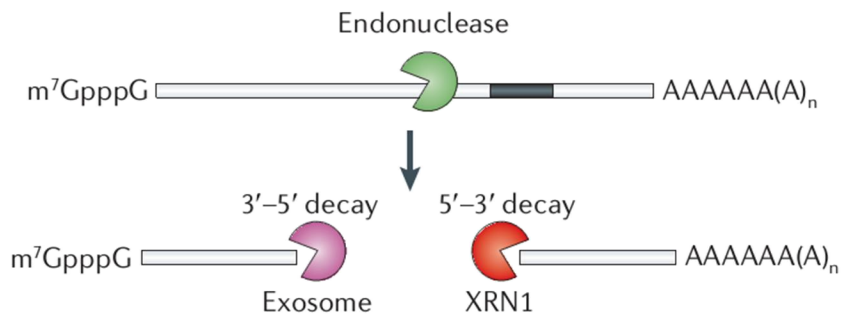
Cells possess numerous mechanisms with which to specifically regulate the decay of different mRNA species. Cis-regulatory elements are sequences and/or structures located within the transcript itself that confer varying “intrinsic” stabilities to the transcript. For example, although histone mRNA does not possess a poly(A) tail, it is stabilized by a stem-loop located in its 3' UTR that blocks degradation of the transcript from the 3' end by acting as a physical barrier, impeding the processing activity of the exosome (Mullen and Marzluff, 2008; Pandey and Marzluff, 1987). The 3' UTR of transferrin receptor mRNA contains IREs (Iron-Responsive Elements) that destabilize the transcript (Casey et al., 1989). In recent years, miRNA and siRNA (small interfering RNA) binding sites have also emerged as well-known and widely-used examples of cis-regulatory elements that modulate the stability of the mRNAs in which they are found (Valencia-Sanchez et al., 2006).

In addition to cis-regulatory elements, there are also trans-acting factors, proteins and/or RNAs that can bind to the transcript, which can affect mRNA stability. Oftentimes, trans-acting factors bind to cis-regulatory elements to mediate the observed basal effect of the cis-regulatory element. The binding sites for these trans-acting factors are commonly found in the 3' UTR (UnTranslated Region) of transcripts where they will not be displaced by translating ribosomes, although ribosome-mediated displacement can itself be a regulatory mechanism for targeting certain transcripts for degradation (Garneau et al., 2007). For example, when intracellular iron levels are low, IRE-binding proteins bind to the IREs within the 3' UTR of the transferrin receptor transcript, negating their basally destabilizing effect

**a Exonuclease decay pathways**



**b Endonuclease decay pathways**



**Figure 1. mRNA degradation in eukaryotes.** mRNA in eukaryotes is protected by a 5' cap complex and, for the most part, a 3' poly(A) tail. **(a)** Exonucleolytic degradation generally begins with deadenylation by the Ccr4-Not complex, the Pan2-Pan3 complex, or PARN. This is followed by decapping of the transcript by Dcp2 or Nudt16 and/or by degradation of the mRNA from the exposed 3' end by the exosome. In the case of decapped transcripts, processing of the mRNA from the 5' end is performed by Xrn1. **(b)** Endonucleolytic cleavage leaves two fragments, one with its 3' end exposed and the other with its 5' end exposed. These fragments are then processed by the exosome in a 3' to 5' manner or by Xrn1 in a 5' to 3' manner. (Schoenberg and Maquat, 2012)

and helping to maintain iron homeostasis by upregulating transferrin receptors to increase iron uptake (Casey et al., 1989). Similarly, miRNA and siRNA bind to their respective binding sites by base complementarity along with the RISC (RNA-Induced Silencing Complex) which can then cleaves the target transcript endonucleolytically, leading to its degradation (Carthew and Sontheimer, 2009).

This interplay between cis-regulatory elements and trans-acting factors allows cells to specifically regulate the stability of various transcripts by degrading unwanted transcripts that might intrinsically be stable or protecting needed transcripts that might intrinsically be unstable. In addition to the simple examples above, there are numerous cases of indirect, and more subtle, interactions between cis-regulatory elements and trans-acting factors regulating mRNA stability. As an example of competition between trans-acting factors, both HuR and AUF1 (AU-rich element RNA-binding protein 1) compete for binding to the 3' UTR of the JunD transcript whose stability is then determined by which of the factors binds (Zou et al., 2010). As another example, the p27 mRNA 3' UTR contains binding sites for both miR-221 and miR-222; however, due to a hairpin that forms, neither site is accessible and the transcript remains stable (Kedde et al., 2010). Upon binding of the trans-acting factor, PUM1, the hairpin structure melts, exposing the miRNA binding sites and destabilizing the transcript.

The above was but a very terse sampling of various regulatory modes that govern mRNA stability; far more modes that regulate stability exist (i.e.: no-go decay, nonsense-mediated decay). There is no general mechanism that explains how cis-regulatory elements and trans-acting factors interact, with the RNA degradation machinery or with each other, to elicit the desired effect. In fact, it is still not well-established in many cases how cis-

regulatory elements and trans-acting factors interact with the degradation machinery to ultimately affect degradation.

### **3.3 AU-rich elements**

Among the many cis-regulatory elements identified in the 3' UTR of cellular transcripts, AREs (AU-Rich Elements) are one of the best-studied and are commonly associated with transcript instability. As is often the case, unfortunately, there is no black-and-white descriptor that defines all AREs; however, there are three broad classes of AREs: class I and II AREs contain AUUUA pentamers and at least two overlapping UUAUUUA(A/U)(A/U) nonamers, respectively, in the context of a U-rich region while class III AREs do not contain AUUUA motifs, but consist of U-rich or AU-rich regions (Chen and Shyu, 1995). The behaviour of AREs, as well as the trans-acting factors that bind to them, can vary from transcript to transcript based on the context of the surrounding sequence (Winzen et al., 2004).

The instability conferred by AREs to their host transcripts is hypothesized to be mediated by trans-acting factors such as the RNA-binding proteins AUF1, CUG-BP, KSRP, RHAU, and TTP. It is believed that these factors recruit the RNA degradation machinery by interacting both with the target mRNA and components of the RNA degradation machinery. For example, AUF1 has been shown to interact with the exosome (Chen et al., 2001), CUG-BP has been shown to interact with PARN (Moraes et al., 2006), KSRP and RHAU have been shown to interact with both the exosome and PARN (Gherzi et al., 2004; Tran et al., 2004), and TTP has been shown to interact with the decapping enzyme, the exosome, and Ccr4 (Lykke-Andersen and Wagner, 2005).

In addition to these destabilizing ARE-binding proteins, there are also factors that can stabilize ARE-containing transcripts such as HuR and nucleolin. For example, on Bcl-2 transcripts, nucleolin competes with AUF1 for binding to the AREs present in the transcript's 3' UTR (Ishimaru et al., 2010). The stability of the transcript is thus determined by whether or not nucleolin could prevent AUF1 from binding to the mRNA and recruiting the RNA degradation machinery.

### **3.4 The RNA-binding protein, HuR**

The Hu (human antigen) family of proteins consists of HuB, HuC, HuD, and HuR. Their analogue, ELAV (Embryonic Lethal Abnormal Vision), was originally discovered in *Drosophila* and was so named due its requirement during embryonic development and its continued role in the visual system (Campos et al., 1987). Of particular interest, HuR, a 36 kDa protein also known as ELAVL1 (ELAV-like 1), is the only member of the Hu family to be ubiquitously expressed throughout the body. Since its discovery, much research has been conducted on its various roles and its regulation.

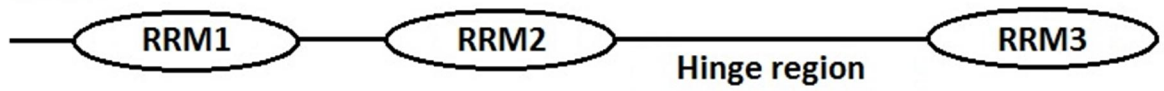
Among the numerous functions that it serves within the cell, HuR has primarily been studied in the context of its ability to bind to and regulate a diverse set of RNAs at various stages of their life-cycle. Although primarily nuclear, HuR is known to shuttle between the nucleus and the cytoplasm, allowing it to perform its regulatory duties in both cellular compartments (Kim and Gorospe, 2008). It has been shown to be involved in the regulation of alternative splicing (Zhou et al., 2011), nuclear-cytoplasmic RNA shuttling (Doller et al., 2008), the regulation of RNA stability (Brennan and Steitz, 2001; Fan and Steitz, 1998), and the regulation of translation (Durie et al., 2011; Yu et al., 2013).

HuR's RNA-binding capability arises from the three RRM (RNA Recognition Motifs) it possesses (Fig. 2). Although all three RRMs have been implicated in binding to AREs, RRM 3 has also been shown to interact with the poly(A) tail of certain transcripts (Ma et al., 1997). The differential usage of these RRMs, as well as their post-translational modifications, allows for HuR to distinguish between AREs in various contexts. Furthermore, between RRMs 2 and 3, there is a hinge region that contains a HNS (HuR Nucleocytoplasmic Shuttling sequence) motif. The HNS possesses both NLS (Nuclear Localization Signal) and NES (Nuclear Export Signal) activity (Gallouzi and Steitz, 2001). The shuttling activity has been shown to be mediated by interaction with transportin-2, a transport protein involved in nucleocytoplasmic shuttling. In addition to this pathway, HuR also makes use of the CRM1 transport system through interaction of RRM 3 and the hinge region with pp32 and APRIL.

As previously mentioned, HuR is commonly thought of as a stabilizing trans-acting factor. Like nucleolin, HuR is thought to exert its stabilizing effect on transcripts primarily through competition with destabilizing trans-acting factors. For example, in the case of the JunD and COX-2 transcripts, HuR competes with AUF1 and miR-16, respectively, for binding to their 3' UTRs (Young et al., 2012; Zou et al., 2010). In both cases, binding of HuR leads to stabilization of the mRNA. This is not always the case, however, and p16 serves as a counterexample. Binding of HuR to the 3' UTR of the p16 mRNA promotes the binding of AUF1 to the transcript as well, leading to its destabilization (Chang et al., 2010).

As its stability-modulating effect occurs in the cytoplasm, altering HuR's localization affects its ability to influence RNA stability. For example, it has been shown that activation of the p38 MAPK pathway can lead to an accumulation of HuR in the

**HuR**



**Figure 2. The protein domains of HuR.** HuR contains three RRM (RNA Recognition Motifs) that give it the ability to bind to AREs (AU-Rich Elements). Through differential usage and post-translational modification of these RRMs, HuR is able to distinguish between AREs in various contexts. RRM 3 is also known to interact with the poly(A) tail of certain transcripts. Between RRM 2 and RRM 3, there exists a hinge region that contains a HNS (HuR Nucleocytoplasmic Shuttling sequence) motif that exhibits both NLS (Nuclear Localization Signal) and NES (Nuclear Export Signal) activity. Phosphorylation of residues within this hinge region regulates HuR's localization. HuR's nucleocytoplasmic shuttling is mediated by both the transportin-2 and CRM1 transport systems.

cytoplasm, resulting in the stabilization of genes such as SMN (Farooq et al., 2009). HuR has been shown to be phosphorylated by a number of kinases. In particular, phosphorylation of S202 or S242 within the hinge region by Cdk1 was shown to increase HuR's nuclear localization while phosphorylation of S221, also in the hinge region, by PKC  $\alpha$  or  $\delta$  was shown to promote its cytoplasmic relocation (Kim et al., 2008a; Kim et al., 2008b). In addition, HuR's localization can also be regulated by cleavage of the protein. It has been shown that caspase-mediated cleavage of HuR into two cleavage products, HuR-CP1 and HuR-CP2 (HuR cleavage product 1 and 2, respectively), during muscle differentiation leads to HuR accumulation in the cytoplasm (Beauchamp et al., 2010). The larger of these fragments, HuR-CP1, binds to transportin-2 and inhibits its ability to import full-length HuR, leading to the observed cytoplasmic accumulation. This accumulation has been shown to be important for myogenesis by stabilizing the myogenic factors myogenin and MyoD whose transcripts contain AREs.

The levels of HuR protein also affect its ability to modulate RNA stability. It has been shown that nuclear HuR promotes the alternative processing of its own transcript, resulting in a longer 3' UTR that contains AREs (Dai et al., 2012). This transcript is less stable than its shorter counterpart, resulting in a negative feedback loop that stabilizes HuR levels within the cell.

### **3.5 cIAP1**

The IAP (Inhibitor of Apoptosis Protein) family of proteins consists of, in humans, of NAIP (BIRC1), cIAP1 (Cellular Inhibitor of Apoptosis Protein 1; BIRC2), cIAP2 (BIRC3), XIAP (X-linked Inhibitor of Apoptosis Protein; BIRC4), survivin (BIRC5), BRUCE (BIRC6), KIAP (BIRC7), and ILP2 (BIRC8). IAPs were originally discovered in

baculovirus and were so named for their ability to inhibit apoptosis in infected cells, thus allowing the virus to proliferate (Crook et al., 1993). Of the human IAPs, XIAP is the best characterized and, like its baculovirus counterparts, can inhibit apoptosis by directly binding to and inhibiting the activity of caspases (Scott et al., 2005). Not all IAPs, however, play such a direct role in modulating apoptosis and many play other important roles in the cell, such as survivin's role in cytokinesis (Szafer-Glusman et al., 2011) or cIAP1's role in myogenesis (Enwere et al., 2012).

cIAP1, also known as HIAP2 (Human Inhibitor of Apoptosis Protein 2), is a 69 kDa protein that can also bind caspases; however, it is primarily known for its role in both canonical and non-canonical NF- $\kappa$ B signaling (Mahoney et al., 2008; Zarnegar et al., 2008): a major pathway that plays an integral part in cell proliferation, cell survival, and the immune response. In addition to three BIR (Baculovirus Inhibitor of apoptosis protein Repet) domains, cIAP1 contains a RING (Really Interesting New Gene) domain with E3 ubiquitin ligase activity (Gyrd-Hansen and Meier, 2010). In the canonical NF- $\kappa$ B pathway, cIAP1 generates K63-linked polyubiquitin chains on other members of the signaling complex, forming a scaffold to recruit downstream effectors. In the case of the non-canonical NF- $\kappa$ B pathway, cIAP1 instead generates K48-linked polyubiquitin chains, marking downstream inhibitors of the non-canonical pathway for proteasomal degradation.

The 3' UTR of the cIAP1 mRNA is approximately 500 base pairs (bp) long and contains four AREs (Fig. 7A). It has previously been shown that UV stress causes hnRNP A1 (Heterogenous Nuclear RiboNucleoProtein A1) to relocate from the nucleus to the cytoplasm, where it binds to AREs 3 and 4 in the 3' UTR of cIAP1 mRNA, destabilizing it (Zhao et al., 2009). Furthermore, preliminary data from our laboratory has identified HuR as

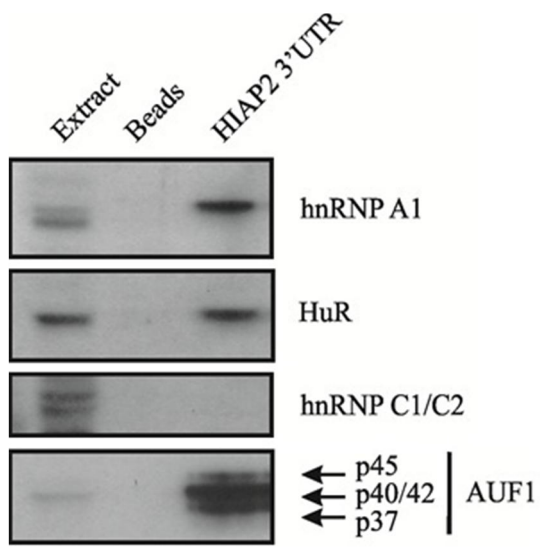
another RNA-binding protein that does interact, either directly or in complex with other factors, with the ARE-containing portion of the cIAP1 3' UTR (Fig. 3); however, the precise manner and location of interaction and its biological consequences remain unknown.

### **3.6 Working hypothesis and objectives**

As previously mentioned, it has been shown the 3' UTR of the cIAP1 mRNA controls its mRNA's stability. Preliminary data further shows that in whole cell lysate, HuR interacts with the cIAP1 mRNA 3' UTR. Given the role HuR and cIAP1 play in myogenesis as well as HuR's preference for binding to AREs and its ability to regulate the mRNA stability of other genes, I hypothesized that HuR could also regulate the stability of cIAP1 mRNA through interactions with one or more of the AREs present in its 3' UTR.

The objectives of my project are:

1. To determine whether endogenous HuR levels affect cIAP1 mRNA stability.
2. To determine whether HuR binds directly to one or more of the AREs present in the cIAP1 mRNA 3' UTR, and if so, which ARE(s) it binds to.
3. To determine whether any effect seen on the steady-state levels and half-life of cIAP1 mRNA as a result of manipulation of HuR levels is mediated through binding to the AREs present in the 3' UTR of the cIAP1 mRNA.



**Figure 3. HuR binds the 3' UTR of cIAP1 mRNA *in vitro*.** The ARE-containing portion of the cIAP1 (also known as HIAP2) 3' UTR interacts with hnRNP A1, HuR, and AUF1, but does not interact with hnRNP C1/C2 as determined by RNA affinity chromatography. The ARE-containing portion of the cIAP1 3' UTR was *in vitro*-synthesized and attached to beads in a column. HeLa cell lysate was passed through the column and proteins that bound to the beads or the 3' UTR fragment were eluted. The eluted proteins were then probed by Western blot for various RNA-binding proteins. (Performed by Dr. Stephen Lewis, unpublished)

## **4. Materials and Methods**

### **4.1 Cell culture and transfection**

C2C12 cells, an immortalized mouse myoblast line, were grown at 37°C in 5% CO<sub>2</sub> in Complete DMEM (10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin in 500 mL HyClone High Glucose DMEM [Thermo Scientific]).

Differentiation was initiated by changing the media to differentiation media (2% HyClone Donor Equine Serum [Thermo Scientific], 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin in 500 mL DMEM [ATCC]) when the cells were 70-80% confluent. Media was replaced with fresh differentiation media daily for the duration of the five day differentiation time-course.

U2OS cells, an immortalized human osteosarcoma line, were grown at 37°C in 5% CO<sub>2</sub> in Complete DMEM.

For plasmid transfection, U2OS cells were seeded in a total volume of 1 mL at  $2.0 \times 10^5$  cells/well in 12-well culture plates. The following day, the plasmid transfection mixture was prepared (75 µL jetPrime™ buffer, 0.75 µg plasmid, 1.5 µL jetPrime™ reagent [Polyplus Transfection] per well; for a list of plasmids used, please see the appendix) and incubated for 10 min. before 75 µL of mixture was pipetted into each well. The following day, cells were processed for Western blot analysis or quantitative reverse transcription PCR (qRT-PCR).

For siRNA transfection, U2OS cells were seeded in a total volume of 800 µL at  $1.0 \times 10^5$  cells/well in 12-well plates (1.5 mL at  $2.0 \times 10^5$  cells/well in 6-well plates; at least 6 wells were seeded for one S10 preparation). The following day, the siRNA transfection mixture

was prepared (200/500  $\mu\text{L}$  OPTI-MEM, 1/2  $\mu\text{L}$  siRNA, 2/4  $\mu\text{L}$  Lipofectamine® RNAiMax reagent [Life Technologies] per well for 12- and 6-well plates, respectively; for a list of siRNAs used, please see the appendix) and incubated for 20 min. before 200  $\mu\text{L}$  of mixture was pipetted into each well (500  $\mu\text{L}$  for 6-well plates). Three days later, cells were processed for Western blot analysis, qRT-PCR, or S10 lysate (6-well format).

For the genetic rescue experiment, U2OS cells were seeded in a total volume of 800  $\mu\text{L}$  at  $1.0 \times 10^5$  cells/well in 12-well plates. The following day, siRNA transfection was performed as described above. The next day, plasmid transfection was performed as described above. Two days later, cells were processed for Western blot analysis or qRT-PCR.

For the ELISA experiment with FLAG-HuR overexpression, U2OS cells were seeded in a total volume of 1 mL at  $1.0 \times 10^5$  cells/well in 12-well plates. The following day, plasmid transfection was performed as described above to overexpress FLAG-HuR or FLAG control. The next day, the media was removed and replaced with 1 mL of fresh Complete DMEM. The transfection mixture was prepared (75  $\mu\text{L}$  jetPrime™ buffer, 0.188  $\mu\text{g}$  CAT reporter plasmid, 0.375  $\mu\text{g}$   $\beta$ -Gal plasmid, 1.5  $\mu\text{L}$  jetPrime™ reagent per well) and incubated for 10 min. before 75  $\mu\text{L}$  of mixture was pipetted into each well. The following day, cells were processed for ELISA. For the ELISA experiment without FLAG-HuR overexpression, only the second plasmid transfection was performed.

#### **4.2 Immunofluorescence**

Cells were washed in the well with PBS prior to fixing with 1 mL 3.7% formaldehyde in PBS for 15 min. on a shaker. After washing with PBS two times, cells were permeabilized, blocked, and stained overnight at 4°C on a shaker in 0.3% Triton X-100, 0.5% BSA, and

1:1000 mouse anti-HuR antibody in PBS. The following day, cells were washed with 0.2% Triton X-100 in PBS three times for a total of 15 min., then stained with 1:2000 Alexa Fluor® 488-conjugated anti-mouse antibody in 0.2% Triton X-100 in PBS for 1 h on a shaker. After washing three times with 0.2% Triton X-100 in PBS for a total of 15 min., samples were stained with 1 µg/mL Hoechst 33342 in PBS for 10 min. on a shaker. After washing three times in PBS for a total of 15 min., samples were imaged using an IX51® inverted microscope (Olympus) using an X-Cite® 120Q (Lumen Dynamics) as an excitation light source.

### **4.3 Western blot**

Cells were washed in the well with PBS prior to the addition of 50 µL RIPA lysis buffer (50 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% SDS, 5 g/L deoxycholate; 1 mM PMSF and 10 µg/mL leupeptin were added fresh before use) and subsequently incubated for 15 min. on a shaker. The wells were scraped to detach any remaining cells and the lysate was collected and centrifuged at 13000 RPM in a tabletop centrifuge for 15 min. at 4°C. Protein was quantified in technical duplicate in a 96-well plate by DC™ protein assay (Bio-Rad Laboratories) according to the manufacturer's instructions and stored at -20°C.

Samples containing equal amounts of protein were prepared with Laemmli buffer (Bio-Rad Laboratories) supplemented with 5% β-mercaptoethanol, denatured by boiling for 2 min., and loaded onto a 10% SDS-polyacrylamide gel (0.375 M Tris, 0.1% SDS, 10% acrylamide [diluted from 30% 37.5: 1 acrylamide/bis solution, Bio-Rad Laboratories]). The resolved proteins were transferred onto a 0.45 µm PVDF membrane (transfer buffer: 25 mM Tris, 192 mM glycine, 15% methanol) using the XCell SureLock™ Mini-Cell transfer apparatus (Life Technologies), transferring for 1.5 h at ~30 V. The membrane was blocked with

Odyssey® blocking buffer (LI-COR Biosciences) for 20 min. on a shaker before being incubated with primary antibody in Odyssey® blocking buffer overnight at 4°C on a shaker (for a list of antibodies used, please see the appendix). Membranes were washed three times with 0.1% PBST for 15 min. total and then incubated with secondary antibody in Odyssey® blocking buffer for 1 h on a shaker. After washing again with 0.1% PBST for 15 min., membranes were imaged using the Odyssey® imaging system (LI-COR Biosciences).

#### **4.4 mRNA half-life measurement**

Actinomycin D (Sigma-Aldrich), dissolved in anhydrous ethanol, was added to each well at various times to a final concentration of 5 µg/mL. After the chase period, cells were processed for qRT-PCR.

Curve-fitting was performed using Microsoft Excel 2010 by fitting an exponential function,  $y = y_0 * e^{-kx}$ , to the data without forcing a specific y-intercept. The half-life was calculated as  $t_{1/2} = \frac{\ln 2}{k}$ .

#### **4.5 Quantitative reverse transcription PCR**

Cells were lysed by the addition of 300 µL of RNAzol® RT (Molecular Research Centre) followed by a 5 min. incubation on a shaker. The lysate was collected and 120 µL of RNase-free water was added. After mixing by inversion for 10 s, the mixture was allowed to stand for 15 min. The mixture was then centrifuged at 13000 RPM in a tabletop centrifuge for 15 min. at 4°C.

For C2C12 samples, the supernatant was collected and mixed with 600 µL acidic phenol:chloroform (Life Technologies) and vortexed for 30 s. The mixture was centrifuged at 13000 RPM in a tabletop centrifuge for 5 min. at 4°C. The aqueous phase was pipetted to

a new tube, 700  $\mu$ L of chloroform was added, the mixture was vortexed for 30 s, and then centrifuged at 13000 RPM in a tabletop centrifuge for 5 min. at 4°C. The aqueous phase was again pipetted to a new tube and RNA precipitation was performed overnight at -20°C by adding 1 mL of anhydrous ethanol. The following day, the mixture was centrifuged at 13000 RPM in a tabletop centrifuge for 30 min. at 4°C.

For U2OS samples, the supernatant was collected and mixed with an equal volume of isopropanol, mixed by inversion for 10 s, and allowed to stand for 10 min.

For both cell types, the supernatant was then discarded, paying attention not to disturb the RNA pellet, and the pellet was washed twice with 500  $\mu$ L of RNase-free 75% ethanol. After the final wash and removal of any remaining ethanol, the pellet was allowed to dry before being resuspended in 20  $\mu$ L of RNase-free water. RNA was quantified by NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and stored at -20°C.

Samples containing equal amounts of RNA were prepared in a total of 8  $\mu$ L each (with RNase-free water). 2  $\mu$ L of qScript<sup>TM</sup> cDNA SuperMix (Quanta BioSciences) was added to each sample, the samples were briefly vortexed, spun down, and RT-PCRed (5 min. at 25°C, 30 min. at 42°C, 5 min. at 85°C). For each transcript of interest, three qPCR reactions (1  $\mu$ L cDNA, 1  $\mu$ L each of 10 mM forward and reverse primer, 7  $\mu$ L RNase-free water, 10  $\mu$ L of SYBR® Green Master Mix [Qiagen] per well) were set up in twin.tec 96-well plates/Masterclear<sup>TM</sup> strips (Eppendorf) using primers specific to that gene (for a list of qPCR primers used, please see the appendix). qPCR was performed (2 min. at 95°C, [10 s at 95°C, 15 s at 56°C] x40) using a Mastercycler ep realplex qPCR machine (Eppendorf) and analyzed using the associated software.

#### **4.6 $\beta$ -Gal assay/CAT ELISA**

For each treatment condition, three wells were prepared (technical triplicate). Cells were washed in the well with PBS prior to the addition of 300  $\mu$ L of Lysis buffer (from the CAT ELISA kit, Roche Applied Science supplemented with 1 mM PMSF and 10  $\mu$ g/mL leupeptin) and subsequent incubation for 15 min. on a shaker. The wells were scraped to detach any remaining cells and the lysate was collected and centrifuged at 13000 RPM in a tabletop centrifuge for 15 min. at 4°C. Lysate was stored at -20°C.

CAT quantification was performed using the CAT ELISA kit according to the manufacturer's instructions with the following modifications: the cell lysate was diluted 1:20 (10  $\mu$ L lysate in 190  $\mu$ L Lysis buffer) and washes were only done three times with 200  $\mu$ L of Wash buffer each. The substrate enhancer was not used.

$\beta$ -Gal activity was, by necessity, quantified in a 96-well plate on the same day lysates were prepared. For each sample, 190  $\mu$ L of Z-buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM Mg<sub>2</sub>SO<sub>4</sub>, 0.35%  $\beta$ -mercaptoethanol, pH 7.0) and 40  $\mu$ L of ONPG solution (68.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 31.6 mM NaH<sub>2</sub>PO<sub>4</sub>, 4 mg/mL ONPG, pH 7.4) were added to a well. Ten  $\mu$ L of cell lysate was added and incubated until the colour of the most intense well appeared fairly yellow. 100  $\mu$ L of 1 M Na<sub>2</sub>CO<sub>3</sub> was then added to each well to stop the reaction. Absorbance was read at 420 nm, aiming for values between 0.1 and 1 for all wells.

For Western blot, the lysate was quantified and processed as described above.

#### **4.7 *In vitro* RNA synthesis**

PCR was performed (95°C for 2 min., [95°C for 20 s, 56°C for 20 s, 70°C for 20 s] x40) using KOD Hot Start polymerase (EMD Millipore) according to the manufacturer's

instructions (with the following modifications: 50 ng of plasmid template, 3  $\mu$ L 25 mM  $\text{MgSO}_4$ , 1.5  $\mu$ L each of 10 mM forward and reverse primer) to amplify DNA segments corresponding to the desired RNA probes from the appropriate CAT reporter plasmids using primers that add a T7 transcription start site to the 5' end (for a list of PCR primers used, please see the appendix). These templates were run on a 1% agarose TAE gel and purified using the UltraClean® 15 DNA purification kit (MO-BIO) according to the manufacturer's instructions.

Synthesis of  $^{32}\text{P}$ -radiolabelled probes from the templates was achieved using the MAXIscript® T7 kit (Life Technologies) according to the manufacturer's instructions with the following modifications: 500 ng of template and 2  $\mu$ L of 3000 Ci/mmol  $\alpha$ - $^{32}\text{P}$  UTP (PerkinElmer) were used and incubation was performed at 37°C for 1 h. The TURBO DNase step was included.

#### **4.8 UV crosslinking of protein-RNA complexes**

*In vitro*-synthesized,  $^{32}\text{P}$ -radiolabeled transcripts from section 4.7 were gel-purified according to the instructions included in the MAXIscript® T7 kit using a denaturing urea gel (5% acrylamide [diluted from 40% 19:1 acrylamide/bis solution, Bio-Rad Laboratories] and 8 M urea in TBE) that was pre-run for 15 min. The gel was exposed to film to identify and excise the full-length probes, which were subsequently cut into small pieces in 300  $\mu$ L of RNase-free water and incubated at 37°C overnight to elute the radiolabeled probes. The following day, eluted probes were separated from the gel pulp by applying the mixture to a pre-filter column (Omega Bio-Tek) and centrifuging for 5 min. Probe quantification was performed by scintillation counter and the probes were subsequently stored at -20°C.

GST and GST-tagged HuR were purified from *E. coli* lysate using Glutathione Sepharose 4B beads (GE Healthcare) and quantified using a BSA standard curve by SDS-PAGE and Coomassie staining. Purified protein was stored at -20°C.

50000 cpm of probe was incubated with recombinant protein and 1 µg of tRNA competitor in a final volume of 20 µL in RNA binding buffer (10 mM Tris-HCl, 1.5 mM MgCl<sub>2</sub>, 150 mM KCl, 0.5 mM DTT, 0.1 mM PMSF, 10 µg/mL leupeptin in RNase-free water) for 30 min. before crosslinking (250 mJ/µm) in a 96-well plate on ice using the FB-UVXL-1000 UV crosslinker (Fisher Scientific). The mixture was then incubated with 2 µL RNase T1/A solution (1 U/µL T1, 10 µg/mL A) for 10 min. followed by 2 µL of 5 mg/mL heparin for 10 min. Samples were then run on a 10% SDS-polyacrylamide gel. The gel was Coomassie stained and then dried on a gel drier for 2 h before being exposed to film overnight at -80°C.

#### **4.9 *In vitro* RNA decay assay**

*In vitro*-synthesized, <sup>32</sup>P-radiolabeled transcripts from section 4.7 were purified using illustra MicroSpin G-50 columns (GE Healthcare) according to the manufacturer's instructions, quantified as above, and stored at -20°C.

To prepare S10 lysate, cells from a 6-well plate (see Cell Culture and Transfection) were washed in the well with PBS prior to being resuspended in PBS by scraping. Cells were pelleted by centrifugation at 500Xg for 5 min. at 4°C and washed twice in PBS before being resuspended in an equal volume of homogenization buffer (10 mM Tris, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl; 1 mM PMSF, 10 µg/mL leupeptin, and 0.5 mM DTT were added fresh before use) and left on ice for 30 min. The suspension was passed through a very fine needle approximately 30 times on ice to lyse the cells. The lysate was centrifuged at 2000 RPM for

10 min. at 4°C. The supernatant was retained and centrifuged again at 10000 RPM for 15 min. at 4°C. Glycerol was added to the supernatant (S10) to a final concentration of 5% V/V. S10 lysate was quantified by DC<sup>TM</sup> protein assay and stored at -80°C.

For every time-course, a reaction mixture was prepared (for each time-point, 10000 cpm of probe, 0.4 µg/µL S10 lysate, 1 mM ATP, 0.1 mM spermadine, 2 mM DTT, 1 U/µL RNasin® Plus RNase Inhibitor [Promega] in a total volume of 20 µL). The reaction was incubated at 37°C. At the specified times, 20 µL of the reaction was removed, mixed with 40 µL of urea loading buffer (10 M urea, 5 mM Tris-borate, 0.1 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue), boiled for 5 min. to stop RNA decay in that aliquot, and placed on ice. At the end of the time-course, samples were run on a denaturing urea gel. The gel was then dried on a gel drier for 2 h before being exposed to a phosphor screen, which was imaged using the Typhoon 9210 phosphorimager (GE Healthcare). Full-length probe was quantified using ImageQuant TL (comes with the Typhoon system).

#### **4.10 Statistical analysis**

Two-tailed Student's t-tests were performed to determine the statistical significance of results using a significance threshold of p-value < 0.05. All values represent the mean of at least three biological replicates ± SEM (standard error of the mean) unless otherwise indicated.

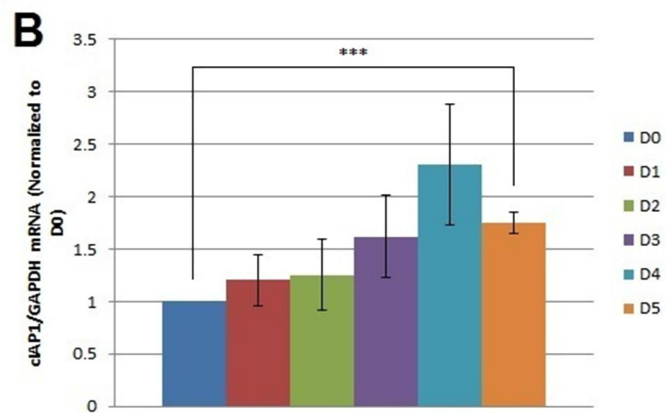
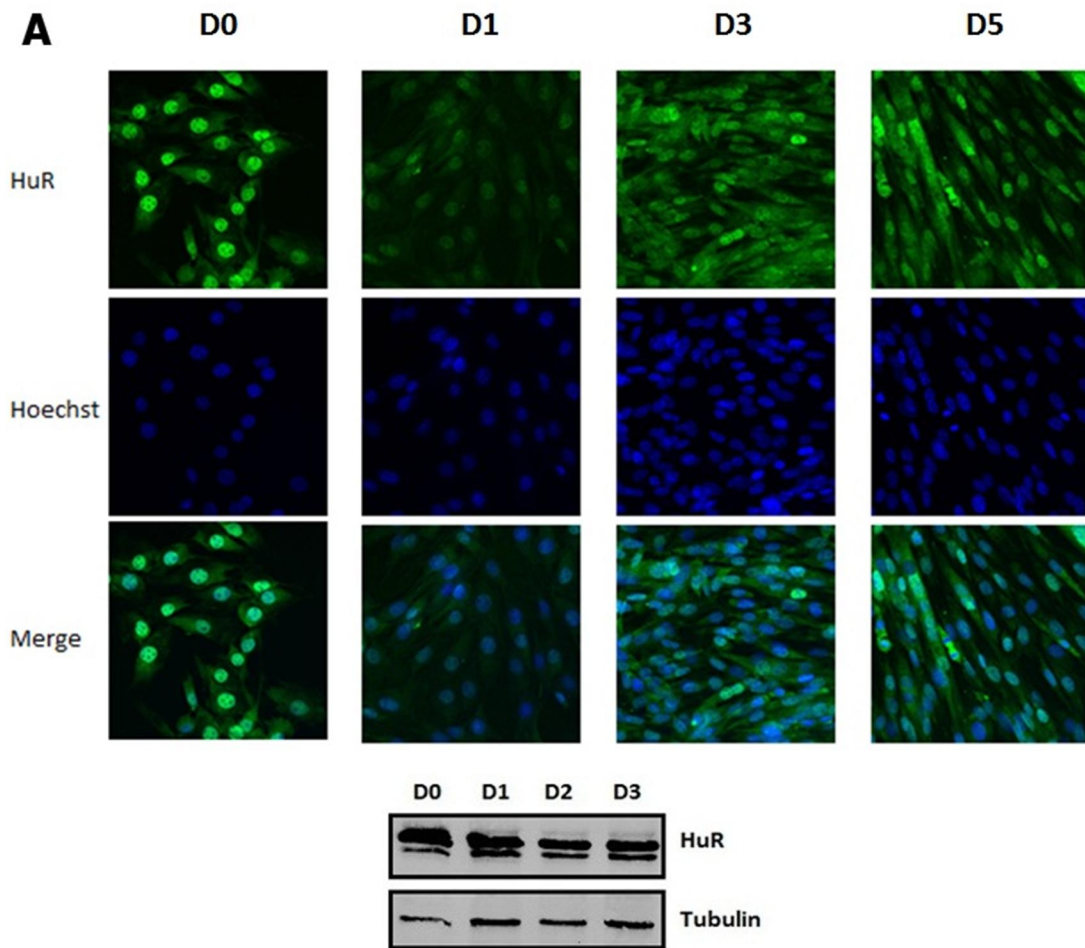
## 5. Results

### 5.1 Correlation between HuR cytoplasmic localization and increased cIAP1 mRNA levels during muscle differentiation

Our preliminary data identified HuR as interacting with the ARE-containing portion of the cIAP1 3' UTR (Fig. 3), suggesting a role for HuR in the regulation of the cIAP1 transcript. Furthermore, previous studies have shown that HuR accumulates in the cytoplasm during myoblast differentiation (Figueroa et al., 2003). As cytoplasmic HuR is known to regulate the levels of ARE-containing transcripts, we wished to determine whether levels of cIAP1 mRNA, which contains four AREs in its 3' UTR, were altered over the course of myogenesis, which one would expect if cIAP1 mRNA was regulated by cytoplasmic HuR. To do so, we chose to utilize the C2C12 immortalized mouse myoblast line. Myogenesis was induced by changing the media the cells were grown in to 2% horse serum differentiation media. Immunofluorescence, RNA, and protein samples were taken daily for immunofluorescence, qRT-PCR (Quantitative Reverse Transcription Polymerase Chain Reaction), and Western blotting, respectively, over the five day differentiation time-course.

Immunofluorescent staining was performed to verify HuR cytoplasmic relocation of HuR during differentiation. Samples were stained with mouse primary anti-HuR antibody and Alexa Fluor® 488-conjugated secondary anti-mouse antibody to visualize HuR and Hoechst stain to visualize the nuclei. As can be seen in the images in Fig. 4A (top panel), HuR gradually relocates from the nucleus to the cytoplasm over the course of differentiation, characterized by the elongation and fusing of myoblasts into myotubes. Total HuR protein levels were not affected, as shown by Western blot (Fig. 4A, bottom panel).

qRT-PCR of the extracted RNA was performed to determine the levels of cIAP1 mRNA throughout differentiation. Total RNA was extracted using RNAzol and acidic phenol:chloroform and reverse transcribed using random hexamer primers. The cDNA product was probed by qPCR for cIAP1 and GAPDH, each in technical triplicate. The Ct values, a logarithmic representation of the quantity of RNA, obtained from the qPCR reactions were converted to the linear scale using prepared standard curves prior to further analysis. We used the average of the cIAP1 and GAPDH values obtained from the technical triplicate. As shown in Fig. 4B, cIAP1 mRNA levels, measured as a ratio of average cIAP1/average GAPDH mRNA and normalized to the day 0 (D0) sample, increases over the course of differentiation ( $p < 0.0001$  between D0 and D5), demonstrating a positive correlation between HuR cytoplasmic accumulation and cIAP1 transcript levels in differentiating C2C12 cells, as expected. The cIAP1/GAPDH mRNA ratio was used to correct for any loading differences between samples. This result is suggestive of cIAP1 mRNA regulation by cytoplasmic HuR.



**Figure 4. cIAP1 mRNA levels increase concomitant to HuR relocalization to the cytoplasm during C2C12 differentiation. (A)** Immunofluorescence was performed on C2C12 samples, showing HuR relocalization to the cytoplasm during differentiation in 2% horse serum. Cells were fixed with 3.7% formaldehyde. Permeabilization, blocking, and staining with primary antibody were performed concurrently with 0.3% Triton X-100, 0.5% BSA, and 1:1000 anti-HuR antibody in PBS. Secondary antibody and Hoechst stain were applied at 1:2000 and 1:10000 dilution, respectively. Samples were imaged using a fluorescence microscope. The bottom panel is a representative Western blot indicating that total HuR levels remain unchanged throughout differentiation. **(B)** Steady-state cIAP1 mRNA levels, measured as a ratio of cIAP1/GAPDH mRNA, increase over the course of C2C12 differentiation in 2% horse serum ( $p < 0.0001$  between D0 and D5), as determined by qRT-PCR. All time-points are normalized to the day 0 (D0) sample, the day the media was changed to differentiation media. The values represent the mean of five (5) independent experiments while the error bars represent SEM (standard error of the mean). (\*\*\*)  $p < 0.001$ )

## 5.2 HuR knockdown reduces steady-state cIAP1 mRNA levels

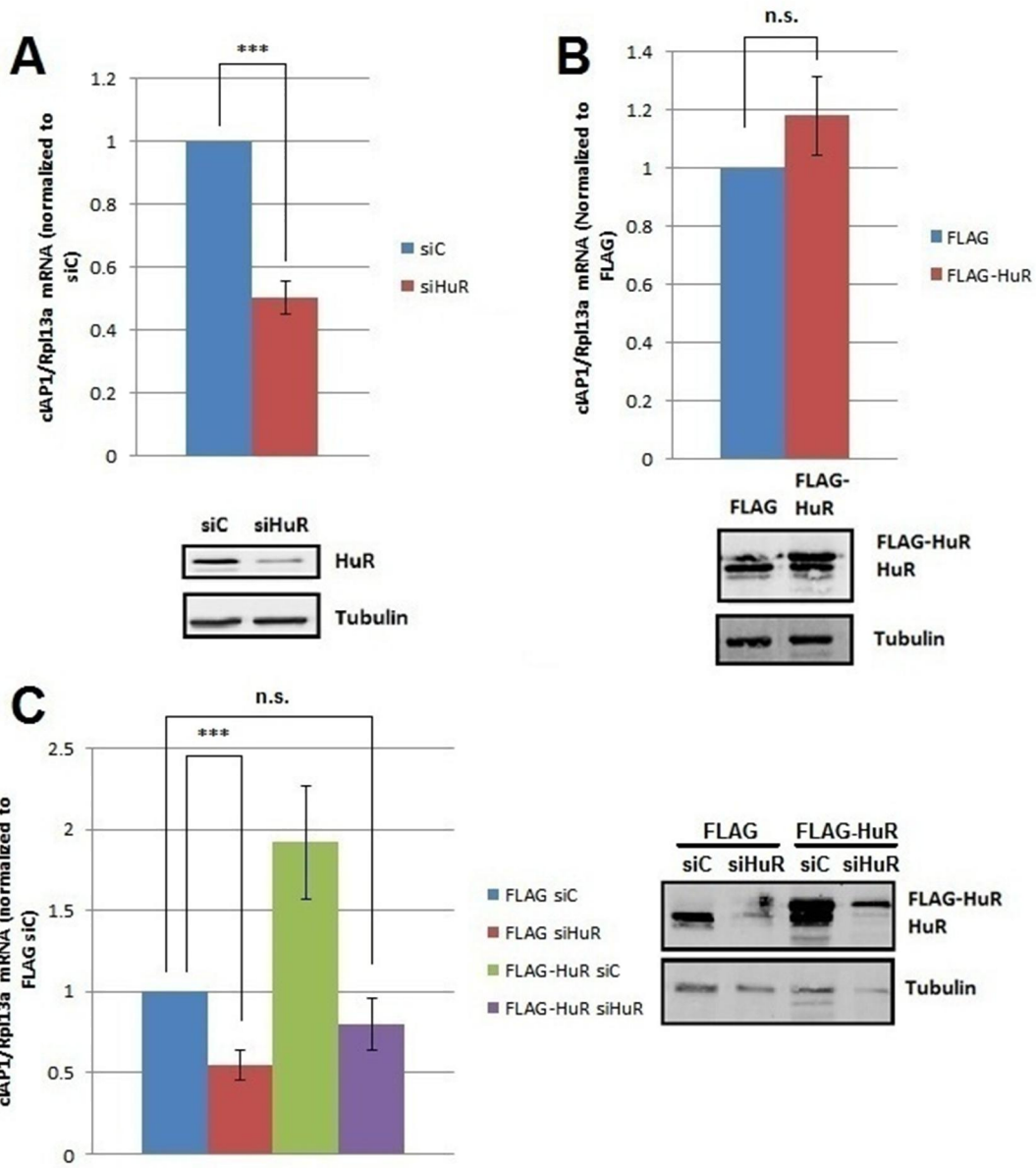
To examine HuR's effect on cIAP1 mRNA in more detail, we chose to work with the U2OS immortalized human osteosarcoma cell line for the remaining cell-based experiments. This was done both to demonstrate the generality of HuR's effect on cIAP1 transcript levels and because U2OS cells were easier to work with due to their high RNA and protein yield.

First, we wished to determine the effect of HuR knockdown on steady-state cIAP1 mRNA levels. Based on our observation that HuR cytoplasmic accumulation correlated with increased cIAP1 mRNA levels, we expected a decrease in cIAP1 mRNA levels in cells depleted of HuR. After a 72 h knockdown of HuR using a custom siRNA targeting the coding region of HuR (siHuR), samples were prepared for qRT-PCR and Western blotting. We used a non-targeting siRNA purchased from Qiagen (siC) as a transfection control. The cDNA product was probed by qPCR for cIAP1 and Rpl13a, each in technical triplicate. As before, the Ct values were converted to the linear scale before further analysis. We then used the average of the cIAP1 and Rpl13a values obtained from the technical triplicate. HuR knockdown for 72 h resulted in a marked decrease in total HuR levels relative to the siC treatment, as shown by Western blot (Fig. 5A, bottom panel). This decline in HuR levels was accompanied by a two-fold reduction in steady-state cIAP1 mRNA levels ( $p < 0.0001$ ), as measured by the average cIAP1/average Rpl13a mRNA ratio (Fig. 5A, top panel). This suggests that HuR positively regulates levels of cIAP1 transcript, possibly by stabilizing it.

We then asked whether overexpression of FLAG-HuR would have the opposite effect on cIAP1 transcript levels as HuR knockdown. To answer this, we overexpressed FLAG-HuR via transient transfection for 24 h before preparing samples for qRT-PCR and Western blotting. To control for transfection-related effects, we utilized the same plasmid

backbone but with only the FLAG tag present. Overexpression of FLAG-HuR for 24 h, however, did not appear to affect cIAP1 transcript levels (Fig. 5B, top panel,  $p = 0.2543$ ). Western blot analysis was used to validate overexpression of FLAG-HuR (Fig. 5B, bottom panel).

Finally, we wondered whether the observed effect of HuR knockdown on cIAP1 transcript level was a result of the downregulation of HuR or of another, non-specific target. We performed a genetic rescue by knocking down HuR for 72 h but reintroducing FLAG-HuR via transient transfection of the FLAG-HuR plasmid 24 h into the knockdown before collecting samples for qRT-PCR and Western blot. If the effect of HuR knockdown on cIAP1 transcript levels is mediated through HuR, we expected that reintroduction of recombinant HuR would abrogate that effect. To control for transfection-related effects, we used siC and the FLAG plasmid. In the HuR knockdown sample transfected with the FLAG control, cIAP1 mRNA levels were significantly reduced ( $\sim 50\%$ ,  $p = 0.0003$ ) compared to the siC/FLAG control (Fig. 5C, left panel), as expected. When FLAG-HuR was reintroduced into the HuR knockdown cells, however, the reduction in cIAP1 mRNA levels was blunted ( $\sim 20\%$ ) and was no longer significant ( $p = 0.2397$ ) compared to the siC/FLAG control. This partial genetic rescue suggests that the downregulation of cIAP1 transcript observed upon HuR knockdown is HuR-specific. Interestingly, cIAP1 transcript levels in the siC/FLAG-HuR sample were significantly elevated relative to the siC/FLAG control ( $p = 0.0224$ ). Western blot analysis was used to validate knockdown and overexpression of endogenous HuR and FLAG-HuR, respectively (Fig. 5C, right panel).



**Figure 5. HuR knockdown reduces steady-state cIAP1 mRNA levels in U2OS cells. (A)**

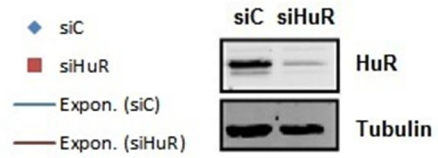
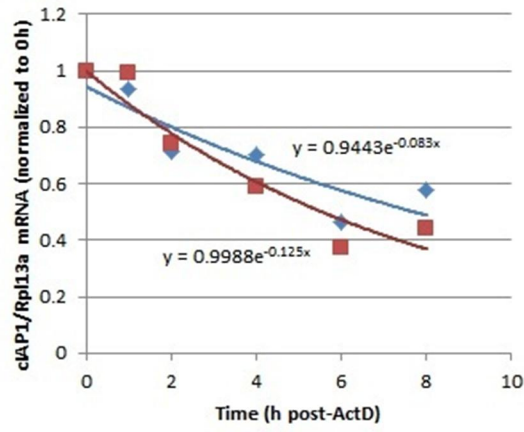
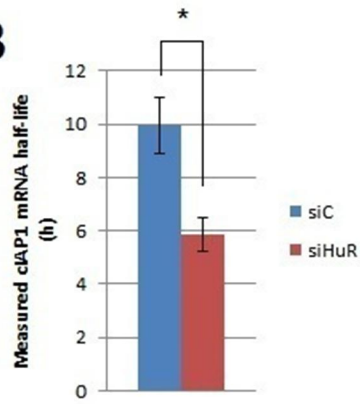
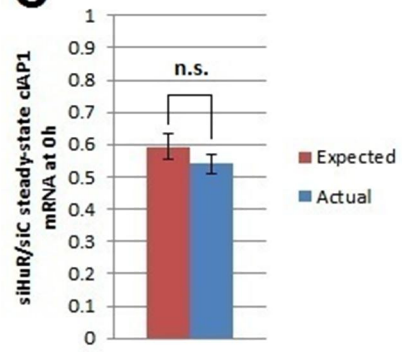
Steady-state cIAP1 mRNA levels, measured as a ratio of cIAP1/Rpl13a mRNA, decrease by approximately two-fold (\*\*  $p < 0.0001$ ) when HuR is knocked down, as determined by qRT-PCR. Knockdown was performed by transfection of a siRNA targeting the coding region of HuR (siHuR) to a final concentration of 100 nM using RNAiMax the day after seeding cells. A non-targeting siRNA purchased from Qiagen (siC) was used as a transfection control. All treatments are normalized to the control siRNA sample. The values represent the mean of eight (8) independent experiments while the error bars represent SEM. The bottom panel is a representative Western blot indicating that total HuR levels were decreased by siHuR treatment. **(B)** Steady-state cIAP1 mRNA levels, as measured by the ratio of cIAP1/Rpl13a mRNA, are not affected by overexpression of FLAG-HuR ( $p = 0.2543$ ), as determined by qRT-PCR. Overexpression was performed using forward transfection of a plasmid containing FLAG-HuR to a final concentration of 0.75  $\mu\text{g}/\text{mL}$  using jetPrime. The same plasmid backbone containing only the FLAG tag was used as a transfection control. All treatments are normalized to the FLAG plasmid sample. The values represent the mean of three (3) independent experiments while the error bars represent SEM. The bottom panel is a representative Western blot indicating that FLAG-HuR was overexpressed. **(C)** Although there is a significant decrease in steady-state cIAP1 transcript level ( $p = 0.0003$ ), measured as a ratio of cIAP1/Rpl13a mRNA, when HuR knockdown cells were transfected with FLAG control, this reduction is diminished and was no longer statistically significant ( $p = 0.2397$ ) relative to the siC/FLAG control when FLAG-HuR is reintroduced, as determined by qRT-PCR. The genetic rescue was performed using forward transfection of siHuR to a final concentration of 100 nM using RNAiMax followed by forward transfection of FLAG-HuR to a final concentration of 0.75  $\mu\text{g}/\text{mL}$  using jetPrime. siC and FLAG were used as transfection controls. All treatments are normalized to the siC- and FLAG-transfected sample. The values represent the mean of seven (7) independent experiments while the error bars represent SEM. The right panel is a representative Western blot indicating that total endogenous HuR levels were decreased by siHuR treatment and that FLAG-HuR was overexpressed. (\*\*\*)  $p < 0.001$ , not significant [n.s.]  $p > 0.05$ )

### **5.3 HuR knockdown destabilizes cIAP1 mRNA**

Up to now, our measurements were of steady-state cIAP1 mRNA levels; however, changes in steady-state levels can arise from changes in the production rate, the decay rate, or both. As HuR is generally known for its stabilizing effect on ARE-containing transcripts, we wished to determine whether the decrease in cIAP1 mRNA steady-state levels observed when HuR is knocked down is due to a decrease in transcript stability. To test this, we decided to measure the stability of the cIAP1 transcript directly using ActD (actinomycin D), an inhibitor of RNA polymerase II. By inhibiting transcription and quantifying the amount of residual cIAP1 transcript as a function of time after ActD treatment, we could determine the rate of degradation absent the confounding effect of mRNA production.

As HuR knockdown resulted in a decrease of steady-state cIAP1 mRNA levels, assuming this effect was mediated by destabilization of the transcript, we expected to observe an increase in the rate of degradation of the cIAP1 mRNA (equivalently, a reduction in its half-life). U2OS cells were treated with siHuR for 72 h to knock down HuR before ActD treatment (one extra well was treated to validate the knockdown by Western blot). siC was used to control for transfection-related effects. After HuR knockdown, the cells were treated with ActD at a final concentration of 5  $\mu\text{g}/\text{mL}$ , shown to inhibit RNA polymerase II, and left to continue growing. At various times after the addition of ActD, samples were prepared for qRT-PCR as described above. Total RNA was extracted using RNeasy and reverse transcribed. The cDNA product was probed by qPCR for cIAP1 and Rpl13a, each in technical triplicate. As before, the Ct values were converted to the linear scale before further analysis. We then used the average of the cIAP1 and Rpl13a values obtained from the technical triplicate.

A representative time-course of an ActD chase is shown in Fig. 6A (left panel). cIAP1 mRNA levels, as measured by the average cIAP1/average Rpl13a ratio and normalized to the 0 h sample, were plotted against the length of time that those samples were grown in ActD. It was assumed that cIAP1 transcript decay follows first-order kinetics (the rate of degradation of a transcript is a constant proportion of the amount of transcript remaining). As such, cIAP1 mRNA levels as a function of time should fit an exponential decay function,  $y = y_0 * e^{-kx}$ , where  $y_0$  is the initial amount of transcript at time 0 and  $k$ , the decay constant, is what we are interested in. As shown in Fig. 6A, we fitted our data to such a function to obtain  $k$  values. The half-life, the time required for a species to decay to 50% of its initial amount, was determined by  $t_{1/2} = \frac{\ln 2}{k}$ . Pooling the calculated half-lives for the various treatment conditions across independent experiments, we observe that when HuR is knocked down, the half-life of cIAP1 mRNA is reduced by approximately 40% (Fig. 6B,  $p = 0.0299$ ). Finally, comparing the decrease in cIAP1 transcript levels we observed in the HuR knockdown samples at the 0 h time-point to the decrease we would expect to see given the measured half-lives, assuming that the rate of production of the transcript remained constant, showed that what we observed was very similar to what we would expect to see (Fig. 6C,  $p = 0.3551$ ). This is consistent with our hypothesis and suggests that the primary cause of reduced steady-state cIAP1 mRNA levels after HuR knockdown is transcript destabilization. Western blot analysis was used to validate knockdown of total HuR (Fig. 6A, right panel).

**A****B****C**

**Figure 6. HuR knockdown reduces cIAP1 mRNA half-life in U2OS cells. (A)** Representative time-course of an ActD (actinomycin D) chase showing the accelerated decay of cIAP1 mRNA, measured as a ratio of cIAP1/Rpl13a mRNA, when HuR is knocked down, as determined by qRT-PCR. Knockdown was performed by forward transfection of siHuR to a final concentration of 100 nM using RNAiMax. siC was used as a transfection control. All time-points are normalized to the 0 h sample, the time ActD was added to the media (to a final concentration of 5 µg/mL), of their respective treatment. The right panel is a representative Western blot indicating that total HuR levels were decreased by siHuR treatment. **(B)** The half-life of cIAP1 mRNA ( $t_{1/2} = \frac{\ln 2}{k}$ ), as determined by fitting exponential decay functions ( $y = y_0 * e^{-kx}$ ) to the time-courses, is reduced by approximately 40% when HuR is knocked down ( $p = 0.0299$ ). The values represent the mean of three (3) independent experiments while the error bars represent SEM. **(C)** Expected vs. observed decrease in steady-state cIAP1 mRNA levels, measured as a ratio of siHuR/siC cIAP1 transcript levels, suggests that mRNA stability is the primary contributing factor affecting cIAP1 mRNA levels when HuR is knocked down ( $p = 0.3551$ ). The expected steady-state cIAP1 mRNA levels were calculated using the measured half-lives, assuming first-order decay kinetics and no change in the rate of production of cIAP1 mRNA, while the 0 h samples were used to calculate the observed decrease. The values represent the mean of three (3) independent experiments while the error bars represent SEM. (\*  $p < 0.05$ )

#### **5.4 HuR binds specifically to the second ARE *in vitro***

Next, we wished to determine the specific cis-regulatory element(s) responsible for the observed effect of HuR protein on the cIAP1 transcript. Our first step was to determine whether HuR could bind the cIAP1 mRNA directly, and if so, the location of binding. Our preliminary data showed that HuR interacts with the ARE-containing portion of the cIAP1 3' UTR (H2B) using RNA affinity chromatography (Fig. 3). Although it remained unknown whether this interaction was direct or not, we nevertheless used this segment as a starting point. Since HuR is known to associate with AREs (Ma et al., 1997), we decided to examine whether HuR interacts with one or more of the four AREs present in the cIAP1 3' UTR.

To this end, we used two segments of the cIAP1 3' UTR, as depicted in Fig. 7A. The H2B fragment represents the second quarter of the cIAP1 3' UTR and contains all four of the AREs and so was expected to interact with HuR. The various mutants represent disrupting mutations made to one or more of the AREs in the context of the H2B fragment (for a list of ARE mutations, please see the appendix). If HuR binds to any of the AREs, we would expect mutation of those AREs to disrupt binding. As H2A and the H2B mutant with all four AREs mutated (mut. 1234) both do not contain AREs, we did not expect HuR to bind to either fragment.

PCR was performed on plasmids containing the wild-type or various mutants of the cIAP1 3' UTR using primers to specifically amplify the H2A and H2B fragments. The forward primer in these pairs contained a T7 transcriptional promoter at the 5' end, allowing us to use these short DNA amplicons as the templates for *in vitro* transcription reactions using <sup>32</sup>P-radiolabeled UTP. The resulting <sup>32</sup>P-radiolabeled RNA probes were incubated with increasing amounts of bacterially-derived GST-HuR and UV crosslinked, covalently

binding the interacting portion of the ribonucleoprotein complex. Bacterially-derived GST was used to demonstrate the binding specificity of the HuR portion of GST-HuR. Subsequent treatment with RNase T1/A digested non-interacting portions of the transcript, leaving only a short stretch of nucleotides protected from digestion by the bound protein. Proteins that interact with a probe are essentially radiolabeled. Binding between GST-HuR and a radiolabeled probe was thus detected by separating the reaction on a SDS-polyacrylamide gel and looking for radiolabeled GST-HuR by exposing the gel to film.

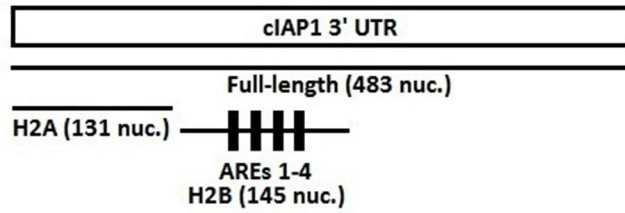
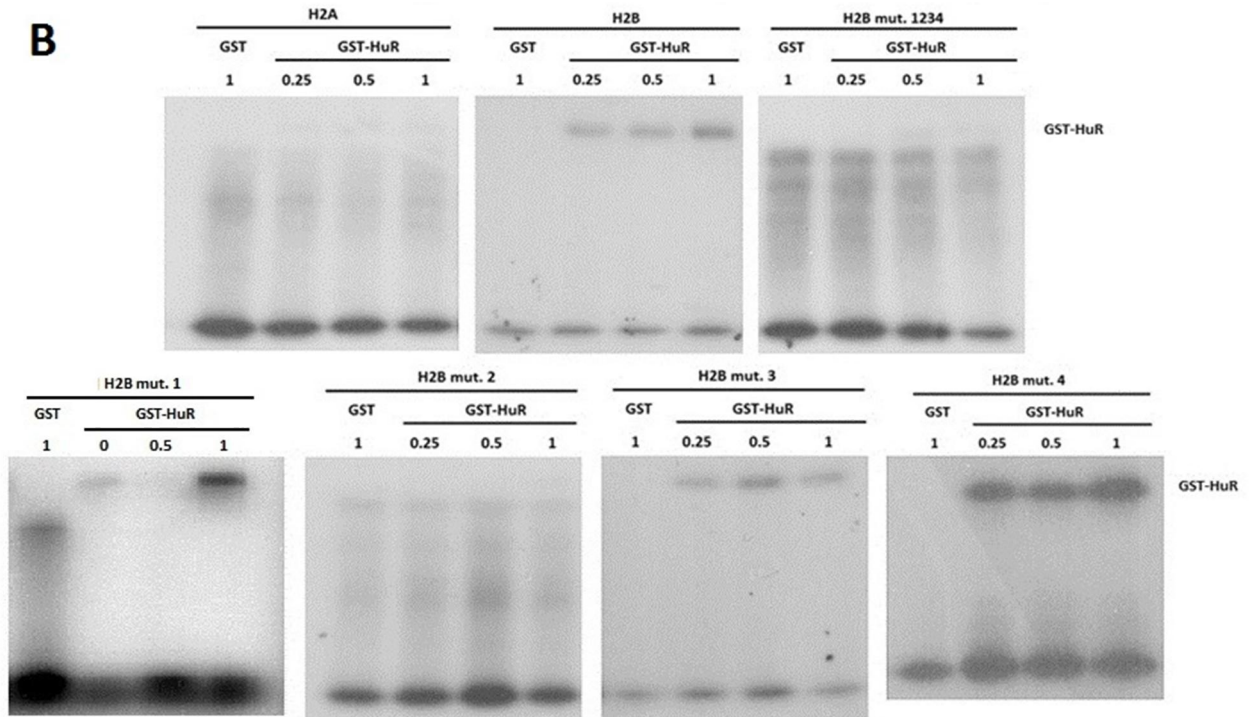
As expected, GST did not bind to any of the probes, supporting the specificity of the GST-HuR interactions (Fig. 7B). Also as expected, GST-HuR showed binding to the H2B fragment but not to the H2A and H2B mut. 1234 fragments. Of the four single ARE mutants, only mut. 2 lost the ability to bind HuR, suggesting that HuR binds to the second ARE within the cIAP1 3' UTR.

**A**

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UAAAGAAAAUAGUCUAUUAUUUUAACCGCAUAAAAAGGUCUUUAAAAUUAUGUUGAACACUUGAAGCCAUCUAAA
GUAAAAAGGGAAUUAUGAGUUUUUCAAUUAGUAACAUUCAUGUUCUAGUCUGCUUUGGUACUAAUAAUCUUGUUUC
UGAAAAGAUGGUAUCAUAUAUUUAUCUAAUCUGUUUAUUUACAAGGGAAGAUUUAUGUUUGGUGAACAUAUA
UAGUAUGUAUGUGUACCUAAGGGAGUAGUGUCACUGCUUGUUUUGCAUCAUUUCAGGAGUUACUGGAUUUGUUGUU
CUUUCAGAAAGCUUUGAAUACUAAAUUUAUGUGUAGAAAAGAACUGGAAACCAGGAACUCUGGAGUUCAUCAGAGU
UAUGGUGCCGAAUUGUCUUUGGUCUUUACUUGUGUUUAAAAUAAGGAUUUUUCUUUAUUUCUCCCCUAGU
UUGUGAGAAACAUCUCAUAAAGUGCUUU

```

**B**

**Figure 7. HuR binds specifically to the second ARE in the 3' UTR of cIAP1 mRNA *in vitro*.**

**(A)** Sequence and schematic representation of the cIAP1 3' UTR and the H2A/H2B fragments used as probes for UV crosslinking. The full-length cIAP1 3' UTR is 483 nucleotides (nt) long while the H2A and H2B fragments span from position 4 to position 134 (131 nt) and from position 118 to position 262 (145 nt), respectively. The H2B fragment contains all four AREs that are present in the cIAP1 3' UTR. The H2B mutants used in this experiment have the indicated ARE(s) disrupted. **(B)** UV crosslinking of GST-HuR to *in vitro*-synthesized, <sup>32</sup>P-radiolabelled probes shows binding to the second ARE of the cIAP1 3' UTR. UV crosslinking was performed by incubating 50000 counts per minute (cpm) of the indicated radiolabeled probe with the indicated amount of bacterially-derived GST-HuR (in µg) prior to covalently linking interacting complexes by UV irradiation and separation by SDS-PAGE. Bacterially-derived GST was used as a specificity control.

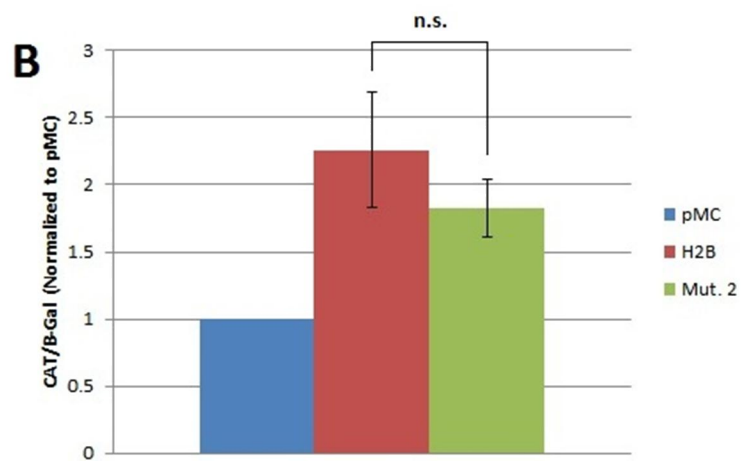
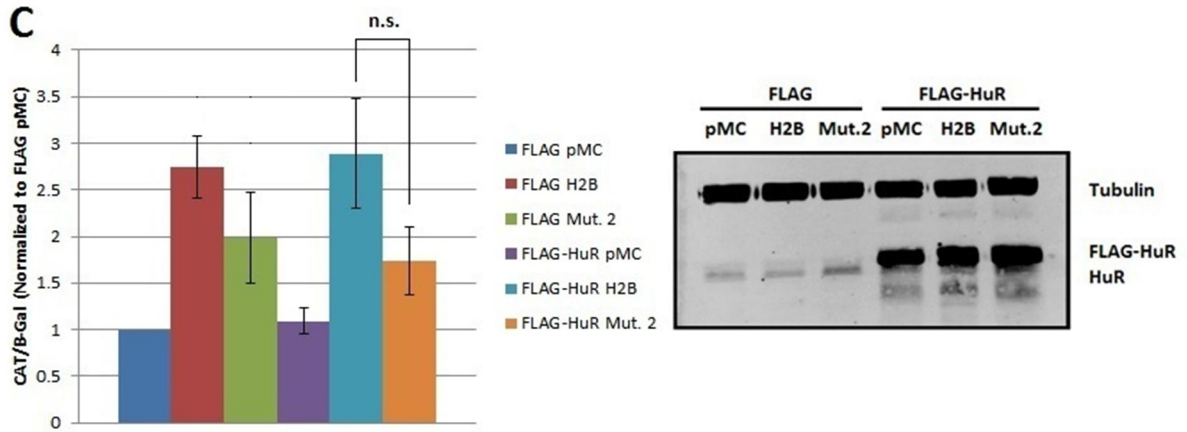
## 5.5 Mutation of the second ARE does not affect reporter protein levels

Having established that HuR protein binds to the second ARE within the cIAP1 3' UTR, we wished to determine whether this particular binding event is responsible for the regulation of cIAP1 mRNA stability by HuR. We decided to use a CAT (Chloramphenicol AcetylTransferase) reporter system to assess the role played by the second ARE of the cIAP1 3' UTR. Although not a direct measurement of transcript stability, it is assumed that any changes in transcript stability would result in changes in the steady-state levels of that transcript and, as a result, the protein it encodes.

To determine the effect of the second ARE on transcript stability, reporter constructs composed of either the H2B fragment or the mut. 2 variant appended to the CAT coding region were used (Fig. 8A). If the H2B fragment confers instability that is abrogated by HuR binding to the second ARE, one would expect the construct containing the mut. 2 fragment to be less stable due to its inability to bind HuR, and thus yield less protein product than the wild-type H2B construct. U2OS cells were cotransfected with plasmids containing the reporter constructs and a plasmid containing a CMV promoter-driven  $\beta$ -Gal ( $\beta$ -galactosidase) gene, used as a control for transfection efficiency and loading. Each treatment was performed in technical triplicate, for which the average CAT and  $\beta$ -Gal values of the three wells were used. After 24 h of expression, samples were prepared for ELISA. CAT protein levels, measured as a ratio of average CAT/average  $\beta$ -Gal protein, did not vary significantly between the H2B and mut.2 reporters (Fig. 8B,  $p = 0.3933$ ).

Following this unexpected observation we wondered if overexpressing FLAG-HuR might lead to a greater discrepancy between the behaviours of the two constructs. As HuR is, under normal conditions, primarily nuclear while its stabilizing effect is thought to take

place in the cytoplasm, it is possible that there is not enough cytoplasmic HuR to fully affect the flood of HuR-sensitive transcripts resulting from overexpression of the reporters. As such, we expected that when FLAG-HuR was overexpressed prior to performing the CAT reporter assay, that there would be an increase in H2B reporter levels but no increase in mut. 2 reporter levels relative to the FLAG-transfection controls. FLAG-HuR, or FLAG, was transfected 24 h prior to cotransfection of reporter and  $\beta$ -Gal plasmids. A plasmid containing a CAT reporter lacking any 3' UTR fragment (pMC) was also included to control for effects specific to the CAT coding region itself. 24 h after cotransfection, samples were prepared for ELISA. As expected, overexpression of FLAG-HuR, as shown by Western blot (Fig. 8C, right panel), did not alter CAT expression levels of the pMC samples relative to the FLAG control (Fig. 8C, left panel,  $p = 0.5449$ ). Unexpectedly, however, overexpression of FLAG-HuR did not result in a statistically significant change in CAT protein levels between the two reporters ( $p = 0.1474$ ). The difference between both reporters remained non-significant when FLAG was overexpressed ( $p = 0.2431$ ).

**A****C**

**Figure 8. Mutation of the second ARE does not affect the levels of CAT reporter protein in U2OS cells. (A)** Schematic of the CAT reporter construct used. The reporter construct was generated by appending the H2B fragment of the cIAP1 3' UTR, or the variant with the second ARE mutated (mut. 2), to the CAT coding region. **(B)** In the context of the H2B fragment, levels of the CAT reporter protein, as measured by CAT/ $\beta$ -Gal protein, are not affected by mutation of the second ARE ( $p = 0.3933$ ), as determined by ELISA. Expression of the reporter plasmids was performed using forward transfection of two plasmids, one containing the CAT reporter to a final concentration of  $0.188 \mu\text{g/mL}$  and the other containing  $\beta$ -Gal to a final concentration of  $0.375 \mu\text{g/mL}$ , using jetPrime. A plasmid containing a CAT reporter lacking any 3' UTR fragment (pMC) was used as a normalization reference. All values are normalized to the pMC sample. The values represent the mean of five (5) independent experiments while the error bars represent SEM. **(C)** The difference between the H2B and mut. 2 reporter proteins, as measured by CAT/ $\beta$ -Gal protein, remains non-significant after overexpression of FLAG-HuR ( $p = 0.1474$ ), as determined by ELISA. Expression of plasmids was performed using forward transfection of FLAG-HuR to a final concentration of  $0.75 \mu\text{g/mL}$  using jetPrime followed by forward transfection of both the CAT reporter to a final concentration of  $0.188 \mu\text{g/mL}$  and  $\beta$ -Gal to a final concentration of  $0.375 \mu\text{g/mL}$ , using jetPrime. FLAG and pMC were used as transfection and specificity controls, respectively. All treatments are normalized to the FLAG- and pMC-transfected sample. The values represent the mean of four (4) independent experiments while the error bars represent SEM. The right panel is a representative Western blot indicating that FLAG-HuR was overexpressed.

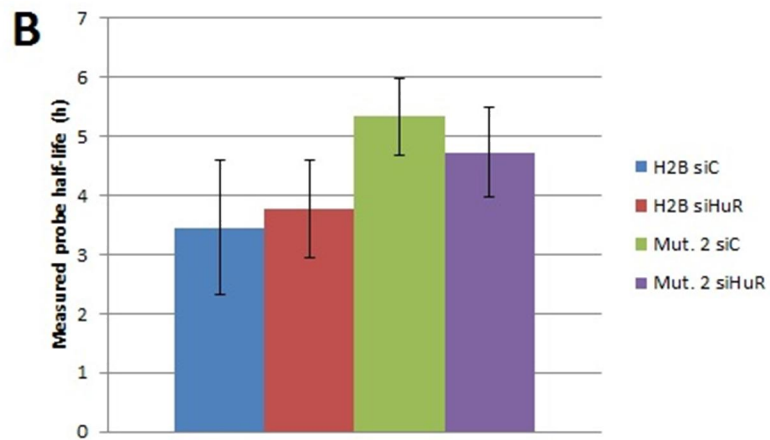
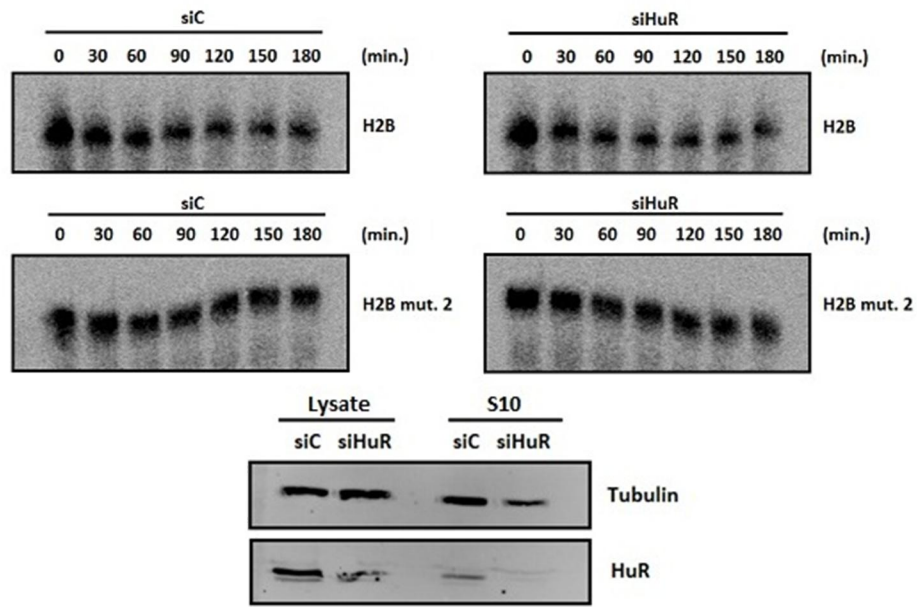
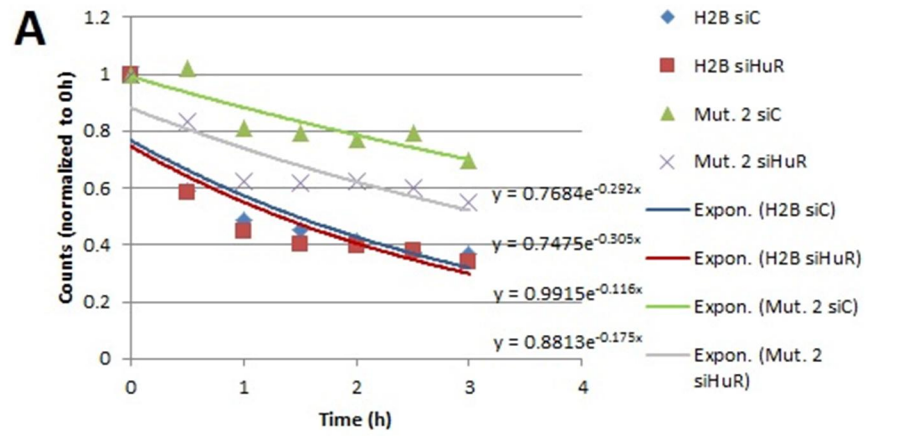
## 5.6 Neither HuR nor the second ARE affect the stability of probes *in vitro*

Having seen no difference between the expression of H2B and mut. 2 CAT reporters, we decided to directly measure the effect of the second ARE on transcript stability, eliminating confounding factors such as possible changes in translation. At the same time, we wished to probe the interaction between HuR and the second ARE, linking the effect observed with HuR knockdown to the binding of HuR to the ARE.

To accomplish this, we decided to use an *in vitro* RNA decay assay. S10 lysate contains functional proteins and is commonly used for *in vitro* translation assays. As such, we decided to adapt the protocol to determine the rate of degradation of various RNA species in S10 lysate. After a 72 h HuR knockdown, U2OS cells were carefully lysed in non-denaturing conditions and processed to obtain S10 lysate. siC was used as a transfection control. Western blot analysis was used to validate knockdown of HuR in the S10 lysate (Fig. 9A, bottom panel). *In vitro*-synthesized, <sup>32</sup>P-radiolabeled H2B and mut. 2 probes were prepared as described in the UV crosslinking experiment. Equal counts of radiolabeled probe were allowed to decay in S10 lysate for varying lengths of time before stopping the reaction with urea loading buffer. RNA in the reaction was separated by denaturing urea gel, which was subsequently exposed to a phosphor screen and imaged by phosphorimager to visualize and quantify the probes. Based on our previous observations in cells, we expected that the half-life of the H2B probe in the S10 lysate prepared from siHuR-treated cells should be lower than in S10 prepared from siC-treated cells. The half-life of the mut. 2 probe, unable to bind HuR, was not expected to be affected by HuR knockdown.

A representative time-course of an *in vitro* RNA decay assay is shown in Fig. 9A (top and middle panels). Probe quantity, as measured by counts and normalized to the 0 h

sample, was plotted against the duration of the reaction, yielding a plot similar to the one obtained in the half-life experiment (Fig. 6A, left panel). Similarly, assuming that probe decay follows first-order kinetics, we fit individual time-courses with an exponential decay function and calculated their half-lives. Pooling the calculated half-lives of various conditions across independent experiments, we observe that HuR knockdown does not alter the half-life of H2B nor that of mut. 2 relative to siC control in S10 lysate (Fig. 9B,  $p = 0.8369$  for H2B,  $p = 0.6120$  for mut. 2). Furthermore, the mutation of ARE 2 does not affect the half-life of the probe, both in siC and siHuR S10, relative to wild-type H2B probe ( $p = 0.3120$  for siC,  $p = 0.4852$  for siHuR).



**Figure 9. Neither HuR nor the second ARE affect the half-life of probes *in vitro*. (A)**

Representative time-course of an *in vitro* RNA decay assay, both quantified (top panel) and as visualized by phosphorimager (middle panels). Knockdown prior to S10 lysate preparation was performed by forward transfection of siHuR to a final concentration of 100 nM using RNAiMax. siC was used as transfection control. The decay assay was performed by incubating, for various lengths of time, 10000 cpm of *in vitro*-synthesized, <sup>32</sup>P-radiolabelled H2B or mut. 2 probe with S10 lysate (at a final concentration of 0.4 µg/µL) prepared from siC- or siHuR-treated U2OS cells. Visualization and quantification of the probes was performed using a phosphorimager. All time-points are normalized to the 0 h sample, the time probes were introduced into the S10 lysate, of their respective treatment. The bottom panel is a Western blot indicating that HuR levels in the S10 lysate were decreased by siHuR treatment. **(B)** The half-life of both probes ( $t_{1/2} = \frac{\ln 2}{k}$ ), as determined by fitting exponential decay functions ( $y = y_0 * e^{-kx}$ ) to a time-course, is unchanged in the HuR knockdown S10 relative to the siC S10 (p = 0.8369 for H2B, p = 0.6120 for mut. 2). Furthermore, mutation of the second ARE of H2B does not alter the half-life of H2B (p = 0.3120 for siC, p = 0.4852 for siHuR), regardless of the S10 used. The H2B values represent the mean of three (3) independent experiments, the mut. 2 values represent the mean of two (2) independent experiments, and the error bars represent SEM.

## 6. Discussion

### 6.1 General discussion

HuR is involved in many aspects of the mRNA life-cycle, from splicing to decay. It has been implicated in the regulation of a collection of genes, a regulon, involved in cell survival, proliferation, migration, angiogenesis, and inflammation (Simone and Keene, 2013). In particular, HuR has been shown to increase the translation of the XIAP mRNA (survival) through an IRES (Internal Ribosome Entry Site) element in the 5' UTR (Durie et al., 2011) and the transcript stability of cyclins A and B1 (Wang et al., 2000),  $\beta$ -actin (Dormoy-Raclet et al., 2007), COX-2 (Young et al., 2012), and TLR4 (Lin et al., 2006). Given its ubiquitous expression throughout the body and the oncogenic nature of its regulon, it is not surprising that elevated cytoplasmic HuR levels have been associated with many cancers (Brosens et al., 2008; Erkinheimo et al., 2003; Miyata et al., 2013). Elevated expression of cIAP1 is also associated with cancer (Che et al., 2012) as it affects survival, proliferation, and inflammation through its effect on NF- $\kappa$ B signaling (Gyrd-Hansen and Meier, 2010).

Preliminary data from our laboratory indicated that HuR interacts with the H2B fragment (Fig. 3). Given HuR's known role in regulating mRNA stability, we asked whether HuR regulates cIAP1 mRNA stability.

In this study, we began by examining the behaviour of cIAP1 mRNA levels in differentiating C2C12 mouse myoblasts. It has been shown that during myogenesis HuR accumulates in the cytoplasm and stabilizes certain ARE-containing transcripts (Figuroa et al., 2003; van der Giessen et al., 2003), making this an excellent system in which to determine whether cytoplasmic HuR affects the levels of the ARE-containing cIAP1

transcript. We show a positive correlation between cytoplasmic accumulation of HuR during myogenesis and increased expression of cIAP1 mRNA (Fig. 4), suggesting that cytoplasmic HuR does indeed regulate cIAP1 mRNA. Previous work from the Korneluk lab demonstrates that downregulation of cIAP1 in C2C12 cells attenuates myogenesis (Enwere et al., 2012), supporting our observation and providing a physiological reason for the upregulation of cIAP1 during differentiation. Interestingly, this same study found that primary mouse myoblasts exhibited the opposite response: downregulation of cIAP1 led to more pronounced muscle formation.

Next, we transiently knocked down or overexpressed HuR in U2OS cells to determine the effect of HuR on steady-state cIAP1 mRNA levels. HuR knockdown resulted in an approximately two-fold decrease in the levels of cIAP1 transcript (Fig. 5A), as expected given HuR's accepted role as a stabilizer of ARE-containing transcripts. Overexpression of FLAG-HuR, however, did not affect cIAP1 mRNA levels (Fig. 5B). One possible explanation is that there is already sufficient HuR present to affect all of the cIAP1 transcripts so that overexpression of yet more HuR would not exhibit any additional effect.

To demonstrate that the decrease in steady-state levels of cIAP1 transcript in response to HuR knockdown was not an off-target effect of transfecting siHuR, we performed a genetic rescue by overexpressing FLAG-HuR in HuR-knockdown U2OS cells. We observed a significant decrease in cIAP1 transcript levels when FLAG was overexpressed to rescue the effect of HuR knockdown (~50% decrease, consistent with the effect of HuR knockdown alone; Fig. 5C); however, when FLAG-HuR was used for the rescue, the decrease in cIAP1 transcript levels was no longer statistically significant (~20% decrease). In both cases, statistical significance was determined relative to the siC-treated,

FLAG-transfected baseline. Unfortunately, the difference between the siHuR-treated, FLAG-transfected and siHuR-treated, FLAG-HuR transfected samples, a more rigorous measure of the effect of FLAG-HuR rescue, was not statistically significant. This is indicative of a partial rescue whereby the effect of the rescue is dampened so as to be more difficult to discern. Such a partial rescue could arise from a mechanism as simple as imperfect transfection efficiency. Suppose that the probability of successfully transfecting a given cell with plasmid is independent of whether that cell has been successfully transfected with siRNA and that the plasmid transfection efficiency is approximately 60%. Under these conditions, we would expect only 60% of siHuR-transfected cells to subsequently be transfected with FLAG-HuR plasmid. As the effect to be rescued is generated by HuR knockdown, assuming that overexpression of FLAG-HuR alone has no effect on cIAP1 transcript levels, this would suggest that only around 60% of the aggregate effect observed with HuR knockdown could be rescued by reintroducing FLAG-HuR.

As mRNA steady-state levels are affected by both the rate of decay as well as the rate of transcription, we sought to verify that the downregulation of cIAP1 mRNA levels observed after HuR knockdown was caused by destabilization of the transcript and not a decrease in the rate of transcription. To accomplish this, we used actinomycin D, a fast-acting DNA intercalating agent that inhibits RNA polymerase II at concentrations greater than 1  $\mu\text{g}/\text{mL}$  (Bensaude, 2011), to inhibit the transcription of mRNA so that we could track the decay of cIAP1 mRNA in U2OS cells. We show that the cIAP1 transcript is destabilized by approximately 40% in response to HuR knockdown (Fig. 6B), in line with the observed decrease in steady-state cIAP1 mRNA levels (Fig. 6C).

The 3' UTR of the cIAP1 mRNA is approximately 500 nt long and contains four AREs (Fig. 7A). We wished to determine the region of this 3' UTR that is responsible for the observed HuR-dependent effect. Previous work from our laboratory has shown that the cIAP1 transcript is destabilized by the accumulation of hnRNP A1 in the cytoplasm and its subsequent binding to the third and fourth AREs in the cIAP1 3' UTR during UV irradiation (Zhao et al., 2009). Additionally, it was shown that the H2B fragment was sufficient to confer this destabilizing effect to a CAT reporter construct. As such, we decided to test for direct binding by HuR to the H2A, H2B, and various mutant H2B fragments using *in vitro* UV crosslinking. Using bacterially-derived GST-HuR, we show that HuR binds directly to the second ARE in the context of the H2B fragment (Fig. 7B). Our preliminary data also shows that AUF1, a trans-acting factor known to bind and destabilize ARE-containing transcripts (Gratacos and Brewer, 2010), interacts with the H2B fragment (Fig. 3), although the precise location of interaction is unknown. Therefore, a possible mechanism to explain HuR's stabilizing effect is that binding of HuR to the second ARE disrupts, perhaps by competition, the interaction between hnRNP A1 and the third or fourth AREs or between AUF1 and the cIAP1 3' UTR.

Next, we wished to determine whether or not the second ARE, in the context of the H2B fragment, plays a role in regulating the stability of the transcript. To do so, we measured, in U2OS cells, the CAT expression of reporter constructs consisting of either the H2B fragment or the mut. 2 variant appended to the CAT coding region. Our results indicated that the disruption of the second ARE did not significantly affect the expression of CAT protein (Fig. 8B). One possible explanation for this unexpected observation is that the regulatory element affects mRNA stability and protein expression in opposite directions,

possibly by modulating translation efficiency in addition to mRNA stability. An example of this type of behaviour is DDB2, whose transcript contains a regulatory element in its 3' UTR that destabilizes the transcript but increases its protein expression through an unknown mechanism (Melanson et al., 2013). In fact, the aforementioned paper also suggests that such an element also exists within the cIAP1 3' UTR. It is possible that the destabilizing element within the H2B fragment is such an element. If so, mutation of the second ARE would disrupt the fragment's destabilizing element, stabilizing the transcript relative to the wild-type but also disrupting the mechanism that is responsible for increased protein expression, yielding no net effect on protein expression.

The introduction of confounding factors downstream of mRNA stability, such as translation efficiency, was a result of using protein expression as an indirect measure of mRNA stability. This problem could be circumvented by directly measuring the CAT mRNA levels. Unfortunately, the use of the CAT qPCR primers yielded background Ct values similar to the experimental values (data not shown).

As HuR has been shown to be predominantly nuclear under basal conditions, it is also possible that we observed no difference in CAT reporter expression between H2B and mut. 2 constructs because endogenous cytoplasmic HuR levels were inadequate. In this scenario, the flood of HuR-binding H2B reporter transcripts overwhelms the levels of cytoplasmic HuR, causing HuR to become limiting, resulting in the majority of H2B reporter transcripts not being bound by HuR anyway. In this way, the HuR-binding status of only a small fraction of the mut. 2 reporter transcripts would be affected relative to if they had been H2B reporter transcripts instead, dampening the observed aggregate effect of mutating the HuR-binding ARE. We decided to overexpress FLAG-HuR in the hopes of

exacerbating any differences in the expression of the two CAT reporter constructs. When FLAG-HuR was overexpressed, neither the expression of the H2B reporter nor that of the mut. 2 reporter is affected relative to their FLAG control (Fig. 8C).

To reduce the number of confounding factors affecting our results, we turned from measuring the protein expression of CAT reporter constructs and decided to directly measure the stability of the RNA fragments. To do so, we made use of an *in vitro* RNA decay assay. Incubation of equal counts of radiolabeled H2B or mut. 2 probe in S10 lysate prepared from siC- or siHuR-treated U2OS cells indicated no differences between the stabilities of both probes in both conditions (Fig. 9B). One possible explanation stems from the probes' lack of both a 5' m<sup>7</sup>G cap and a 3' poly(A) tail, a trait of the MAXIscript® *in vitro* transcription reaction used to synthesize the probes. It is believed that deadenylation is the rate-limiting step in the degradation of many mRNAs. As AREs have been implicated in stimulating deadenylation (Xu et al., 1997), it is possible that their destabilizing effect is a result of accelerating this rate-limiting step. As a result, a probe lacking a poly(A) tail would not be expected to be destabilized further by AREs via this mechanism, explaining why there appeared to be no difference in half-life between H2B and mut. 2 probes. Additionally, without the destabilizing effect of the AREs, HuR no longer has a destabilizing effect to inhibit, accounting for the observed HuR-independence of the half-life of both probes.

Along these lines, it is also possible that the second ARE and binding of HuR to the second ARE play no role in the stability of the transcript. Instead, another interaction involving HuR, possibly in the second half of the cIAP1 3' UTR or even the coding region or the 5' UTR, could be responsible for the observed HuR-mediated effect on cIAP1 mRNA stability. This would explain the results obtained from both the CAT reporter assay as well

as the *in vitro* RNA decay assay. It has previously been shown that HuR can also regulate the expression of c-fms transcript via its non-ARE-containing 3' UTR (Woo et al., 2009). Although it was not shown that this regulation was a result of changes in mRNA stability, it nevertheless leaves open the possibility that non-ARE-containing portions of the cIAP1 transcript are responsible for the observed HuR-dependent effect.

Overall, we have identified HuR as a trans-acting factor that stabilizes the cIAP1 transcript and shown that HuR binds, *in vitro*, directly to the second ARE in the context of the H2B fragment, although we have not yet been able to link the binding of HuR to the second ARE to the stabilizing effect we observed in U2OS cells. These results are in line with HuR's known roles as a regulator of ARE-containing transcript stability and as the controller of a regulon involved in cell survival, proliferation, migration, angiogenesis, and inflammation (Simone and Keene, 2013).

## **6.2 Conclusion**

We started our study with the hypothesis that HuR regulates the stability of the cIAP1 mRNA through the AREs present in the transcript's 3' UTR. This hypothesis was supported by our initial observation of a positive correlation between cIAP1 transcript levels and HuR cytoplasmic localization. We showed that, in U2OS cells, downregulation of HuR does indeed decrease the steady-state levels of cIAP1 transcript and followed by verifying that this decrease was mediated by destabilization of the transcript and not a change in the rate of its production. We found that *in vitro* HuR binds directly to the second ARE within the cIAP1 3' UTR in the context of the H2B fragment; however, it still remains to be determined whether or not this binding is responsible for the effect of HuR on transcript

stability observed in cells. Further experiments will be required to establish the mechanism responsible for HuR's stabilizing effect.

### **6.3 Limitations of the study**

We have shown that HuR affects the stability of cIAP1 mRNA in U2OS cells and binds directly to the second ARE of the cIAP1 3' UTR in the context of the H2B fragment *in vitro*. There are, however, a number of caveats associated with the experiments performed. Additionally, a few experiments could be performed to confirm our findings and increase our understanding of this regulatory process.

The first of these caveats is the use of a ratio instead of a direct measurement as a measure of the levels of a species-of-interest (i.e.: cIAP1/Rpl13a). The use of such ratios assumes that the standardization factor (i.e.: Rpl13a) is more-or-less unaffected by the variable (i.e.: changes in HuR level). Although it would have been better to directly measure levels of the species-of-interest, the use of the ratio is necessary to control for differences in the amount of cDNA loaded into the qPCR reaction. Although we did not perform the experiments necessary to show that the standardization factors we used are indeed unaffected in our experiments, we are able to justify their use. Firstly, both GAPDH and Rpl13a are considered housekeeping genes (genes that are constitutively expressed and perform basic cellular functions) and are frequently used as internal controls (Amabile et al., 2009; Mizuno et al., 2011). Furthermore, transcriptome-wide mapping of HuR binding sites in HeLa cells by PAR-CLIP (PhotoActivatable-Ribonucleoside-enhanced CrossLinking and ImmunoPrecipitation) shows no direct binding between HuR and Rpl13a or GAPDH (Mukherjee et al., 2011), which exhibits a nearly 90% sequence similarity to mouse GAPDH, suggesting that, at the very least, HuR does not exert a direct effect on Rpl13a or

GAPDH mRNA. There have also been studies, albeit in other systems, showing that Rpl13a levels remain steady in numerous conditions (Curtis et al., 2010; Gubern et al., 2009), making it a good candidate for use as a standardization factor. Short of quantifying a pool of potential housekeeping genes followed by statistical analysis, there is no good method for resolving this issue.

Experiments using fragments of the cIAP1 3' UTR also suffer from a simplifying assumption: that binding of HuR to its target is dependent only on local characteristics such as nucleotide sequence and/or local structure. Using shorter fragments may affect the detection of interactions between HuR and the RNA that are dependent on or inhibited by long-distance interactions, such as certain secondary or tertiary RNA structures. Thus, although we have shown that HuR binds directly to the second ARE in the context of the H2B fragment, we may have missed interactions between HuR and the other AREs or observed a binding event that is obstructed in the context of the full-length cIAP1 3' UTR. In the case of hnRNP A1-mediated destabilization of cIAP1 transcript, it was shown that the third and fourth AREs in the context of H2B fragment were sufficient to confer instability to a CAT reporter construct (Zhao et al., 2009), suggesting that this fragment may also be sufficient to confer HuR-mediated stabilization. To further mitigate this issue, we could perform the binding, reporter, and *in vitro* decay experiments using the full-length cIAP1 3' UTR.

Additionally, the *in vitro* binding experiments were performed using GST-HuR purified from *E. coli*. As bacteria lack many of the systems responsible for the post-translational modification of proteins (Sahdev et al., 2008), it is possible that GST-HuR purified from *E. coli* lacks important modifications that can affect its binding properties,

leading to the possibility that the binding event observed in our UV crosslinking experiment does not occur under normal physiological conditions. One possible solution is the use of recombinant proteins purified from a eukaryote, such as *Saccharomyces cerevisiae*. Furthermore, pull-down of HuR and associated RNAs from live cells transfected with reporter constructs containing the wild-type or mutant cIAP1 3' UTR could help validate the dependence of the HuR/cIAP1 mRNA interaction on the second ARE in the context of the full-length cIAP1 3' UTR and under *ex vivo* conditions.

In the CAT reporter experiments, we utilized a second plasmid expressing  $\beta$ -Gal to control for transfection efficiency. Although this technique is commonly used in the literature (Kollias et al., 2006), it assumes that the transfection efficiency of the  $\beta$ -Gal plasmid exhibits a similar linear correlation with the transfection efficiency of the CAT reporter plasmid over all treatment conditions used. The best solution to eliminate the need for this strong assumption is to use a single plasmid that contains both the reporter construct and an exogenous gene that will be used as the transfection control under the control of an unregulated, constitutively-expressing promoter.

In both the CAT reporter experiments and the *in vitro* RNA decay assays, we lacked a positive control to show that the experimental setup was capable of producing and/or detecting ARE-dependent and HuR-mediated transcript stability changes. For example, it is possible that the S10 lysate used in the *in vitro* RNA decay assays just does not contain the activity required to destabilize ARE-containing transcripts. In this case, one would not observe a change in transcript stability after disrupting the AREs, not necessarily because the AREs are not destabilizing, but because the experimental setup was incapable of producing the AREs' destabilizing effect in the first place. In both experiments, a good

positive control would be an ARE-containing 3' UTR upon which HuR is known to exert a stabilizing effect by binding to the ARE, such as the 3' UTR of TNF- $\alpha$  (Dean et al., 2001).

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## **8. Contribution of Collaborators**

Dr. Stephen Lewis performed the experiments and provided the data depicted in Fig. 3.  
Danielle Durie created the FLAG-HuR plasmid used in this study.

## 9. Appendix

### siRNA

siC	Sequence	UUCUCCGAACGUGUCACGUdTdT
	Target	None
	Concentration	100 $\mu$ M (100 nM final)
	Supplier	Qiagen negative control siRNA (Cat. # 1022076)
siHuR	Sequence	AAGUCUGUUCAGCAGCAUUGGUdTdT
	Target	Coding region of the human HuR transcript
	Concentration	100 $\mu$ M (100 nM final)
	Supplier	Dharmacon custom-synthesized

### qPCR primers

clAP1	Forward	TCTGGAGATGATCCATGGGTAGA
	Reverse	TGGCCTTTCATTCGTATCAAGA
	Target	Coding region of the human and mouse clAP1 transcripts
	Supplier	Life Technologies custom-synthesized
GAPDH	Forward	CATGTTCCAGTATGACTCCACTC
	Reverse	GGCCTCACCCATTTGATGT
	Target	Coding region of the mouse GAPDH transcript
	Supplier	Life Technologies custom-synthesized
Rpl13a	Sequences and target site unknown	
	Supplier	Qiagen QuantiTect primer assay (Cat. # QT02321333)

### Antibodies

Mouse monoclonal anti-HuR IgG*	Target	3A2 epitope of human and mouse HuR
	Concentration	1:1000 (immunofluorescence)
	Supplier	Santa Cruz Biotechnology (Cat. # sc-5261)
Mouse monoclonal anti- $\alpha$ tubulin*	Target	DM1A epitope of human and mouse $\alpha$ tubulin
	Concentration	1:10000 (Western blot)
	Supplier	(Cat. # ab7291)
Goat highly cross-adsorbed Alexa Fluor® 488-conjugated anti-mouse IgG	Target	Whole mouse IgG
	Concentration	1:2000 (immunofluorescence)
	Supplier	Life Technologies (Cat. # A-11029)
Goat Alexa Fluor® 750-conjugated anti-mouse IgG	Target	Whole mouse IgG
	Concentration	1:10000 (Western blot)
	Supplier	Life Technologies (Cat. # A-21037)

\* HuR and tubulin primary antibodies were applied simultaneously for Western blot

**PCR Primers (T7 promoter underlined)**

H2A	Forward	<u>TAATACGACTCACTATAGGGCGA</u> AAGAAAAATAGTCTATATTTAAC
	Reverse	TCTAGAGTACCAAAGCAGACTAGAAC
H2B	Forward	<u>TAATACGACTCACTATAGGGCG</u> AGTTCTAGTCTGCTTTGGTAC
	Reverse	TCTAGACAGTGACACTACTCCCTTAGG

**H2B Mutants**

Mutation Site	Original Sequence	Mutated Sequence
ARE 1	AUAU <u>UUU</u> AAU	AUAUAGCAAU
ARE 2	AUUUA	AGCA
ARE 3	AUUUA	AGCA
ARE 4	UAUAUUA	UAUAGCA

**Plasmids**

Construct	Reference
pMC (and variants containing H2A, H2B, mut. 1-4, and mut. 1234.)	Zhao et al., 2009
FLAG-HuR	Danielle Durie (unpublished)
$\beta$ -Gal	Clontech pCMV-LacZ vector (Cat. # 631719)

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Expected completion date	May 2013
Estimated size (number of	75

## **11. Curriculum Vitae**

### **Education**

September 2010 – Present	M. Sc. candidate in Biochemistry, University of Ottawa
September 2005 – April 2010	Honours B. Sc. With Specialization in Biochemistry, University of Ottawa
September 2003 – June 2005	Lisgar Collegiate Institute, Ottawa

### **Awards and Honours**

September 2005 – April 2006	Admission Scholarship, University of Ottawa
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### **Graduate Classes Completed**

BCH 8310	Current topics in RNA molecular biology
BCH 8105	Advanced topics in the molecular biology of human disease II

### **Publications**

Mame Daro Faye, Tyson E. Graber, Peng Liu, Nehal Thakor, Stephen D. Baird, Danielle Durie, and Martin Holcik (2013). Nucleotide composition of cellular IRES defines dependence on NF45 and predicts a post-transcriptional mitotic regulon. *Molecular and Cellular Biology* 33(2), 307-318.