The functional role of the RNA-binding protein HuR in the regulation of muscle cell differentiation

Pascal Beauchamp

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Department of Biochemistry
McGill University
Montréal, Québec
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Résumé

Le développement de tissu musculaire (myogenèse) implique la formation de fibres spécifiques (myotubes) à partir de cellules musculaires (myoblastes). Pour que cela puisse arriver, l'expression séquentielle des Facteurs de Régulation Myogénique (MRFs), tel que MyoD et myogénine, est requise. L'expression de ces MRFs est régulée post-transcriptionnellement par la protéine de liaison à l'ARN HuR qui s'associe avec les régions 3'-non-traduites des ARNm de *MyoD* et *myogénine,* menant à des augmentations significatives de leur demi-vie. Ici, nous montrons que le clivage de HuR par les caspases à l'acide aminé aspartate (D) 226 est un des principaux régulateurs de sa fonction pro-myogénique. Cette activité protéolytique génère deux produits de clivage (CPs), HuR-CP1 et HuR-CP2, qui affectent de façon différente le procédé myogénique. Les myoblastes surexprimant HuR-CP1 ou la forme mutante non-clivable de HuR, HuRD226A, ne sont pas capables de s'engager dans la myogenèse, pendant que de hauts niveaux de HuR-CP2 améliorent la formation de myotubes. HuR-CP2 mais pas –CP1 favorise la myogenèse en stabilisant les ARNm de *MyoD* et *myogénine* à des niveaux semblables à wt-HuR. Inversement, l'effet inhibiteur de HuR-CP1 et HuRD226A dépend de leur capacité à s'associer pendant la myogenèse avec le facteur d'import de HuR, Trn2, menant à l'accumulation de HuR dans le cytoplasme. Ainsi, nous proposons un modèle par lequel le clivage de HuR médié par les caspases génère deux CPs qui collaborent pour réguler la myogenèse; HuR-CP1 en interférant avec l'import de HuR médié par Trn2 et HuR-CP2 en participant à la stabilisation des ARNm encodant les MRFs clés.

Abstract

Muscle tissue development (myogenesis) involves the formation of specific fibers (myotubes) from muscle cells (myoblasts). For this to occur, the sequential expression of Myogenic Regulatory Factors (MRFs), such as MyoD and myogenin, is required. The expression of these MRFs is regulated posttranscriptionally by the RNA-binding protein HuR, whereby HuR associates with the 3'-untranslated regions of *MyoD* and *myogenin* mRNA, leading to a significant increase in their half-lives. Here we show that the cleavage of HuR by caspases at the aspartate (D) 226 residue is one of the main regulators of its pro-myogenic function. This proteolytic activity generates two cleavage products (CPs), HuR-CP1 and HuR-CP2, that differentially affect the myogenic process. Myoblasts overexpressing HuR-CP1 or the non-cleavable mutant of HuR, HuRD226A, are not able to engage myogenesis, while overexpressing HuR-CP2 enhances myotube formation. HuR-CP2 but not -CP1 promotes myogenesis by stabilizing the *MyoD* and *myogenin* mRNAs to the same levels as wt-HuR. Conversely, the inhibitory effects of HuR-CP1 and HuRD226A depend on their abilities to associate during myogenesis with the HuR import receptor, Trn2, leading to HuR accumulation in the cytoplasm. Therefore, we propose a model whereby the caspase-mediated cleavage of HuR generates two CPs that collaborate to regulate myogenesis; HuR-CP1 by interfering with the Trn2-mediated import of HuR and HuR-CP2 by participating in the stabilization of mRNAs encoding key MRFs.

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1. Introduction

1.1 General Introduction

Skeletal muscles are essential for many aspects of life such as breathing, eating, and moving. The process of muscle formation, also called myogenesis or muscle cell differentiation, is tightly regulated, and takes place during the embryonic development stages and during reparation upon injury. Myogenesis is initiated by external signals that result in the cell cycle withdrawal of myoblasts (mononucleated multipotential muscle cell) in order to fuse together and form multinucleated myotubes, also called myofibers. These signals also initiate the expression of key players responsible for the different steps leading to muscle fiber formation. A considerable amount is known about the molecular mechanisms that regulate the expression of the myogenic regulatory factors (MRFs) upon induction of muscle cell differentiation. Though transcription has been shown to be one of the main regulators of MRF expression, posttranscriptional events have recently been demonstrated to play a major role in myogenesis. The latter will be the focus of this thesis in which I will delineate the role of the RNA binding protein HuR in myotube formation.

1.2 Transcriptional regulation of myogenesis

The process of cell differentiation starts from uncommitted embryonic cells that are guided by specific signals to differentiate into adult cells that form part of a

functional tissue or an organ (Asakura et al., 2001; Charge and Rudnicki, 2004; Sabourin and Rudnicki, 2000). During the formation of skeletal muscle, muscle cell differentiation is induced by myogenic differentiation stimuli which cause mononucleated cells committed to myogenic lineage called myoblasts to fuse into multinucleated myotubes (Charge and Rudnicki, 2004). Once they are committed to this process, the myoblasts withdraw from the cell cycle, which causes them to stop proliferating and leads to the formation of myotubes which express several MRFs. MRFs are known to control the expression of muscle specific proteins leading to muscle development, and also influence their own expression. These proteins include myoglobin, which mediates oxygen storage in muscle, and the myosin heavy chain (MyHC), which is one of the main structural muscle proteins (Emerson and Bernstein, 1987; Weller et al., 1986). To produce and maintain viable muscle fibers, it is very important to have a tight regulation, from transcription to translation, of the expression of MRFs. This importance is exemplified by observations that the deregulation of MRFs can prevent the transition from myoblasts to myotubes and even lead to muscle cell transformation (Charge and Rudnicki, 2004; Sabourin and Rudnicki, 2000).

One way to prevent MRF deregulation is by tightly regulating their expression in a time-dependent manner, which allowing the induction of genes required at different stages of the myogenic process. Intriguingly, when muscle differentiation is induced, though a rapid overexpression of the MRFs is observed, this cannot be attributed solely to transcriptional activation (Lassar et al., 1991; Lassar et al., 1994; Sabourin and Rudnicki, 2000). This suggested a possible role for post-transcriptional

regulation during myogenesis. Indeed, recent reports have demonstrated the implication of posttranscriptional regulatory events in myogenesis (Figueroa et al., 2003; Gherzi et al., 2004; van der Giessen and Gallouzi, 2007; Venuti et al., 1995). This level of regulation will be the focus of this thesis in which I will delineate novel molecular pathways by which the posttranscription regulator, HuR, affects myogenesis.

1.2.1 Myogenic regulatory factors (MRF) and their regulation

MRFs include proteins such as MyoD (Myf-3), myogenic factor 5 (Myf-5), myogenin (Myf-1), and MRF4 (Myf-6, herculin) that belong to a family of transcription factors called the basic helix-loop-helix, named after such a structure which binds in a temporal manner to the E-box DNA elements (CANNTG) within muscle-specific promoters, stimulating the expression of pro-myogenic genes (Kaeser and Emerson, 2006). Their general role is to trigger the fusion of myoblasts to form myotubes as part of the temporally regulated events of myogenesis (Campion et al., 1981; Mauro, 1961). Gene inactivation studies in mice determined that two specialized groups of MRFs act during myogenesis (Figure 1.1). The first group, consisting of MyoD and Myf5, acts at the stage of determination to myogenic lineage and the commitment of undifferentiated somite cells into myoblast. The second group, including myogenin and MRF4, is responsible for the transition from proliferative myoblasts to differentiated myotubes.

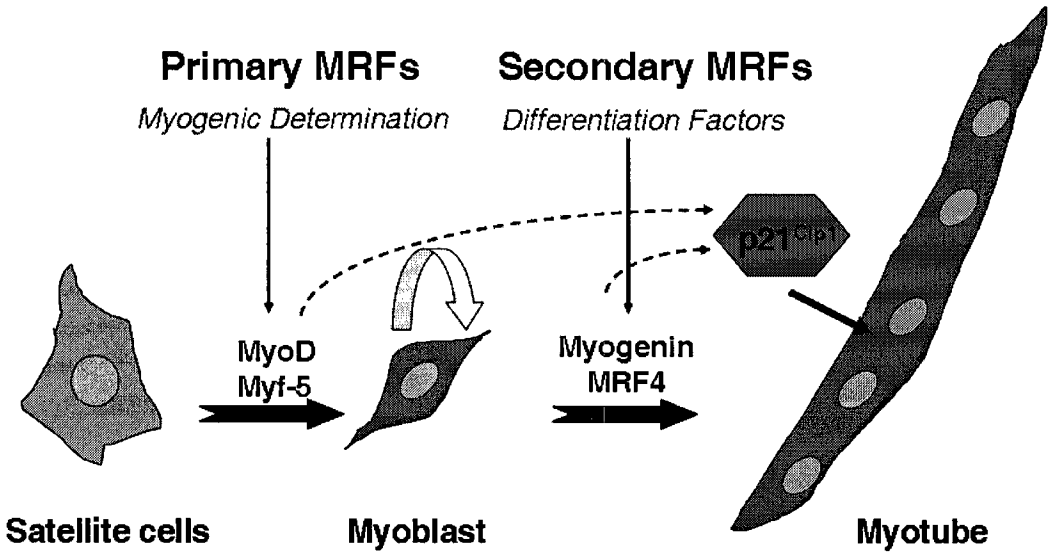


Figure 1.1: MRF overexpression in a chronological order is required during myogenesis. Overexpression of primary MRFs, MyoD and Myf-5, is induced by external differentiation signals provoking the conversion of satellite cells into myoblasts. Then, overexpression of the primary MRFs leads to the overexpression of the secondary MRFs, myogenin and MRF4. This overexpression, associated with $p21^{Clp1}$ expression and cell cycle arrest, is required for the formation of multinucleated myotubes (modified from (Sabourin and Rudnicki, 2000)).

In order for cells to become committed to the myogenic pathway, they must express the primary MRFs at basal levels. Upon activation of myogenesis, MyoD is the first MRF to be upregulated. MyoD protein was found to activate the creatine phosphokinase gene (Lassar et al., 1989), and to bind adjacently to the gene required to express an acetylcholine receptor (Piette et al., 1990). Moreover, MyoD has the ability to promote transcription of its own gene. When inactivation of *myoD* gene was performed in mice, they had a normal phenotype but the expression of Myf-5 had increased by 4-fold (Rudnicki et al., 1992). This suggested the existence of

functional redundancy between some MRFs. However, mice with an inactive *Myf-5*, even though they had normal muscles, did not survive due to a defect in rib formation (Braun et al., 1992). Moreover, when the expression of both MyoD and Myf-5 was suppressed, all mice died at birth due to a formation defect in the skeletal muscle tissue (Rudnicki et al., 1993).

The secondary MRFs act when the proliferative myoblasts become committed to the differentiated phenotype. They are important for terminal muscle differentiation and maintenance (Perry and Rudnick, 2000). Myogenin is not expressed in myoblasts, however MRF4 can be detected in both early and late myogenesis as well as in muscle development and in adult muscle tissue (Hinterberger et al., 1991). Moreover, myogenin and MRF4 are regulators of the expression of genes encoding contractile proteins (Charbonnier et al., 2002; Mak et al., 1992) including those involved in fast and slow fiber differentiation (Hinterberger et al., 1991; Nicolas et al., 2000). The activation of the secondary MRFs is also associated with the expression of the cyclin kinase inhibitor p21, responsible for cell cycle arrest and required for myoblast fusion and myotube formation. Studies performed on both myogenin and MRF4 knockout mice resulted in muscle cells that were expressing the primary MRFs but were unable to differentiate, demonstrating the need for "late" MRFs during the myogenic process (Nabeshima et al., 1993; Olson et al., 1996; Venuti et al., 1995). Furthermore, the muscle deficiencies of those knockout mice were more severe in *myogenin-/-* than in *MRF4-/-* mice. Indeed, all myogenin-/- embryos died perinatally due to the absence of muscle fibers and *MRF4-/-* had

different phenotypes, ranging from complete viability to lethality (Nabeshima et al., 1993; Yoon et al., 1997).

All four MRFs were shown to have the ability to convert most cell types to the myogenic phenotype which expresses MRF and myogenic proteins. MyoD was the first MRF that was demonstrated to have the ability to convert cells such as adipocytes, epithelial cells and osteoblasts to the myoblastic phenotype (Boukamp et al., 1992; Filvaroff and Derynck, 1996; Weintraub et al., 1989). The same results were obtained with the other 3 MRFs. Therefore, the MRFs have important function in the myogenic process and they promote myogenesis by enhancing transcription of one another as well as of muscle specific genes (Braun et al., 1989).

1.2.2 Positive and negative regulation of MRF expression by other factors

MRFs are considered the main regulators of myogenesis, however, some of the factors involved in directly or indirectly regulating their expression are also considered as key promyogenic players. Many of these players are members of the Myocyte Enhancer Factor 2 (MEF2) family of transcription factors which is composed of four MEF2 genes, MEF2A to D (Black and Olson, 1998). Unlike MRFs, they are not restricted to muscle differentiation, as they are expressed predominantly in the development of skeletal, cardiac, and smooth muscles, but also in neuronal cells (Berkes and Tapscott, 2005; Breitbart et al., 1993; Martin et al., 1993; Yu et al., 1992). These genes encode transcription factors containing a MADS (MCM1 agamous deficient serum response factor)-box that specifically binds DNA and the

expression of one of these transcription factors causes an increase in the initiation of myogenesis via MRFs (Kaushal et al., 1994; Molkentin et al., 1995). MEF2s mainly act by binding to AT-rich sequences in the promoter region of *MRF* genes to enhance and maintain their expression in order to increase the rate of transcription as well as myogenesis (Black et al., 1995; Cheng et al., 1993; Molkentin et al., 1995; Yee and Rigby, 1993). One main difference of the MEF2 family of transcription factors compared to MRFs is that even though they can induce MyoD and myogenin expression as well as myogenic conversion in non-muscle cells, they do not activate the expression of muscle specific genes on their own (Kaushal et al., 1994; Leibham et al., 1994; Yee and Rigby, 1993). In fact, their expression is critical in order to assist the MRFs and other muscle specific proteins as shown in an experiment where a mutant form of MEF2A interfering with wt-MEF2A prevented the expression of myogenin and myosin heavy chain (MyHC), a muscle structural protein .

The *Six* family (homologue of the *sine oculis* protein which is required for eye development) represents another set of proteins that is known to have a pro-myogenic function. There are six members of this homeodomain-containing protein family (*Six*1-6) that was first found to have a role in the development of the eye (Pignoni et al., 1997), but it was later demonstrated that their expression was critical during muscle formation (Heanue et al., 1999). More precisely, *Six1* and *Six4* are expressed specifically in skeletal muscles. They were found to bind to a MEF3 motif *in vitro* on the myogenin promoter and mutation of this site inhibits expression of myogenin in mice (Spitz et al., 1998). MEF3 is a consensus sequence (TCAGGTT) found in several skeletal muscle regulatory regions (Spitz et al., 1998). Even though Six 1 and

4 are both involved in myogenesis they seem to have different roles in this process. *Six4* knockout mice are normal at birth and during development (Ozaki et al., 2001). However, *Six1* knockout mice do not have a regular myogenic process as the expression of myogenin and MyoD in limb buds is delayed and they were all observed to die at birth. (Laclef et al., 2003).

The MEF2 and the Six families of proteins are important activators of myogenesis. However, other proteins, identified as antagonist of myogenesis, are considered equally as important. They usually act by binding to E proteins or to protein members of the MyoD family. E proteins are a family of basic helix-loop-helix (bHLH) transcription factors that are required in myogenesis as they heterodimerize with MRFs in order to further activate the transcription of muscle specific gene (Lassar et al., 1991; Murre et al., 1989). Many of these inhibitors of myogenesis have helix-loop-helix domains, including: Id, mTwist, MyoR and Mist-1 (Berkes and Tapscott, 2005). They are characterized by their ability to block the binding of MRFs to E-boxes (CANNTG), a conserved DNA promoter motif which allows for MRFs to activate transcription of their targets. MyoR (myogenic repressor) and Mist-1 (muscle, intestine and stomach expression 1) have basic regions that mediate binding to MRFs to form heterodimers, and although this complex can bind to E-boxes, it is unable to activate transcription of muscle specific genes (Lemercier et al., 1998; Lu et al., 1999). In the case of Id (Inhibitor of differentiation), its heterodimerization with E-proteins prevents the association of E-proteins with MRFs DNA promoter component (Benezra et al., 1990). mTwist (murine Twist) can also inhibit myogenesis by sequestering E-protein from MRFs (Spicer et al., 1996), but

unlike Id, mTwist has a basic region that mediates its binding to MRFs to prevent the association to E-boxes (Hamamori et al., 1997). mTwist can also inhibit differentiation by preventing the trans-activation of MEF2 genes (Spicer et al., 1996). Utimately, positive regulation, performed by protein such as MEF2 and Six and negative regulation, performed by protein such as Id, mTwist, MyoR and Mist-1, allow for tight regulation of MRF activity. Despite the complex network that these factors allow, there still other ways of regulating MRF activity.

1.2.3 Cell-cycle regulation of myogenesis

The cell cycle plays an important role in the regulation of muscle differentiation. As stated earlier, myoblasts must stop proliferating in order to fuse and form myotubes. In fact, for a cell to irreversibly withdraw from the cell-cycle at the G1 phase, multiple regulatory factors must be involved. First, cyclin-dependent kinase (cdk) inhibitors such as $p21^{WAF1}$, p57 and p27 are induced (Cheng et al., 1999; Reynaud et al., 1999), resulting in the arrest of cell cycle progression. When p21 is induced, the action of the "early" MRF MyoD is facilitated (Skapek et al., 1995) and, at the same time, the expression of MRFs enhances the expression of p21. This establishes a feedback loop that is important for the myogenic process, whereby the induction of p21 assists MRF action, and MRFs then in turn enhance p21 expression. One example is of MyoD, which is known to be itself a negative regulator of cell proliferation, that also trans-activates the promoter of p21 upon induction of

myogenesis, which in turn supports cell cycle arrest and myotube formation (Guo et al., 1995; Halevy et al., 1995).

Another pathway that links cell cycle control to myogenesis is that involving p38 Mitogen-Activated Protein Kinase (MAPK). p38 MAPK is known to regulate several processes such as inflammation, cell differentiation, cell growth and cell death (Chang and Karin, 2001; Cocolakis et al., 2001; Nebreda and Porras, 2000; Wada et al., 2008). Once it gets activated by the upstream MAPKs MKK3 and MKK6, it activates other proteins through phosphorylation, including several transcription factors and other kinases (Chang and Karin, 2001). In the context of muscle differentiation, the p38 MAPK pathway collaborates with myogenic specific transcription factors to activate late-transcribed genes (Keren et al., 2006). p38 MAPK was shown to be required in myogenesis by exposing myoblasts to specific p38 MAPK inhibitors, which blocked myogenesis (Cheng et al., 1999; Reynaud et al., 1999). No formation of myotubes was observed and the expression of genes specific to myogenesis, such as p21, myogenin, and MyHC was greatly reduced. In fact, these inhibitors were specifically shown to increase the activity of members of the MEF2 family (MEF2A and MEF2C) by causing their phosphorylation, as well as by inducing the transcription of muscle-specific genes by activating a chromatin remodelling enzyme (Cuenda and Cohen, 1999; Li et al., 2000; Wu et al., 2000; Zetser et al., 1999). Therefore, p38 MAPK has an effect on the transcription of muscle-specific genes by modulating the activity of transcription factors. Though various means exist of influencing the transcription of MRFs, and consequentially,

myogenesis, these alone cannot account for the entire regulation of myogenesis that has been seen, and so non-transcription based mechanisms must also be explored.

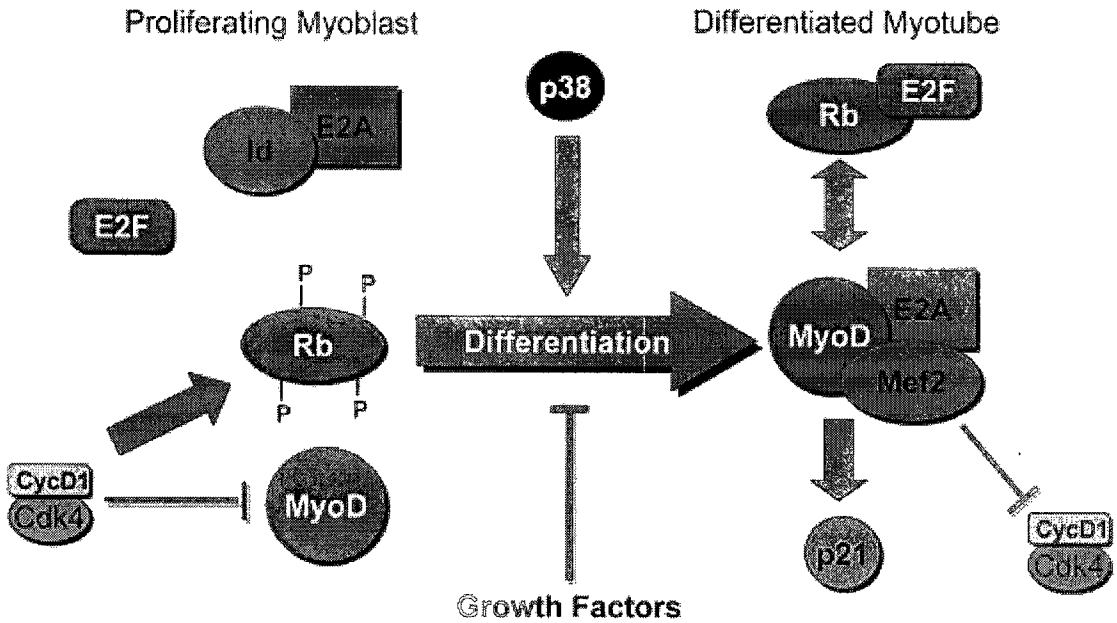


Figure 1.2: Molecular regulation of myogenesis by non-MRFs. Myogenesis is well characterized to be regulated by MRFs. However, other factors have also been shown to have positive or negative role on the myogenic process. Furthermore, cell cycle regulatory elements were also shown to control myogenesis. (Sabourin and Rudnicki, 2000)

1.3 Posttranscriptional regulatory mechanisms in myogenesis

Knowing that transcription alone is not sufficient to maintain the high levels of MRF messages and their respective proteins during the lifespan of a myotube has lead our laboratory and others to suggest that posttrancriptional events are important for muscle development and maintenance (Charge and Rudnicki, 2004; Figueroa et al., 2003; Lassar and Munsterberg, 1994; Lassar et al., 1991; Rudnicki et al., 1992; Sabourin and Rudnicki, 2000; van der Giessen et al., 2003). It is now well-accepted that cis-elements founds in mRNAs encoding different MRFs play an important role in regulating their expression, by associating with trans-acting proteins, called RNA binding proteins (RNA-BPs), such as HuR and KSRP.

1.3.1 AU-rich elements control mRNA stability

One relatively simple way to post-transcriptionally regulate gene expression is to influence the stability of a specific mRNA. Certain sequences exist which destabilize their host mRNA, by decreasing that message's half-life (the time it takes for a specific population of mRNA to get to a level that is 50% of its original amount). One of the best-characterized families of destabilizing sequences is found in the 3'untranslated region (UTR) of mRNAs, and is called the adenine/uridine-rich element (ARE) (Figure 1.3) (Shaw and Kamen, 1986). AREs are composed of several stretches of A and U residues, and promote rapid degradation via several mechanisms (von Roretz and Gallouzi, 2008). They are present on many short lived mRNAs that

encode for critical proteins such as growth factors, cytokines and lymphokines, and allow tight regulation of expression (Barreau et al., 2005).

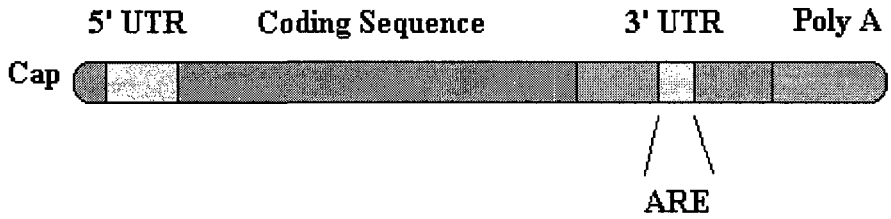


Figure 1.3: The position of an ARE on an mRNA molecule. An mRNA molecule may possess several *cis*-elements that regulate its stability, translation and localization. More precisely, the AU-rich element (ARE), which destabilizes the message in which it lies, is found in the 3'UTR an mRNA molecule (yellow portion).

The first direct evidence identifying the potential destabilizing function of the ARE on an mRNA was obtained when an ARE (an AUUUA motif) located in the 3'UTR of human granulocyte-macrophage-colony-stimulating factor (GM-CSF) mRNA was cloned into the 3'UTR of the very stable β-globin mRNA. Remarkably, the β-globin mRNA was severely destabilized by this AU-rich element (Chen and Shyu, 1995; Shaw and Kamen, 1986). Since this observation, many cellular processes such as cell growth, differentiation and adaptation to extracellular stimuli (Andrews et al., 1987; Brennan and Steitz, 2001; Keene and Tenenbaum, 2002; Wang et al., 2000a) have been shown to depend on ARE-mediated regulation of gene expression.

The stabilization of ARE-containing mRNAs can occur when cells are subjected to stimuli which induce cell death or cell differentiation. During muscle cell differentiation, it has been shown that the ARE-containing 3'UTRs of the messages encoding for MyoD, myogenin, and p21 proteins affect posttranscriptionally the expression of these genes. In fact, it was shown that the stabilities of MyoD, myogenin, and p21 messages actually change during muscle cell differentiation (Figueroa et al., 2003; van der Giessen et al., 2003). The destabilization effect mediated by these AREs is modulated by their association with RNA-BPs that have been demonstrated to play a critical role during myogenesis. Furthermore, since AREs are also known mediators of the cellular mechanisms and regulations of mRNA translation, AREs may also perform these roles during myogenesis based on the binding to RNA-BPs.

1.3.2 The role of KSRP in myogenesis

KH-type splicing regulatory protein (KSRP) is an RNA binding protein that plays a major role during myogenesis. One of its main characteristics is that it contains four RNA binding K homology (KH) motifs that are essential for its function (Adinolfi et al., 1999). It is involved in several cellular processes such as nuclear RNA splicing, cytoplasmic localization of RNA, and the rapid decay of several ARE-containing mRNAs (Gherzi et al., 2004). In addition to binding AREs, the KH domains can also interact with the exosome and poly(A) ribonuclease (PARN) which are involved in ARE-mediated decay (Gherzi et al., 2004; von Roretz and Gallouzi,

2008). Moreover, binding of KSRP to RNA is regulated in a p38 MAPK phosphorylation-dependent manner (Briata et al., 2005). This specific phosphorylation of KSRP inhibits its RNA binding capabilities, thus preventing rapid decay of mRNA, despite the fact that KSRP retains its ability to interact with the mRNA degradation machinery.

In the context of muscle differentiation, KSRP has been shown to interact with the AREs of certain key myogenic factors, including MyoD, myogenin, and p21 (Briata et al., 2005). Knocking down KSRP in myoblasts augments the stability of the messages of myogenin and p21, enhancing the formation of muscle fibers. The ability of KSRP to negatively regulate myogenesis was further defined by studies demonstrating that the overexpression of KSRP inhibits myotube formation, even though there is no effect on stability. In myoblasts KSRP prevents muscle differentiation by minimizing the expression of ARE-containing mRNAs such as MyoD and myogenin, by associating with them and recruiting degradation machineries (Briata et al., 2005). Upon the induction of muscle cell differentiation, KSRP becomes phosphorylated in a p38-dependent manner. This results in the release of these messages, which may then be stabilized by other RNA-BPs. KSRP is therefore well established as a key player in triggering the decay of the mRNAs encoding key MRFs that are required for myogenesis. However, the stabilization of these messages is ensured by HuR that associates with specific AREs located in their 3'UTR.

1.4 The RNA-binding protein HuR

The RNA binding protein HuR was the first posttranscriptional regulator that was shown to be involved in myogenesis (Figueroa et al., 2003; van der Giessen et al., 2003). HuR is a well-known posttranscriptional regulator of mRNAs containing AREs in their 3'UTR. Along with messages involved in myogenesis, HuR regulates through posttranscriptional mechanisms the expression of a wide assortment of genes involved in cell growth, survival and death (Keene, 1999; Lafon et al., 1998; Mazan-Mamczarz et al., 2003; Mazan-Mamczarz et al., 2008; Mazroui et al., 2008; van der Giessen et al., 2003).

1.4.1 Structure and regulation of HuR

HuR (or HuA) is a 32 kDa protein member of the embryonic lethal abnormal vision (ELAV) family of protein (Myer et al., 1997) that also includes HuB, HuC and HuD. Unlike the other members of the family which are primarily localized to the nervous system, HuR is ubiquitously expressed in all tissue (Ma et al., 1996). This family of proteins was first identified in the *Drosophila melanogaster* where their absence resulted in embryonic lethality due to abnormal neuron development (Robinow et al., 1988; Robinow and White, 1991). The mammalian members of the ELAV family were cloned after screening a cDNA library with Hu autoimmune serum (Szabo et al., 1991) that came from a patient suffering of paraneoplastic neurological disorder (PND) (Anderson et al., 1987; Darnell, 1996). Patients that

suffer from PND usually develop small cell lung carcinoma (SCLC) that lead to the formation of tumors that express antibodies against HuB, HuC, or HuD. Even though the tumors remain small due to the immune response, patients die due to neuronal degeneration (Dalmau et al., 1992).

In the case of HuR, although its sequence is similar to the others members of the family it is nonetheless distinct due to its ubiquitous distribution (Ma et al., 1996). All of the members of the ELAV family have three RNA recognition motifs (RRM), which mediate their binding to ARE-containing mRNAs. RRMs 1 and 2 mediate binding of the Hu proteins to ARE, while RRM3 is typically considered to interact with the mRNA poly(A) tail (Ma and Furneaux, 1997). Furthermore, there exists a HuR nucleocytoplasmic shuttling (HNS) motif located in the hinge region between the RRMs 2 and 3, which mediates the shuttling properties of HuR (Figure 1.4).

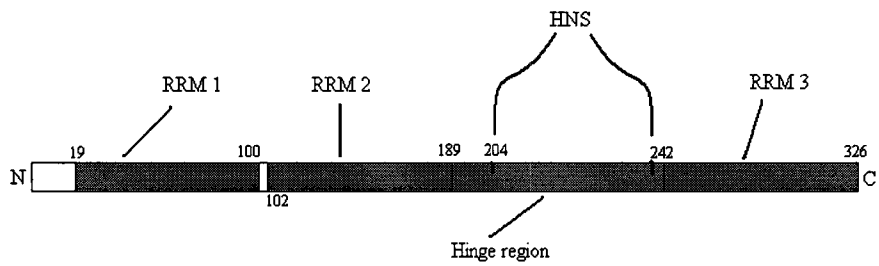


Figure 1.4: Motifs of HuR protein. The RNA-binding protein HuR has 3 RNA recognition motifs (RRMs1-3) that bind to RNA. It also has an HuR nucleocytoplasmic shuttling (HNS) motif, found in the hinge region between RRMs2 and 3.

The exact mechanism regulating the binding of HuR to RNA is not known. Despite this, it has been shown to be regulated at the posttranslational level in certain contexts. Just like several other RNA-binding proteins, the ability of HuR to bind certain mRNAs was shown to be controlled by methylation (Friesen et al., 2001; Li et al., 2002; Liu and Dreyfuss, 1995). One such example is of methylation of HuR in its HNS motif at arginine 217 by the coactivator-associated arginine methyltransferase 1 (CARM1), an arginine methyltransferase, occurs upon stimulation by liposaccharide (LPS) (Li et al., 2002). Moreover, following the stimulation of macrophages with LPS, HuR stabilizes TNFα mRNA (Dean et al., 2001), suggesting that this methylation of HuR seems to be required in order to perform its stabilising task under certain signals (Li et al., 2002).

A second posttranslational modification of HuR, phosphorylation, was also shown to control its function. Phosphorylation of HuR occurs following oxidative stress during cell senescence by Chk2, a cell-cycle checkpoint kinase. This phosphorylation was demonstrated to lead to the dissociation of the complex formed by HuR and the silencing information regulator 2 (Sir2) mRNA, a histone deacetylase acting in transcription, DNA replication, and DNA repair (Blander and Guarente, 2004; Wang et al., 2001).

Yet another posttranslation modification of HuR that may occur is its caspase-mediated cleavage, which will be discussed in a subsequent chapter (Mazroui et al., 2008). In summary, however, posttranslational modifications of HuR allow for this posttranscriptional regulator to adapt its response to a specific cellular context, at the levels of mRNA stability, cellular localization, and translation.

1.4.2 HuR-mediated stabilization of ARE-containing mRNAs

Members of the ELAV family, such as HuR, have been shown to selectively mediate the stabilization of ARE-containing mRNAs. In fact, both UV-crosslinking and gel shift experiments have shown the ability of HuR to bind to AREs and to modulate the stabilization of these messages (Fan et al., 1997; Myer et al., 1997). More precisely, overexpression of HuR was shown to generally protect ARE-containing mRNAs from degradation (Peng et al., 1998). Further analysis of the primary and secondary sequences that are shared between HuR targets permitted the identification of a 17-to-20-base RNA motif that is rich in uracils (U) (Lopez de Silanes et al., 2004). The first ARE-containing mRNA to which HuR was identified to bind was *c-fos* (Ma et al., 1996), though its stabilizing effect was only demonstrated in later studies by correlating ARE binding to the modulation of its stabilization (Fan et al., 1997; Myer et al., 1997). It was also then shown that it is not all of the RRMs that are required for HuR-mediated stabilization, since deleting RRM3 (but only RRM3), allowed for HuR to retain its ability to stabilize ARE-containing mRNAs (Fan and Steitz, 1998).

Though the association of HuR with its mRNA targets is crucial in its stabilizing role, HuR cannot carry out its posttranscriptional regulation without the use of other proteins.

1.4.3 The protein ligands of HuR modulate its nucleocytoplasmic shuttling properties

The role of HuR in the stabilization of ARE-containing messages is one of its most studied functions. It was demonstrated, however, that in order for HuR to stabilize and protect these messages from decay, it must be exported from the nucleus, where it usually localizes, to the cytoplasm (Fan and Steitz, 1998; Keene, 1999). As a result, its shuttling between the nucleus and the cytoplasm must be tightly controlled in order to stabilize mRNA at the right moment. In fact, the localization of HuR is controlled by diverse stimuli, using signalling pathways such as those controlled by the MAPKs, MAPK-activated protein kinase-2 (MK2), AMP-activated kinase (AMPK), cell-cycle checkpoint kinase 2 (Chk2), and protein kinase C (PKC) (Abdelmohsen et al., 2007; Doller et al., 2008a; Doller et al., 2008b; Ming et al., 2001; Subbaramaiah et al., 2003; Wang et al., 2002). If left uncontrolled, a larger amount of HuR is found in the cytoplasm, which may be linked to several types of cancer and correlate with poor survival prognosis in specific cancers such as colon cancer, ovarian carcinoma, and breast carcinoma (Denkert et al., 2004a; Denkert et al., 2004b; Dixon et al., 2001). This raises the possibility of pharmacologically targeting a signalling pathway upstream of HuR to reduce the amount of HuR in the cytoplasm. Another way to possibly find some target to prevent HuR accumulation in the cytoplasm would be to further study the role of HuR ligands in the control of its nucleocytoplasmic localization.

HuR is known to be primarily nuclear, but it has properties that allow it to shuttle back and forth between the nucleus and the cytoplasm. HuR has five previously identified protein ligands: pp32 (PHAP-I; acidic (leucine-rich) nuclear phosphoprotein 32), APRIL (PHAP-II; acidic protein rich in leucine), SETα, SETβ and transportin 2 (Trn2; karyopherin β2b) (Brennan and Steitz, 2001; Fan et al., 2003; Gallouzi et al., 2000; Gallouzi and Steitz, 2001; Jiang et al., 2003). Among them pp32, APRIL and Trn2 are involved in regulating HuR movement inside the cell (Gallouzi et al., 2000; Gallouzi and Steitz, 2001) by binding to the HNS domain of HuR, which is involved in its cellular movement and interacting with transporters of the nuclear transport system. Hence, familiarity with the nuclear transport system is needed to better understand the role of HuR ligands and localization as they influence how HuR performs its tasks.

In vertebrates, the nuclear pore complex (NPC) permits the bidirectional movement in and out of the nucleus, allowing for the export and import of molecules (Dworetzky and Feldherr, 1988). The NPC allows two modes of transport: passive, energy-independent diffusion, and active, ATP-dependent opening and closing (Rout and Wente, 1994). When found in a closed conformation, the NPC can still allow the passage of molecules no larger than 40 kDa, such as ions, metabolite and small proteins, via diffusion using passive transport (Miller et al., 1991).

On the other hand, active nuclear transport, used by proteins and RNA molecules to be exported or imported, involves transport receptors (exportin and

importin). During export, an exportin protein first bind to nuclear export signal (NES) on the cargo and to Ran-GTP in the nucleus allowing it to engage in multiple low-affinity interactions with phenylalanine-glycine (FG)-rich repeats on the NPC in an energy independent way. Then, the complex passes through the nuclear pore and gets into the cytoplasm. When the cargo gets to the cytoplasm, it dissociates and Ran-GTP is hydrolyzed by the GTPase-activating protein (GAP) producing Ran-GDP that is sent back to the nucleus (Stade et al., 1997). For import process, a component of the import complex (Importin ß), carrying its cargo through interaction with nuclear localising signal (NLS), binds to cytoplasmic fibrils and enters the nucleus through the nuclear pore (Bayliss et al., 2000). After this in the nucleus, in an energy-dependent stage, Ran-GTP causes a conformational change in the complex leading to the dissociation of the cargo from the transport adaptors (Powers et al., 1997; Fornerod et al., 1997; Stade et al., 1997). The asymmetric distribution of RanGTP and RanGDP (Ran gradient) regulates the pick-up and delivery of cargo (Figure 1.5). Hence, the nuclear import and export pathways are similar, however one difference is that RanGTP is involved in the Exportin/cargo recognition and is in fact involved in the export complex, while RanGTP only serves to dissociate the prefabricated import complex (Gorlich et al., 1997).

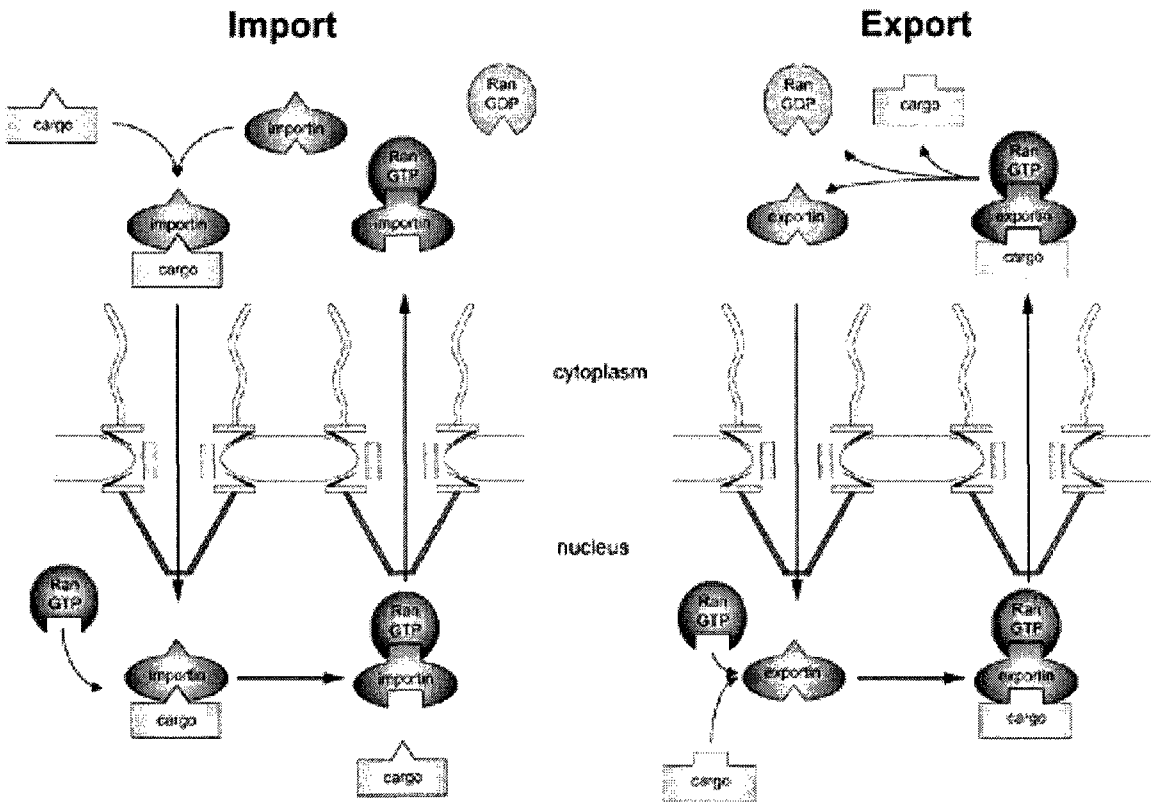


Figure 1.5: Active nuclear transport. Active nuclear transport of a cargo requires the binding to an importin (for import, left) or an exportin (for export, right) receptor. Furthermore, a gradient of RanGTP/RanGDP is required for functional trafficking, where RanGTP is mainly found in nucleus, and RanGDP in the cytoplasm. As well, RanGTP needs to be hydrolyzed in order to release its cargo in the cytoplasm (Köser et al., 2005).

Often a cargo has a preferred importin or exportin, however in several cases a cargo can use more than one pathway. More specifically, in the case of HuR, it was identified to have a minimum two transport pathways that are independent of each other (Gallouzi and Steitz, 2001). Even though HuR has no typical nuclear localising

signal (NLS) and no typical nuclear export signal (NES), the binding of its HNS domain to ligand with import or export signals permits its nucleocytoplasmic movement. The transport pathways used by HuR were identified using the 16 amino acid cell permeable domain of Antennapedia (AP) peptide that allows for rapid cellular uptake of recombinant proteins into most cell types with a high efficiency (>90%) (van der Giessen et al., 2003). Fusion of AP to the NES sequence of CRM1 or to the HNS domain of HuR, competes with the interactions of HuR ligands responsible for its export and import respectively (Brennan and Steitz, 2001; Gallouzi and Steitz, 2001; Rebane et al., 2004; van der Giessen and Gallouzi, 2007).

One of the pathway discovered involved CRM1 (chromosomal region maintenance protein 1). The CRM1 transporter is an export factor of the nucleus that is known to export RNA-BP to the cytoplasm via interaction with leucine rich nuclear export signals (NES). Among the proteins that bind to CRM1 there are pp32 and APRIL, which each contain a NES and also bind to HuR, which itself does *not* have a NES. The interaction between HuR and pp32 or APRIL allows HuR to be exported via the CRM1 pathway by forming a complex of three proteins (Figure 1.6). In fact, it was demonstrated by using Leptomycin B, a drug that specifically inhibits the CRM1 export pathway, that pp32 and APRIL were accumulating in the nucleus and they were associating with increased amounts of HuR. This showed that the association of HuR with export factors is required to be exported using the CRM1 pathway (Brennan et al., 2000). Hence, pp32 and APRIL, are important HuR ligands that regulate its nucleocytoplasmic localization.

The second pathway discovered for the export of HuR was an unknown export pathway. Originally, Trn 2 was thought to be an export factor of mRNA with the help of TAP export factor, but it was later identified, *in vivo* and *in vitro* to be involved in the import of HuR along with Transportin 1 (Trn1 or karyopherin β2A) (Figure 1.6), both members of the karyopherin family (Guttinger et al., 2004; Rebane et al., 2004).

Quite a few RNA-BPs, including HuR, are known to have the ability to mediate both their import and export by binding to different transport factors. Transportin 1 and Transportin 2, which are more than 80% identical, have been shown to interact with HuR and to mediate its import. More precisely, Trn1 and Trn2 were shown to bind specifically but with distinct affinity with the M9 region of hnRNP A1 and the HNS domain of HuR. (Guttinger et al., 2004; Rebane et al., 2004). It was also suggested that the function of Trn1 and Trn2 as import factor of HuR and hnRNPA1 was redundant. Recently however, Trn2 was shown to be a modulator of myogenesis and the specific import factor responsible for the nuclear localization of HuR early on during this process (van der Giessen and Gallouzi, 2007). What is known about its function during myogenesis is discussed in the next chapter, but it is worth mentioning that its role in most processes is still elusive.

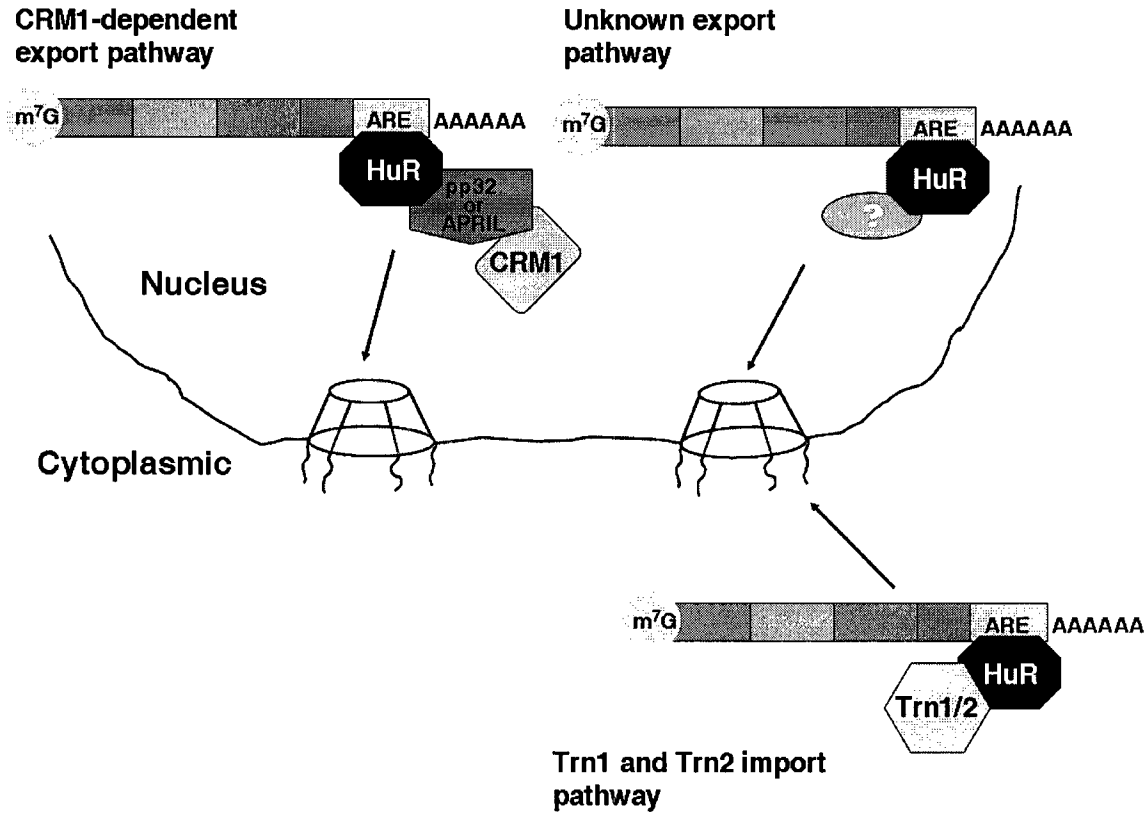


Figure 1.6: Nuclear transport of HuR. HuR can be exported from the nucleus and imported to the nucleus via two pathways. It can be exported via the CRM1-dependent pathway through its association with pp32 or APRIL, or alternatively via an unknown pathway. Its import can be mediated by associating with Trn1 or Trn2.

1.5 The role of HuR in Myogenesis

In order for myogenesis to occur, high levels of MRF proteins must be expressed, and intriguingly, this is managed during the induction of differentiation without an increase in the rate of transcription (Sabourin and Rudnicki, 2000). This is explained by the increased stability of the MRF, *MyoD* and *myogenin* mRNAs upon induction of myogenesis. This increase in the stability of these mRNAs is the result of the binding of HuR to AU-rich sequence in the 3' of these messages (Figure 1.7). This ultimately makes HuR an important regulator of myogenesis.

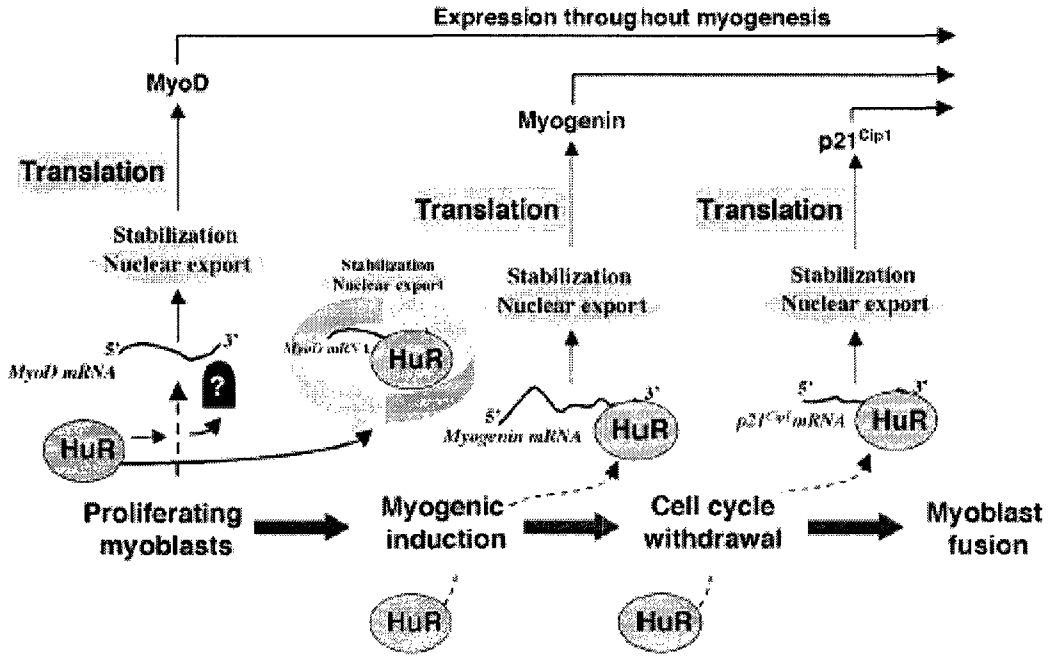


Figure 1.7: Model for the involvement of HuR during myogenesis. The temporal progression of the myoblast-to-myotube transition involves the expression of *MyoD*, *myogenin* and *p21Cip1* mRNAs in a sequential manner. HuR is expressed at a high level throughout the differentiation process. In proliferating myoblasts, HuR indirectly affects the expression of *MyoD* mRNA. Nevertheless, this message is exported and translated. Likewise, in myoblasts that are committed to differentiation (myogenic induction), MyoD expression is enhanced (gray circular arrows) and its message associates with HuR. Thus, HuR enters into play at that time, ensuring the stabilization and export of *MyoD*. Both *myogenin* and *p21Cip1* mRNAs associate with HuR when they are expressed during myogenesis. The induction of myogenin correlates with the upregulation of the cell cycle inhibitor p21Cip1. HuR does not affect the expression of *p21Cip1* mRNA although their interaction correlates with *p21Cip1* mRNA stabilization and rapid export to the cytoplasm. HuR may serve as an escort that ensures that all messages required for myogenesis arrive safely at their cytoplasmic destination. (van der Giessen et al., 2003)

1.5.1 HuR stabilizes promyogenic messages during myogenesis

The importance of HuR in the myogenic process was confirmed by knockdown experiments where reducing the levels of HuR in myoblasts prevented muscle differentiation. However, the myogenic process was totally recovered when HuR expression was rescued (van der Giessen et al., 2003), and was further shown that HuR achieves this by stabilizing the ARE-containing *MyoD*, *myogenin* and *p21* messages (Figueroa et al., 2003; van der Giessen et al., 2003).

The stabilization of *MyoD*, *myogenin* and *p21* mRNAs upon induction of differentiation is mainly due to the stabilization of the AREs in their 3' UTR by HuR (Charge and Rudnicki, 2004; Figueroa et al., 2003). HuR was identified to interact with these sequences by gel shift and by reverse transcribed-PCR of RNA that were co-immunoprecipitated with HuR. By overexpressing HuR in cells, it was found to have a stabilizing effect on *MyoD* and *myogenin* mRNA upon induction of myogenesis (Figueroa et al., 2003; van der Giessen et al., 2003). In fact, the half-life of *MyoD* and *myogenin* mRNAs were shown to almost double in all cases when HuR was overexpressed (Figueroa et al., 2003).

Furthermore, it was demonstrated that the stabilization of *MyoD* and *myogenin* mRNA by HuR during myogenesis could be the result of its accumulation in the cytoplasm (van der Giessen and Gallouzi, 2007). Therefore, regulation of HuR transport system might be important in order to perform its task.

1.5.2 The Transportin 2-mediated cytoplasmic accumulation of HuR and its impact on myogenesis

HuR is known to primarily localize in the nucleus in proliferating myoblasts but upon induction of myogenesis, it becomes more cytoplasmic and this is not associated with a change in its level of expression (van der Giessen et al., 2003). Moreover, this cytoplasmic accumulation correlates with the association of *HuR* with $p21^{CIP1}$ and *myogenin* mRNAs in late differentiation, when these messages are stabilized and expressed (van der Giessen et al., 2003). This suggests a link between HuR localization and its ability to stabilize messages.

It has been shown that HuR accumulation in the cytoplasm occurs in the same time frame as the disruption of the HuR-Transportin 2 complex. This occurs late in muscle differentiation, as shown by co-immunoprecipitating HuR with anti-Trn2 antibody at different days of myogenesis (van der Giessen and Gallouzi, 2007). In fact, the import factor Trn2 is required for HuR nuclear localization in myoblasts and during early differentiation. This was shown by knocking down Trn2 expression in myoblasts, a condition under which HuR accumulates in the cytoplasm and stabilizes *MyoD* and *myogenin* mRNA (van der Giessen and Gallouzi, 2007). Therefore, it seems that relocalization of HuR to the cytoplasm is an important event during myogenesis to stabilize myogenic messages and it is of great importance to understand the mechanism by which HuR-Trn2 complex can be disrupted *in vivo*.

HuR is not only observed to accumulate in the cytoplasm during the myogenic process but also during cell responses to a variety of stresses. Indeed, in the context of cell death following stresses such as ultraviolet radiation (UV), starvation, and proteasome inhibition, no significant difference in HuR expression was observed, though a significant accumulation of HuR in the cytoplasm was shown (Mazroui et al., 2008; Wang et al., 2000a). It was observed that the cytoplasmic accumulation of HuR is controlled by several stress-activated pathways (Lopez de Silanes et al., 2005). Among them, we note the activity of the signalling molecules p38 MAPK and extracellular signal-related protein kinase (ERK) (Tran et al., 2003; Yang et al., 2004). Other proteins, such as AMP-activated protein kinase (AMPK), have the opposite effect, *reducing* the cytoplasmic localization of HuR (Wang et al., 2002).

The import factors that have been shown to bring HuR from the cytoplasm to the nucleus are members of the karyopherin family: Trn1 and Trn2 (Gallouzi and Steitz, 2001; Guttinger et al., 2004; Lee et al., 2006; Rebane et al., 2004). Both of them were first thought to be redundant HuR import factors that bind to the HNS domain of HuR with different affinities (Guttinger et al., 2004; Rebane et al., 2004). More recently however, it was shown that in cell culture during myogenesis, HuR was exclusively interacting with Trn2 during the formation of myotubes and that Trn2 assists in the rapid import of HuR into the nucleus (van der Giessen and Gallouzi, 2007). In fact, in myoblasts and during the early steps of myogenesis, HuR is brought back to the nucleus by Trn2 via the interaction with the HNS domain of HuR, while later during the differentiation process the HuR-Trn2 complex is disrupted and HuR accumulate in the cytoplasm (van der Giessen and Gallouzi, 2007). In this context,

Trn2 seems to act as a regulator of myogenesis by preventing the rapid accumulation of HuR in the cytoplasm, hence preventing premature formation of myotubes. The exact mechanism used by Trn2 to manage this regulation during muscle cell differentiation is still unknown however.

One recent observation of HuR processing may add another layer of complexity to this system. Our group recently showed that during cell death, HuR is cleaved by caspase-3 and -7 at the aspartic acid residue of HuR (D226), which is in the HNS motif of HuR (Mazroui et al., 2008). Since this domain is responsible for the formation of the HuR-Trn2 complex, it is quite reasonable to speculate that caspase-mediated cleavage may be an important posttranslational modification of HuR that influences both its cellular localization, and its association with Trn2 during myogenesis.

1.6 The role of caspases in myogenesis

Caspases, a specific family of cysteinyl aspartyl proteases, are best known to be the downstream effectors of apoptotic cellular death. Even though their roles are well characterized during apoptosis, they are also known to have important functions in other cellular contexts. Intriguingly, some work has linked caspase activity to myogenesis (Fernando et al., 2002).

Caspases normally exist in a procaspase form, but once processed (by another caspase), they cleave proteins at aspartic acid residues. There are two classes of caspases involved in apoptosis: the initiator caspases (caspase-2,-8,-9, and -10) and

the effector caspases (caspase-3,-6, and -7) (Yuan and Horvitz, 2004). They are each part of a cascade that first requires the activation of initiator caspases that lead to the activation of effector caspases. During myogenesis however, myotubes are resistant to apoptosis. Despite this, both processes have many similarities at the cellular and molecular levels. These include the disassembly/reorganization of actin fibers, as well as the expression of myosin light chain (Fernando et al., 2002). Furthermore, when the myogenic process is initiated, cellular mechanisms that regulate other processes such as the p38 subfamily of the mitogen-activated protein kinase (MAPK) family are also activated, (Juo et al., 1997; Molkentin et al., 1995; Puri et al., 2000; Zetser et al., 2001; Zetser et al., 1999). The interplay between these processes suggested that players of one, such as effectors of apoptosis could impact another, such as myogenesis.

1.6.1 The role of caspase 3 in myogenesis

While defining the signal cascades involved in the activation of the MAPK pathway in both apoptosis and muscle differentiation, several groups have demonstrated that the pro-apoptotic factors p53, Rb (retinoblastoma), and caspase 3 are required for the progression of muscle cell differentiation (Fernando et al., 2002; Huh et al., 2004; Porrello et al., 2000). More specifically, in the case of caspase 3, its inhibition leads to a significant decrease in the level of muscle differentiation (Fernando et al., 2002). In fact, the promyogenic role of caspase 3 involves its activation, through cleavage, of Mammalian Sterile Twenty-like kinase (MST1).

MST1 plays an important role in myogenesis because its activation is required for the initiation of myogenesis. Hence, the activation of MST1 is a critical means for caspase 3 to induce myogenesis (Fernando et al., 2002).

Intriguingly, in a model for muscle wasting (cachexia), caspase 3, along with caspases 1, 6, 8 and 9, have been shown to be active (Belizario et al., 2001). Cachexia is a condition associated with the excessive deterioration of skeletal muscles, and is mediated by ubiquitin and proteosome pathways (Di Marco et al., 2005). The aforementioned caspases are cleaved and activated and play a role in the muscle wasting, creating an interesting link between muscle development and wasting.

In the well-characterized context of cellular death, caspase 3 is an effector caspase which acts by proteolytically cleaving several proteins to activate them and modulate their proapoptotic activity (Blanc et al., 2000; Boulares et al., 1999; Le Rhun et al., 1998; Nicholson et al., 1995; Tewari et al., 1995; Wang et al., 1996). During apoptosis, caspase 3 activity has also been specifically linked to the activation of p38 MAPK and JNK pathways (Cardone et al., 1997; Chaudhary et al., 1999; Frasch et al., 1998). Both of these pathways overlap with myogenesis and are considered important regulators of it (discussed earlier). Simultaneously, there exists the possibility that caspase 3 may cleave other important myogenic proteins that are still yet to be discovered (Mukasa et al., 1999).

1.6.2 Caspase-dependent cleavage of HuR upon stress

When cells are stressed and the apoptotic process is activated, HuR is known to accumulate in the cytoplasm (as seen previously) (Mazroui et al., 2008; Wang et al., 2000b). Cytoplasmic HuR is known to promote apoptosis, and also associates with pp32/PHAPI, which has been shown to enhance the activity of the apoptosome. The apoptosome consists of heptamerized Apaf-1, which occurs in the presence of cytochrome c, and this structure allows for procaspase 9 to self-cleave, generating active caspase 9. The initiator caspase 9 then proceeds to activate caspases 3 and 7, which are effectors of apoptosis (Adrain and Martin, 2001). During this stress response, HuR itself is cleaved by caspases 3 and 7 at an aspartic acid residue (D226) at $MGVD_{226}$ site which lies in its HNS domain (Mazroui et al., 2008) (Figure 1.8). What is interesting about this cleavage is the significance it holds during apoptosis, since the use of a non-cleavable isoform (HuRD226A) delays caspase-dependent apoptosis. (Mazroui et al., 2008).

Caspases play an important role during myogenesis and muscle wasting, along with the cleavage of HuR during stress response. Since both HuR and caspase 3 have been shown to affect myogenesis, I have decided to determine if the caspase-mediated cleavage of HuR occurs during myogenesis, and how this may influence muscle differentiation.

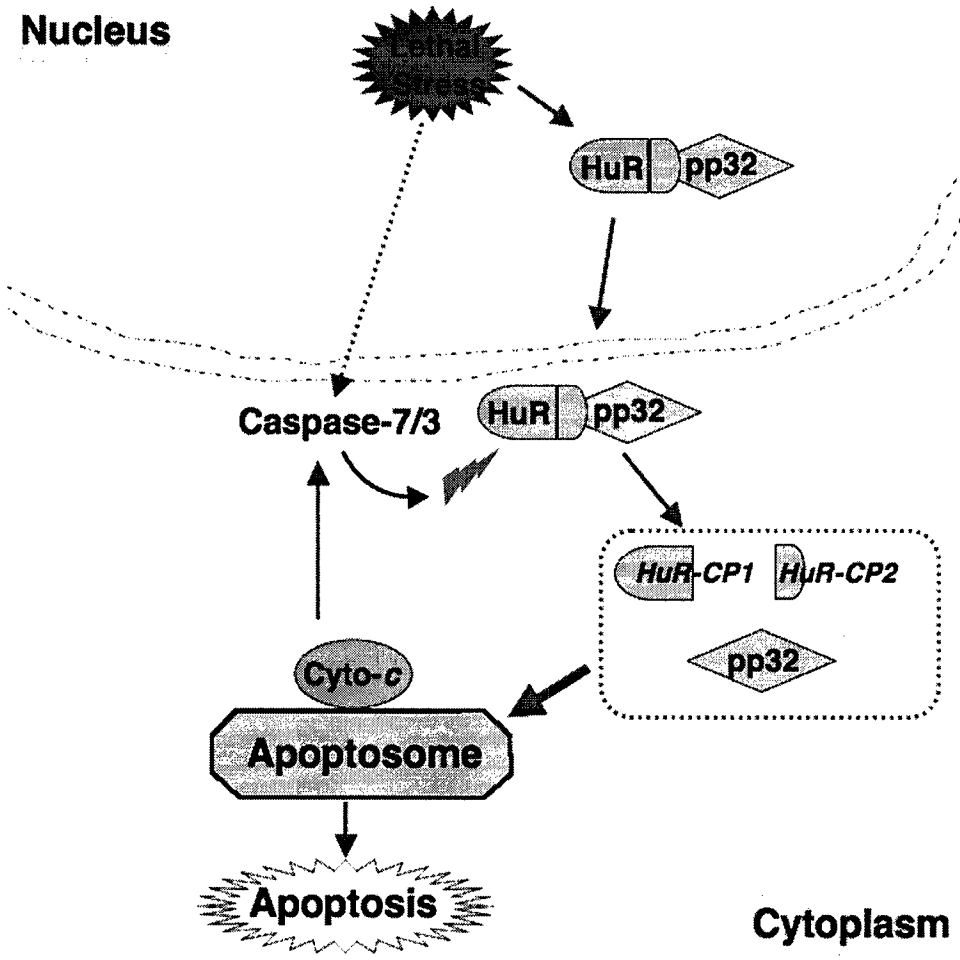


Figure 1.8: HuR cleavage upon stress in HeLa cells. When cells are subjected to stress that leads to apoptosis, HuR travels to the cytoplasm together with its ligand pp32/PHAP-1 where it is cleaved by caspase-7 and -3 producing HuR-CP1 and HuR-CP2. HuR cleavage contributes to the increase in apoptosome activity performed by pp32/PHAP1 (Mazroui et al., 2008)

1.7 Rationale

The protein HuR is a well known RNA-BP that has nucleocytoplasmic shuttling abilities and that can also stabilize ARE-containing mRNAs. In the context of muscular differentiation, HuR stabilizes messages of important myogenic factors (MyoD, myogenin, and p21) and it is essential for myogenesis to occur. Moreover, the nuclear import factor Trn2 was identified as responsible for HuR localization in the nucleus of myoblast prior to differentiation and early during the myogenic process. Then, later during myogenesis, upon disruption of HuR-Trn2 complex, HuR was observed to accumulate in the cytoplasm.

Recently, we observed that the rapid cytoplasmic translocation of HuR also occurs in cells undergoing apoptosis in response to several stresses (Mazroui et al., 2008). Indeed, under lethal conditions, cells initiate the caspase 3/7-mediated cleavage of HuR at the Asp (D) 226 residue, generating two cleavage products (CPs): HuR-CP1 and HuR-CP2. In HeLa cells, these cleavage products collaborate with the HuR protein ligand pp32/PHAP-I to enhance apoptosome activity and apoptosis, while overexpression of the non-cleavable mutant of HuR, HuRD226A, delayed this fatal outcome. The fact that apoptotic players such as p53 and caspase 3 have been shown to affect myogenesis (Fernando et al., 2002; Huh et al., 2004; Porrello et al., 2000) raises the possibility that HuR cleavage could be part of the regulatory mechanisms that affect its pro-myogenic function. In this thesis, I investigate the influence that the caspase dependent cleavage of HuR has on muscle cell differentiation, how the regulation of HuR/Trn2 interplay may also be involved in the observed responses.

2. Results

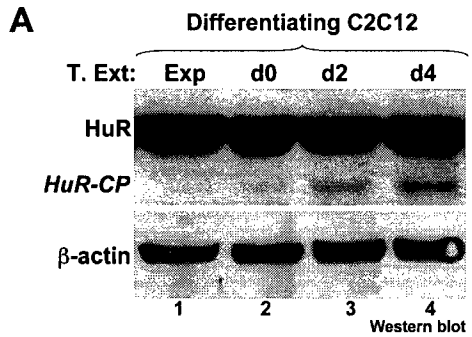
2.1 The caspase-mediated cleavage of HuR is a cytoplasmic event that occurs during myogenesis

It has been shown that caspase-mediated cleavage plays an important role in regulating the activity of some pro-myogenic factors (Fernando et al., 2002). We and others have demonstrated that HuR, which is cleaved by caspases-3 and -7 during apoptosis (Mazroui et al., 2008), is one of the key players during the formation of muscle fibers (Figueroa et al., 2003; van der Giessen et al., 2003; van der Giessen and Gallouzi, 2007). To assess whether HuR could be cleaved by caspases during myogenesis, we performed a Western blot analysis on total extracts from differentiating C2C12 cells (mice embryonic muscle cells) (van der Giessen and Gallouzi, 2007). Using the anti-HuR monoclonal antibody (Gallouzi et al., 2000), we detected a second band at 24kDa, under the 32kDa band of wt-HuR, which began appearing at day 0 of myogenesis with a maximum intensity level at day 4 (Figure 2.1A). The size of this band corresponded to the size of the HuR cleavage product (HuR-CP) previously seen in HeLa cells during caspase-induced stress response (Mazroui et al., 2008). Treating C2C12 cells with the Pan-caspase inhibitor zVAD (Chipuk and Green, 2004) caused a significant reduction in the intensity of the HuR-CP band (Figure 2.1B), suggesting that one or more members of the caspase family are responsible for the generation of this CP.

Next, we determined the cellular location where the cleavage of HuR occurs, during myogenesis. Nuclear and cytoplasmic fractions were prepared from differentiating C2C12 cells and used for Western blot analysis. Using the anti-HuR antibody we detected the 24kDa band in the cytoplasmic fraction as soon as the differentiation process was engaged (Figure 2.1C), but never in the nuclear fraction. Immunofluorescence for HuR in zVAD-treated C2C12 cells at day 2 of differentiation showed that blocking caspase activity also resulted in the nuclear sequestration of HuR (Figure 2.1D). These results argued for a direct relationship between the cytoplasmic localization of HuR that begins at the early steps of muscle fibers formation (van der Giessen and Gallouzi, 2007), and its caspase-mediated cleavage.

Figure 2.1: During myogenesis, HuR is cleaved by caspases in the cytoplasm

(A) Western blot analyses were performed using 100 μg of total protein extracts from exponentially growing (Exp) and differentiating (Day d0 to d4) C2C12 cells. The blot was probed with monoclonal antibody (3A2) against HuR and β-actin as a loading control. **(B)** C2C12 myotubes on Day 3 of differentiation were treated with the pan-caspase inhibitor zVAD for 16 hours and total cell extracts were prepared on Day 4 of differentiation for SDS-PAGE, followed by western blotting to probe for HuR and β-actin. **(C)** Nuclear/cytoplasmic fractionation was performed during C2C12 differentiation, and western blotting analysis was performed on 100 μg of cell extract for each time point. Membranes were probed with antibodies against HuR, β-actin, and hnRNP A1, a nuclear marker for accurate fractionation. **(D)** C2C12 cells treated with DMSO or zVAD upon induction of differentiation were fixed at Day 2, and analyzed by immunofluorescence staining for DAPI and against HuR. Representative images of two independent experiments are shown. Bars, 20 μm.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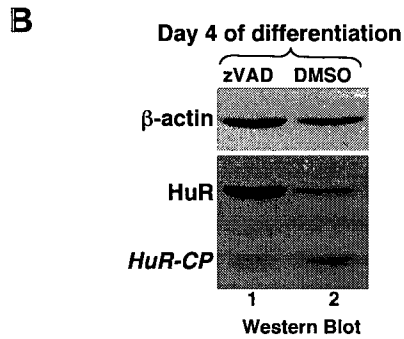
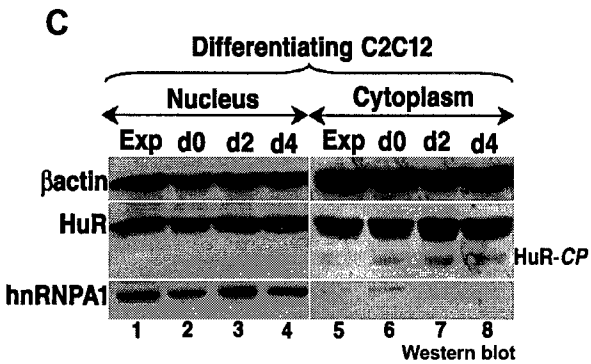
Western blot


B
Day 4 of differentiation
zVAD DMSO
β-actin
HuR
HuR-CP
1 2
Western Blot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Differentiating C2C12
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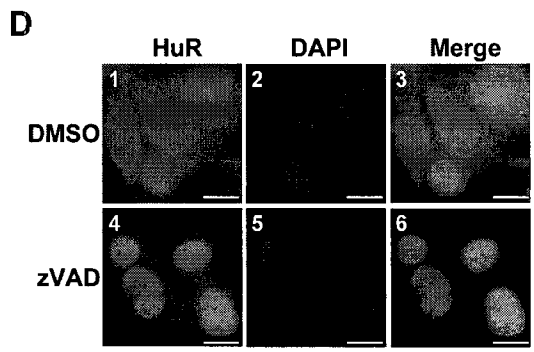
hnRNPA1
1 2 3 4 5 6 7 8
Western blot


D
HuR
DAPI
Merge
DMSO
zVAD
1
2
3
4
5
6

2.2 The caspase-dependent cleavage of HuR occurs during myogenesis at aspartic acid 226 and it is required for proper myogenic differentiation.

The previous study on HuR cleavage during apoptosis has indicated that although the C-terminal region of the human HuR harbours three potential cleavage sites (D226, D254 and D256) only the D226 residue was targeted by caspase-3 and -7 in HeLa cells undergoing apoptosis (Mazroui et al., 2008). Since these three D residues were also conserved in the murine HuR (Figure 2.2A) we decided to replace each of them by Alanine (A) and assess which one of these mutants could prevent the generation of HuR-CPs during myogenesis. Transfection of the GFP-tagged isoforms of these mutants (Figure 2.2B) into C2C12 cells that were induced for differentiation yielded high levels of GFP-HuR-CP in all cases except the D226A mutant (Figure 2.3A). This result showed that, as in HeLa cells, the cleavage of HuR by caspases during muscle cell differentiation occurs at the D226 residue. Next we investigated the impact of the D226A mutation on the pro-myogenic function of HuR. Each of the D-to-A HuR mutants were transfected into differentiating myoblasts, and through immunofluorescence (Figure 2.3B) and fusion index analysis (Coletti et al., 2002) (Figure 2.3C), we saw that only the non-cleavable mutant of HuR, caused a severe reduction in differentiation (Figure 2.3B). Fusion index is a quantitative measure of myogenesis, by calculating the percentage myoblasts that have differentiated. It consists of the ratio of nuclei found in myotube and the total number of nuclei found in a microscopic field. Western blotting on extracts treated in this same way also showed that with transient transfection of the GFP-HuRD226A mutant, the protein

level of myoglobin, a marker of differentiation (van der Giessen et al., 2003), was significantly reduced (Figure 2.3D). Together these observations clearly indicated that the caspase-mediated cleavage of HuR at the D226 residue is involved myotube formation.

Figure 2.2: The potential caspase cleavage sites of HuR are conserved between mouse and human.

(A) ClustalW was used to perform an alignment of human HuR (hHuR; Accession Number NM_001419) and mouse HuR (mHuR; Accession Number NM_010485) amino acid sequences to demonstrate that the cleavable D226 residue is conserved between the species, as well as the other theoretical sites D254 and D256. **(B)** Schematic representation of GFP-tagged wild type HuR (GFP-wt-HuR) and GFP-tagged HuR mutants (GFP-HuRD226A, GFP-HuRD254A, GFP-HuRD256A).

A

```
mouse          1 MSNGYEDHMAEDCRDDIGRTNLIVNYLPQNMTQEELRSLFSSIGEVESAKLIRDKVAGHS
Human          1 MSNGYEDHMAEDCRGDIGRTNLIVNYLPQNMTQDELRSLFSSIGEVESAKLIRDKVAGHS
                 ********************* ********************* **************************

mouse         61 LGYGFVNYVTAKDAERAISTLNGLRLQSKTIKVSYARPSSEVIKDANLYISGLPRTMTQK
Human         61 LGYGFVNYVTAKDAERAINTLNGLRLQSKTIKVSYARPSSEVIKDANLYISGLPRTMTQK
                 ****************** ****************************************

mouse        121 DVEDMFSRFGRIINSRVLVDQTTGLSRGVAFIRFDKRSEAEEAITSFNGHKPPGSSEPIT
Human        121 DVEDMFSRFGRIINSRVLVDQTTGLSRGVAFIRFDKRSEAEEAITSFNGHKPPGSSEPIT
                 ************************************************************

mouse        181 VKFAANPNQNKNMALLSQLYHSPARRFGGPVHHQAQRFRFSPMGVDHMSGISGVNVPGNA
Human        181 VKFAANPNQNKNVALLSQLYHSPARRFGGPVHHQAQRFRFSPMGVDHMSGLSGVNVPGNA
                 ************ ************************************** *********

mouse        241 SSGWCIFIYNLGQDADEGILWQMFGPFGAVTNVKVIRDFNTNKCKGFGFVTMTNYEEAAM
Human        241 SSGWCIFIYNLGQDADEGILWQMFGPFGAVTNVKVIRDFNTNKCKGFGFVTMTNYEEAAM
                 ************************************************************

mouse        301 AIASLNGYRLGDKILQVSFKTNKSHK
Human        301 AIASLNGYRLGDKILQVSFKTNKSHK
                 **************************
```

B

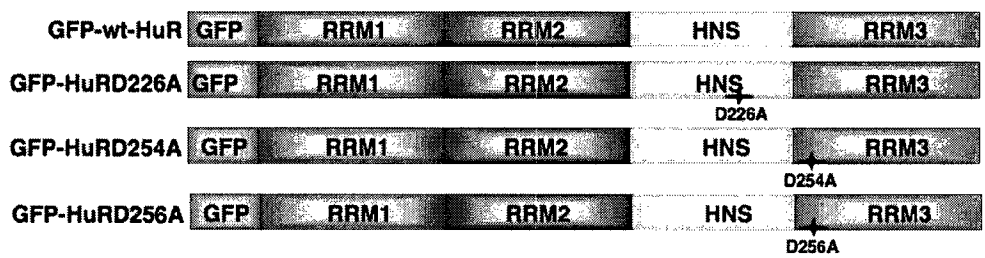
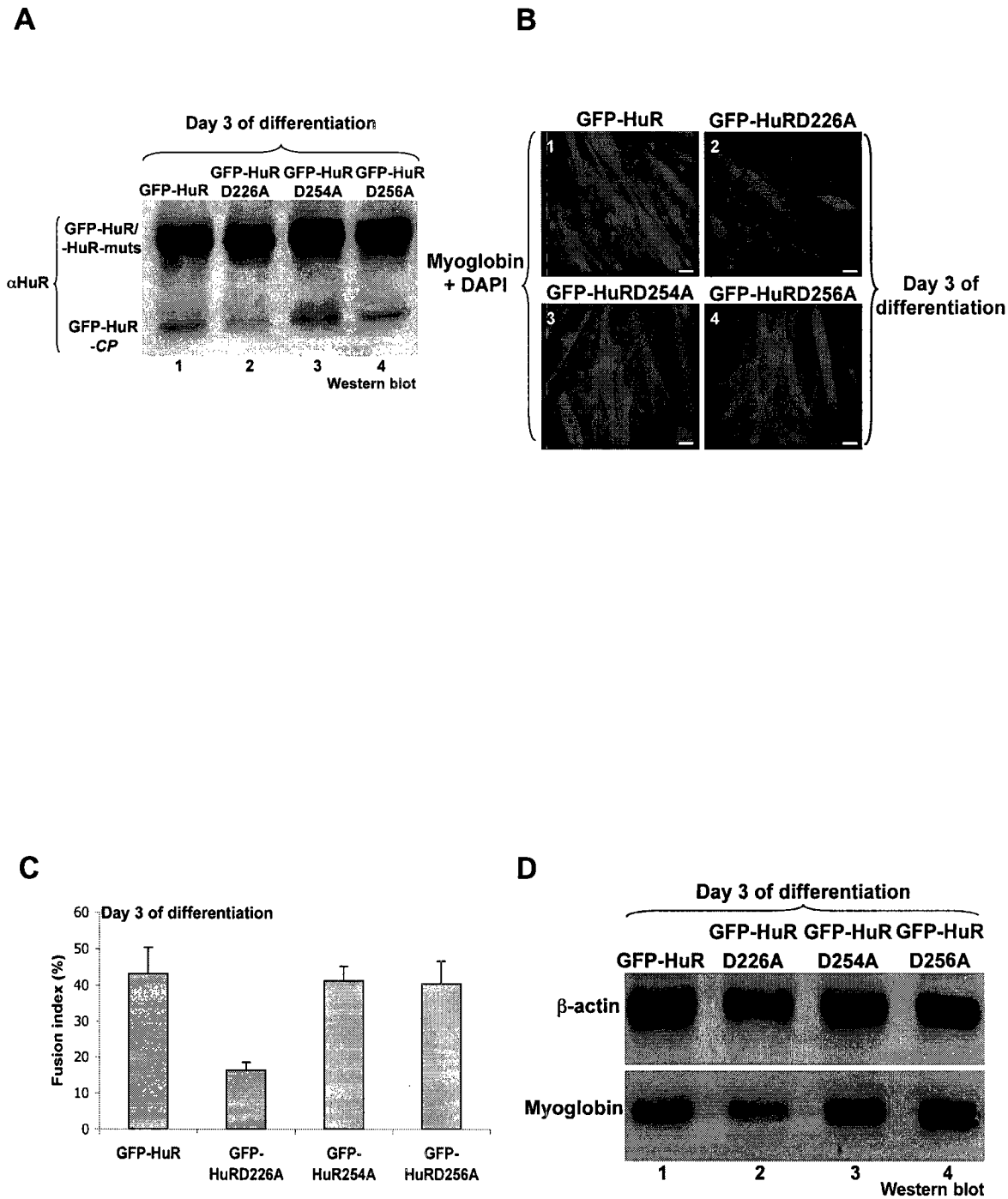


Figure 2.3: The cleavage of HuR at D226 is required for proper myogenesis

(A) C2C12 cells were transfected with GFP-HuR mutants as indicated, and total cell extracts were run on SDS-PAGE for western blot analysis. Probing the membrane with an antibody for HuR demonstrated expression of the transfected mutants (upper band) as well as their cleavage products (lower band). (B) Exponentially growing C2C12 cells were transfected with the GFP-HuR mutants described in Fig. 1D, fixed on Day 3 of differentiation, and immunofluorescence was performed using DAPI and an antibody against myoglobin to stain differentiated myotubes. Representative images of three independent experiments are shown for each HuR mutant. Bars, 20 μm. (C) Immunofluorescence staining with the anti-myoglobin antibody was used to calculate the fusion index (percent differentiation) of the myotubes after each transfection. The fusion index is determined by dividing the number of nuclei in myotubes by the total number of nuclei in a given microscopic field. Error bars represent the Standard Error of the Mean (SEM) of three independent experiments. (D) C2C12 cells were transfected with the indicated GFP-HuR mutants, and total cell extracts from Day 3 of differentiation were run on SDS-PAGE for western blot analysis using antibodies against myoglobin and β-actin. Representative western blots of three independent experiments are shown.


A
Day 3 of differentiation
GFP-HuR
GFP-HuR D226A
GFP-HuR D254A
GFP-HuR D256A
αHuR
GFP-HuR/ -HuR-muts
GFP-HuR -CP
1
2
3
4
Western blot
B
Myoglobin + DAPI
GFP-HuR
GFP-HuRD226A
GFP-HuRD254A
GFP-HuRD256A
Day 3 of differentiation
C
Day 3 of differentiation
Fusion index (%)
60
50
40
30
20
10
0
GFP-HuR
GFP-HuRD226A
GFP-HuR254A
GFP-HuRD256A
D
Day 3 of differentiation
GFP-HuR
GFP-HuR D226A
GFP-HuR D254A
GFP-HuR D256A
β-actin
Myoglobin
1
2
3
4
Western blot

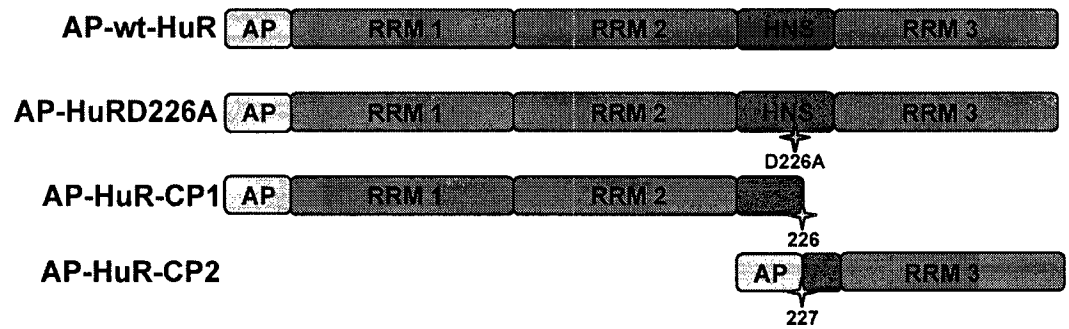
2.3 HuR-CP1 or HuRD226A inhibit myotube formation, while HuR or HuR-CP2 enhances myogenesis

During apoptosis in HeLa cells, we previously observed that a non-cleavable variant of HuR, HuRD226A, as well as the two HuR CPs play an important roles in modulating apoptosome activation (Mazroui et al., 2008). To investigate the effect of these HuR isoforms on the process of muscle differentiation, we overexpressed HuR, HuRD226A, and the HuR cleavage products, CP-1 and -2 in myoblast (C2C12) cells. To achieve high cellular uptake of these isoforms, they were conjugated to the cell-permeable peptide Antennapedia (AP) (Gallouzi and Steitz, 2001) (Figure 2.4A), which allowed their rapid and efficient uptake (>90%) into muscle cells (Figure 2.4B). Upon induction of differentiation, myotube formation appeared to be affected by the presence of these proteins, as assessed by the fusion index of the myotubes (Figure 2.5A-B). Moreover, there was a small but reproducible inhibition of the expression levels of myogenin and myosin heavy chain (Figure 2.5C). More precisely, we observed that myoblast fusion and myotube formation were significantly reduced in the presence of either AP-HCP1-GST or AP-HuRD226A-GST up to day 3 of differentiation, when compared to control AP-GST (Figure 2.5). In contrast, a slight increase in the fusion index was observed when cells were treated with either AP-HuR-GST or AP-HCP2-GST (Figure 2.5A-B). Therefore, the overexpression of HuR mutants during myogenesis demonstrates that they have a potential role in this process.

Figure 2.4: C2C12 cells uptake the AP-conjugated HuR isoforms with high efficiency.

(A) Schematic of AP-tagged (C-terminus) and GST-tagged (N-terminus) wild type HuR (HuR), non-cleavable HuR (HuRD226A), HuR-CP1, and HuR-CP2. **(B)** C2C12 cells were treated twice with AP-GST, AP-HuR-GST, AP-HuR-CP1-GST, AP-HuR-CP2-GST, AP-HuRD226A-GST both 24 h before the induction of differentiation, and also at the induction of differentiation (day 0) when cells reached 100% confluency. The expression of AP-recombinant proteins was monitored by western blot using an antibody against GST, on total cell extracts from day 1 of differentiation. Representative western blots of two independent experiments are shown.

A


AP-wt-HuR
AP
RRM 1
RRM 2
HNS
RRM 3
AP-HuRD226A
D226A
AP-HuR-CP1
226
AP-HuR-CP2
227

B

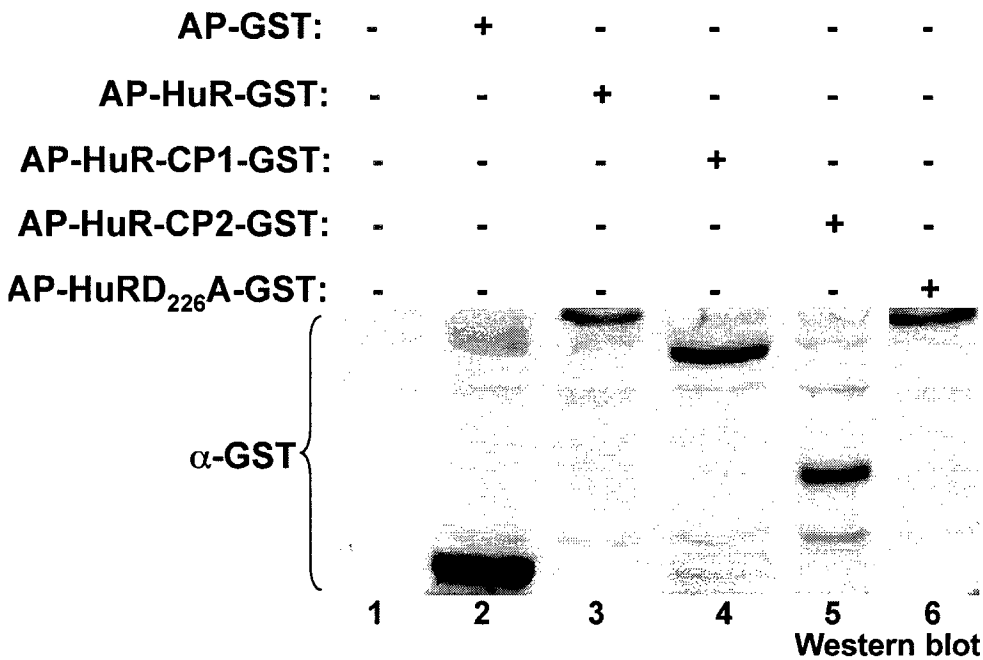
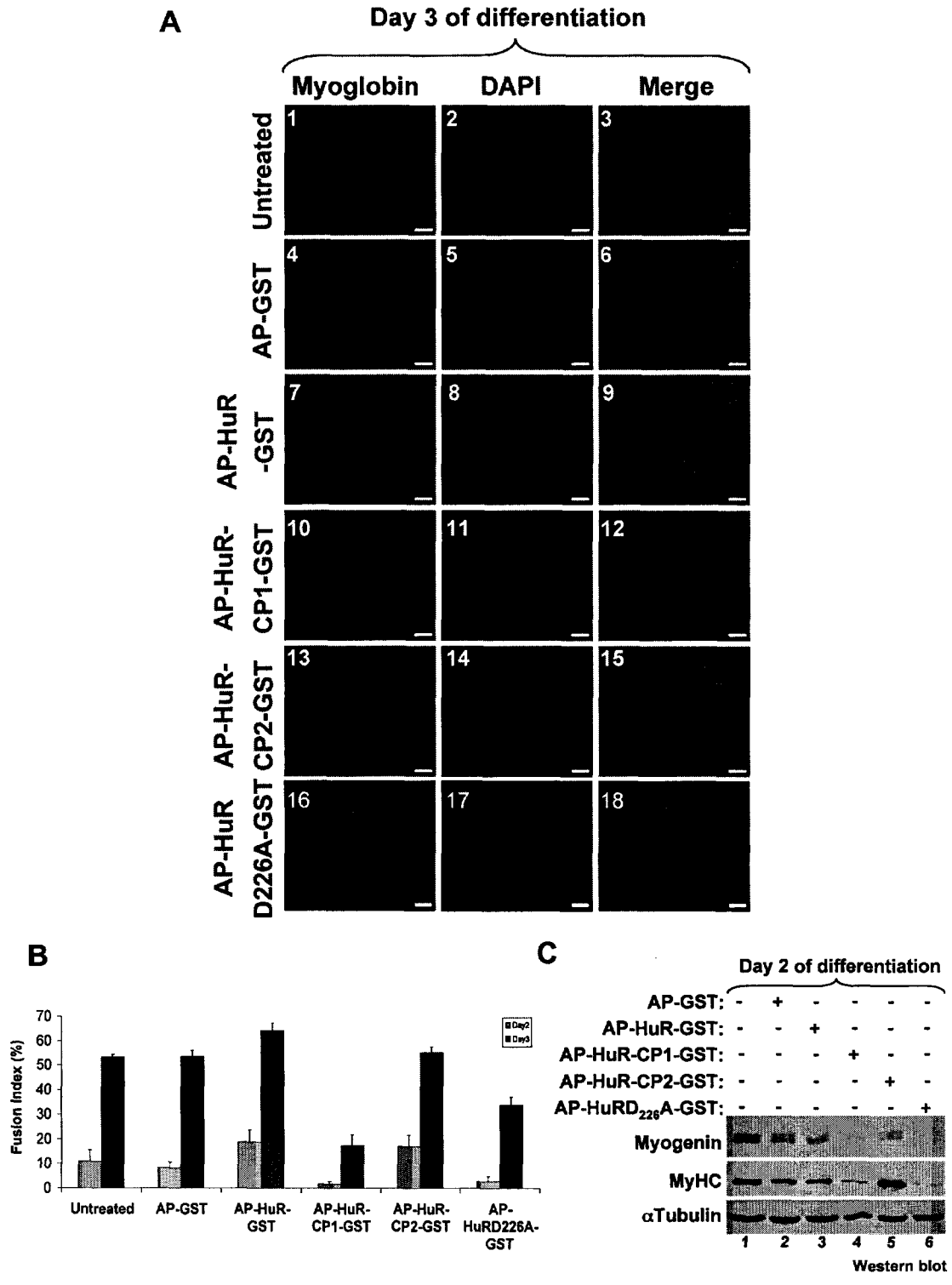

AP-GST: - + - - - -
AP-HuR-GST: - - + - - -
AP-HuR-CP1-GST: - - - + - -
AP-HuR-CP2-GST: - - - - + -
AP-HuRD226A-GST: - - - - - +
α-GST
1 2 3 4 5 6
Western blot

Figure 2.5: Muscle cell differentiation is inhibited by the overexpression of HuRD226A or HuR-CP1, and promoted by wild type HuR or HuR-CP2.

(A) C2C12 cells were treated with AP-GST, AP-HuR-GST, AP-HuR-CP1-GST, AP-HuR-CP2-GST, AP-HuRD226A-GST as described in figure 2.4. The AP-conjugated protein-treated cells were fixed and immunofluorescence staining was performed with DAPI and antibodies against myoglobin on C2C12 cells at days 2 and 3 of differentiation. A single representative field for each cell treatment at day 3 of differentiation is shown. Bars, 20 μm. (B) The fusion index is shown and was calculated as described in Figure 2.1. Error bars represent the SEM of two independent experiments. (C) C2C12 cells were treated with the different AP-conjugated recombinant proteins as described above. Cells were collected at day 2 of differentiation and western blot analysis was performed on the cell extracts. The blot was probed for myogenin, myosin heavy chain (MyHC), and α-Tubulin. Representative Western blots of two independent experiments are shown.


A
Day 3 of differentiation
Myoglobin
DAPI
Merge
Untreated
AP-GST
AP-HuR -GST
AP-HuR-CP1-GST
AP-HuR-CP2-GST
AP-HuR D226A-GST
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
B
Fusion Index (%)
70
60
50
40
30
20
10
0
Day2
Day3
Untreated
AP-GST
AP-HuR-GST
AP-HuR-CP1-GST
AP-HuR-CP2-GST
AP-HuRD226A-GST
C
Day 2 of differentiation
AP-GST: - + - - - -
AP-HuR-GST: - - + - - -
AP-HuR-CP1-GST: - - - + - -
AP-HuR-CP2-GST: - - - - + -
AP-HuRD226A-GST: - - - - - +
Myogenin
MyHC
αTubulin
1 2 3 4 5 6
Western blot

2.4 The cleavage products of HuR, CP1 and CP2, execute different functions during myotube formation

The murine HuR has the same molecular weight (32 kDa) than its human counterpart. Hence, we concluded that the caspase-mediated cleavage of HuR in differentiating muscle cells generates two HuR CPs, HuR-CP1 (24kDa) and HuR-CP2 (8kDa). In order to define the functional relevance of HuR CPs in myogenesis, we performed rescue experiments using the four HuR isoforms, wt-HuR, HuRD226A, HuR-CP1 and HuR-CP2, on muscle cells depleted of endogenous HuR by small interfering RNA (siRNA) (van der Giessen et al., 2003). To achieve an efficient rescue we used the AP-conjugated HuR isoforms described above (Figure 2.4A). As expected using siRNA duplexes against HuR mRNAs (siRNA-HuR) and control (siRNA-Ctr) (van der Giessen et al., 2003), we observed that the depletion of HuR expression by ~80% (Figure 2.6) prevented muscle cell differentiation (Figure 2.7A, compare panels 1, 2). These HuR-depleted myoblasts were then treated with 50 nM of each AP-conjugated isoform alone or in combination, both, one day before, and on the day of, differentiation induction (Figure 2.7). Phase contrast images (Figure 2.7A) and immunofloresence experiments (Figure 2.7B) showed that HuRD226A failed to rescue myoblast fusion in siRNA-HuR-treated cells. This demonstrated the implication of caspase-mediated cleavage in the pro-myogenic function of HuR. Using the AP-conjugated HuR CPs in the same experiments, we observed that while HuR-CP1 did not reestablish the myotube formation in siRNA-HuR-treated cells, HuR-CP2, alone or with -CP1, promoted myotube formation similarly to wt-HuR (Figure 2.7C). These observations demonstrated that during myogenesis, HuR-CP2

participates in the promotion of muscle fiber formation. Moreover, when wt-HuR was added in combination with HuRD226A, no rescue occurred (Figure 2.7A panel 14 and Figure 2.7B panel 40), showing the dominant-negative role of HuRD226A. This was also seen when HuRD226A was added to siRNA-Ctr-treated cells (Figures 2.7A panel 9, 2.7B panel 37 and 2C). Additionally, while HuR-CP1 alone caused no rescue, HuR-CP2 not only gave rise to myotubes in siRNA-HuR-treated cells but also enhanced, although slightly, the fusion efficiency of siRNA-Ctr-treated myoblasts (Figures 2.7A panels 5-6, 2.7B panels 21-22 and 2.7C). These data and the fact that HuR-CP1 alone prevented muscle cell fusion in control cells (Figures 2.7A, panel 5 and 2.7B, panels 19-21) clearly indicated that both HuR-CP1 and -CP2 play different roles in the myogenic process with HuR-CP2 being the activator and HuR-CP1 being the moderator. Therefore, it was not surprising to see that introducing the moderator HuR-CP1 at early stages of myogenesis [a time when endogenous HuR is not normally cleaved (Figures 2.1A and C)] causes a defect in muscle fiber formation (Figure 2.5). This could explain in part why under physiological conditions, muscle cells trigger the cleavage of HuR only later during the myogenic process (day 3-4) (Figure 2.1A), when HuR is more needed in the cytoplasm to participate in myotube formation (Figueroa et al., 2003; van der Giessen et al., 2003; van der Giessen and Gallouzi, 2007).

Figure 2.6: siRNA-mediated knockdown of HuR.

C2C12 cells treated with siRNA-HuR or siRNA-Ctr as described (van der Giessen et al., 2003) were induced for differentiation. 5 μg of total cell extracts were collected on Day 0 of differentiation and used for western blot analysis with antibodies against HuR, and α–Tubulin as a loading control. Representative western blots of three independent experiments are shown.

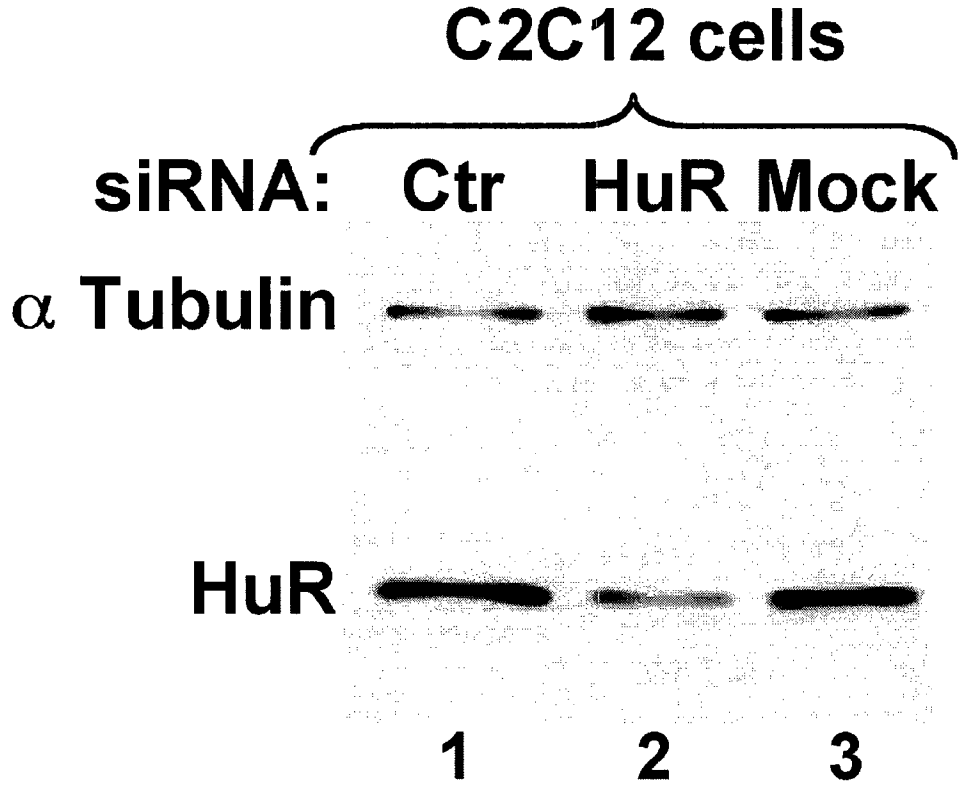
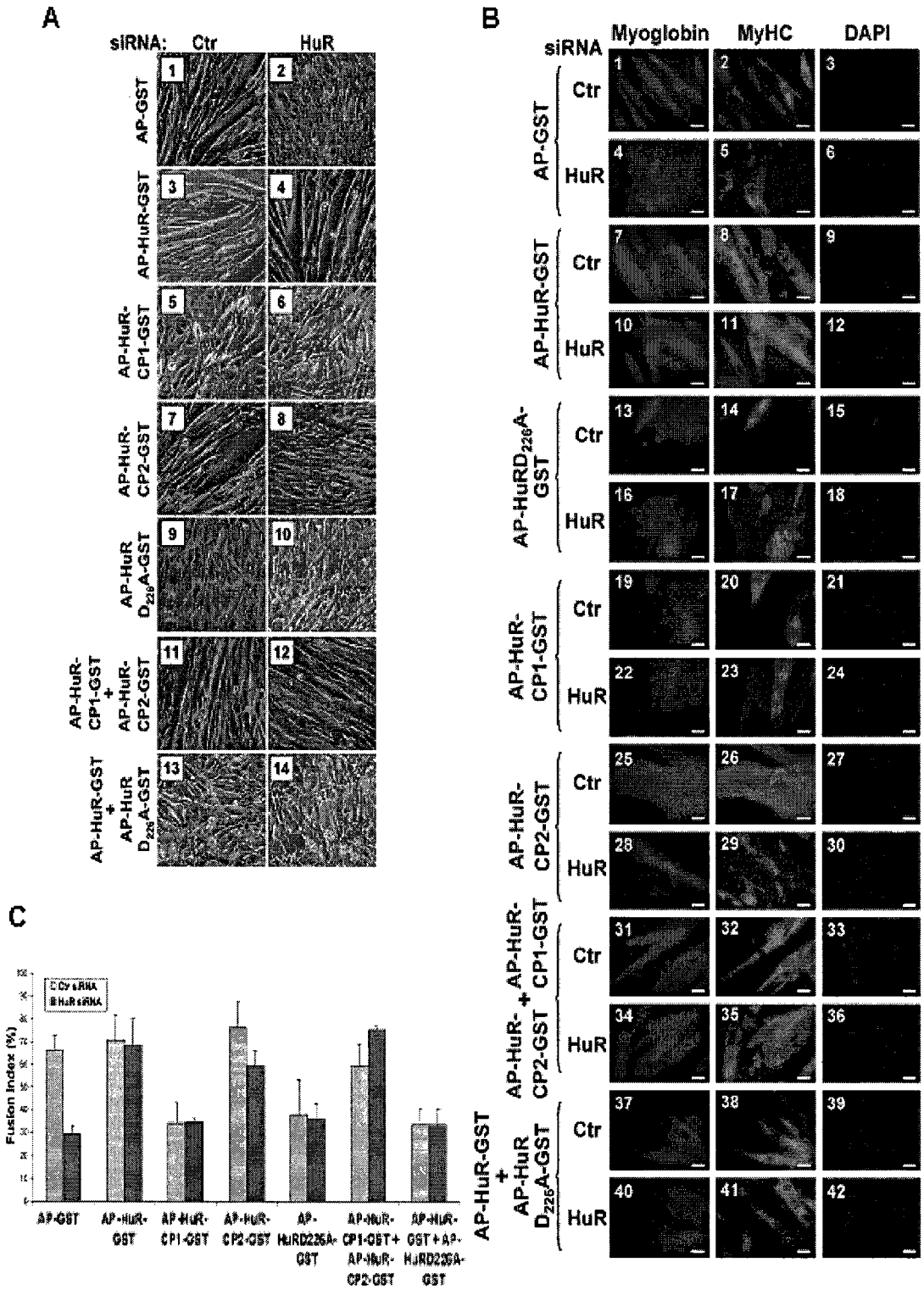

C2C12 cells
siRNA: Ctr HuR Mock
α Tubulin
HuR
1 2 3

Figure 2.7: HuR-CP2 but not HuR-CP1 or HuRD226A reestablished the myogenic process in muscle cells lacking endogenous HuR.

(A to C) AP-conjugated recombinant proteins (Figure 2.4) alone or in combination were added twice to HuR- and control (Ctr)-siRNA-treated C2C12 cells (24 h prior to differentiation initiation and at the induction of differentiation). AP-GST and -HuR-GST were used respectively as negative and positive controls. **(A)** Phase-contrast pictures of a single representative field of view for each cell treatment on day 3 of differentiation. **(B and C)** HuR- and control (Ctr)-siRNA treated cells were fixed and immunofluorescence staining was performed for DAPI and against myoglobin and the myosin heavy chain (MyHC) on the C2C12 cells at day 3 of differentiation. **(B)** A single representative field for each cell treatment is shown. Bars, 20 μm. **(C)** The fusion index is shown and it was calculated as described in Figure 2.1. Error bars represent the SEM of two independent experiments.


A
siRNA: Ctr HuR
AP-GST
AP-HuR-GST
AP-HuR-CP1-GST
AP-HuR-CP2-GST
AP-HuR D228A-GST
AP-HuR-CP1-GST + AP-HuR-CP2-GST
AP-HuR-GST + AP-HuR D228A-GST
B
siRNA
Myoglobin
MyHC
DAPI
Ctr
HuR
AP-GST
AP-HuR-GST
AP-HuRD226A-GST
AP-HuR-CP1-GST
AP-HuR-CP2-GST
AP-HuR-CP1-GST + AP-HuR-CP2-GST
AP-HuR-GST + AP-HuR D226A-GST
C
Ctr siRNA
HuR siRNA
Fusion Index (%)
AP-GST
AP-HuR-GST
AP-HuR-CP1-GST
AP-HuR-CP2-GST
AP-HuRD226A-GST
AP-HuR-CP1-GST + AP-HuR-CP2-GST
AP-HuR-GST + AP-HuRD226A-GST

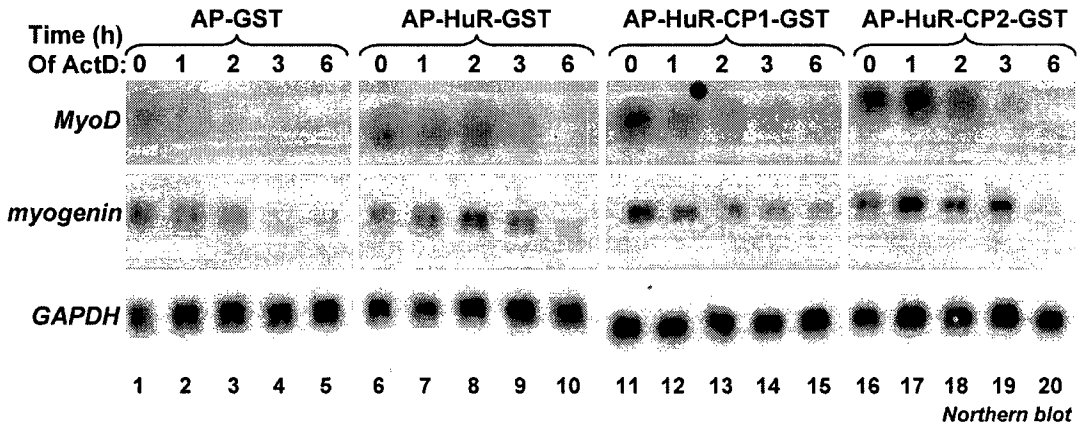
2.5 HuR-CP2 but not -CP1, stabilizes *MyoD* and *myogenin* mRNAs to the same levels as wt-HuR

The fact that only a small portion of cytoplasmic HuR is cleaved raises the possibility that the role of HuR CPs is to help the remaining non-cleaved HuR execute its pro-myogenic function. It has been demonstrated that HuR promotes myogenesis by stabilizing the *MyoD* and *myogenin* messages (Figueroa et al., 2003; van der Giessen and Gallouzi, 2007), and so we assessed the effect HuR-CPs can have on the fate of these mRNAs during myogenesis. We performed an Actinomycin D (ActD) [inhibitor of pol-II-mediated transcription (Dormoy-Raclet et al., 2007)] pulse chase experiment on differentiating C2C12 cells in the presence of AP-conjugated wt-HuR, HuR-CP1, HuR-CP2, and GST as a control (Figure 2.8). We observed that the half-lives of these messages have increased by ~120 min (*MyoD*) and ~180 min (*myogenin*) in the presence of wt-HuR or HuR-CP2, and by only ~60 min in the presence of HuR-CP1 (Figures 2.8A-C). These results indicated that one of the main functions of HuR-CPs, particularly -CP2, is to participate in the HuR-mediated stabilization of its mRNA targets during myogenesis.

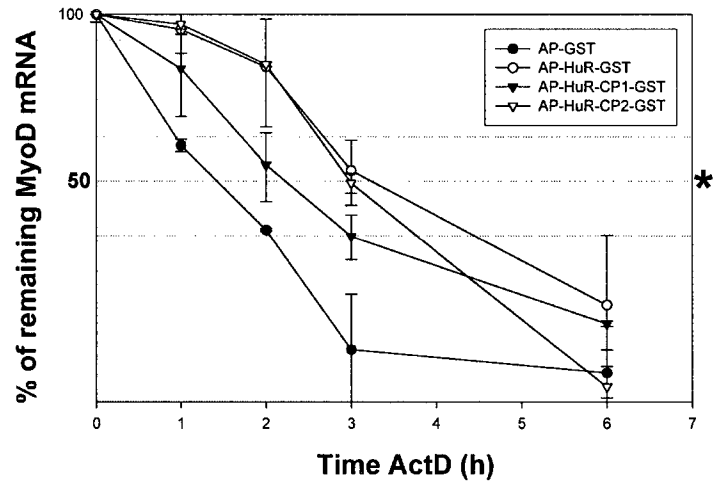
Figure 2.8: HuR-CP2, but not –CP1, mediates *MyoD* and *myogenin* mRNA stability at the same levels as wt-HuR.

(A) Differentiation was induced in C2C12 cells, which were then treated, 16h later, with AP-GST, -HuR-GST, -HuR-CP1-GST, or -HuR-CP2-GST. Approximately 1h after AP-treatment, cells were treated with actinomycin D (ActD) for 0, 1, 2, 3, or 6h, immediately after which total RNA was collected. Northern blot analysis was performed on the RNA extracts to assess the stability of *MyoD* and *myogenin* mRNA using radiolabeled probes. *GAPDH* was used as a loading control. Representative northern blots of two independent experiments are shown. The white lines indicate that intervening lanes have been spliced out. **(B and C)** Stability of *MyoD* (B) and *myogenin* (C) mRNA in cells treated as described in A were quantified using the Image Quant software (Molecular Dynamics, USA) to evaluate band intensities. The percent remaining of *MyoD* and *myogenin* mRNAs was defined as the relative intensity of each message to *GAPDH* bands at each time point and was calculated as a percent of the abundance of each message at 0 h of ActD treatment, and plotted on a logarithmic curve. The star (*) at the left of each plot indicates the line of the 50% remaining mRNA relative to the original abundance of message, and therefore the half-life of the message under the different treatments. Error bars represent the SEM of two independent experiments.

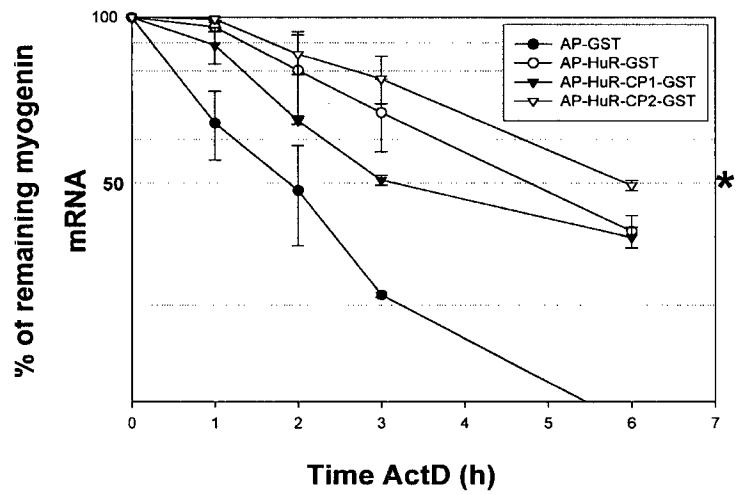
A


Time (h)
Of ActD:
AP-GST
AP-HuR-GST
AP-HuR-CP1-GST
AP-HuR-CP2-GST
0 1 2 3 6
MyoD
myogenin
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Northern blot

B


% of remaining MyoD mRNA
100
50
0 1 2 3 4 5 6 7
Time ActD (h)
AP-GST
AP-HuR-GST
AP-HuR-CP1-GST
AP-HuR-CP2-GST
*

C


% ot remaining myogenin mRNA
100
50
0 1 2 3 4 5 6 7
Time ActD (h)
AP-GST
AP-HuR-GST
AP-HuR-CP1-GST
AP-HuR-CP2-GST
*

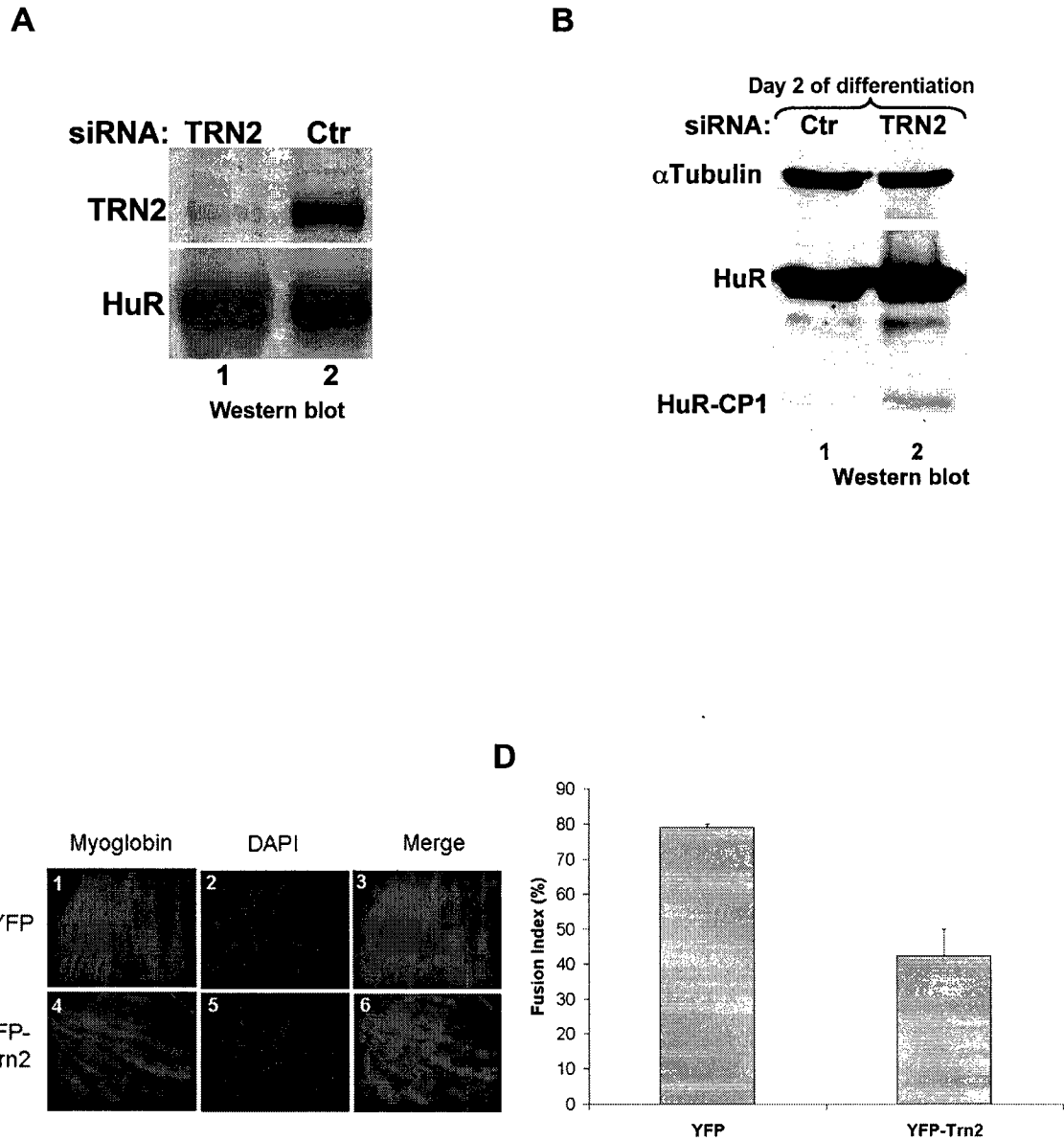
2.6 HuR-CP1 and HuRD226A, but not HuR-CP2, interacts with the import receptor Trn 2

One of the key events that has recently been shown to play an important role in the pro-myogenic function of HuR is the disruption of its association with the import receptor Trn2 (van der Giessen and Gallouzi, 2007). It was observed that in proliferative myoblasts and before the formation of myotubes (day 0 to 2), Trn2 acts as a myogenic moderator by ensuring the presence of HuR in the nucleus, where it is needed for a currently unknown function. Then during cell fusion and myotubes formation HuR becomes cytoplasmic as a result of its release from the HuR-Trn2 complex leading to the maintenance of the HuR-mediated stabilization of *MyoD* and *myogenin* mRNAs. Our experiments described in Figure 2.1 showed that physiologically the generation of HuR-CP1 and -CP2 (Figures 2.1A, C) coincides with the dissociation between HuR and Trn2 (van der Giessen and Gallouzi, 2007). To assess the link between this dissociation and the caspase-mediated cleavage of HuR, we followed the appearance of HuR-CP1 in differentiating muscle cells that were lacking Trn2 expression. Western blot analysis on total extracts from cells treated with specific siRNA duplexes against *Trn2* mRNAs showed that the depletion of Trn2 yielded >50% increase in the cleavage of HuR (Figures 2.9A-B). As previously demonstrated (van der Giessen and Gallouzi, 2007) this effect correlated with a significant increase in the efficiency of muscle cell differentiation (Figure 2.15A, compare panels 1 and 4). Furthermore, when overexpressed in myoblast, Trn2 causes an important decrease in the fusion index of cell transfected with YFP-Trn2

compared to those transfected with the control YFP (Figure 2.9C-D). Of note, we observed that Trn2 overexpressing cells generated a shorter myotubes with less fused nuclei when compared to the control (Figure 2.9C-D). Hence, it is possible that under normal conditions the maintenance of the HuR-Trn2 complex plays an important role in preventing the early cleavage of HuR during the myogenic process.

Figure 2.9: Knocking down Trn2 expression enhances caspase-dependent cleavage of HuR while overexpressing it represses differentiation.

(A-B) C2C12 myoblasts were treated with TRN2 siRNA duplex or control siRNA (Ctr) and differentiation was induced for 2 days when 100 % confluency was reached for (B). **(A)** Cells were collected the day after Trn2 knockdown and Western blot analysis was performed using anti-Trn2 and HuR antibodies. HuR was used as loading control. **(B)** Cells were collected at day 2 of differentiation and Western blot analysis was performed using anti-αTubulin and HuR antibodies. Anti-αTubulin was used as a loading control. Representative Western blots of two independent experiments are shown. **(C)** C2C12 myoblasts were transfected twice with YFP or YFP-Trn2, once at 50% confluency and the second time at 75% confluency, before inducing differentiation the day after. Cells were fixed at day 2 of differentiation and DAPI as well as immunofluorescence staining was performed using anti-myoglobin. A single representative field for each cell treatment is shown. Bars, 20 μm. **(D)** The fusion index is shown, calculated as described in Figure 2.1. Error bars represent the SEM of two independent experiments.


A
siRNA: TRN2 Ctr
TRN2
HuR
1 2
Western blot
B
Day 2 of differentiation
siRNA: Ctr TRN2
αTubulin
HuR
HuR-CP1
1 2
Western blot
C
Myoglobin
DAPI
Merge
YFP
YFP-Trn2
1
2
3
4
5
6
D
Fusion Index (%)
90
80
70
60
50
40
30
20
10
0
YFP
YFP-Trn2

Our previous study has shown that the pro-apoptotic effect of HuR is mediated by the association of the apoptosome activator pp32/PHAP-I with HuR-CP2 but not -CP1 (Mazroui et al., 2008). Therefore, it is possible that the moderatory effect of Trn2 at early stages of myogenesis could involve its association with one or both of HuR-CPs. To test this possibility, we followed the interaction between Trn2 and all HuR isoforms. We performed co-immunoprecipitation experiments using anti-Trn2 antibody on total cell extracts of differentiating muscle cells that were previously transfected with GFP-tagged HuR isoforms (Figure 2.10). Western blot analysis using the anti-HuR antibody (3A2) showed that both HuRD226A and HuR-CP1 interact with Trn2 to a greater extent than wt-HuR (Figure 2.11A). Since the epitope recognized by the 3A2 antibody is located in the RRM1 motif of HuR (Gallouzi et al., 2000), HuR-CP2 could not be detected in this experiment (Figure 2.11A lower panel). Western blotting using anti-GFP antibody was then used in the Trn2-immunoprecitation experiments described above, and we did not detect any interaction between HuR-CP2 and Trn2 (Figure 2.11B, lanes 5-8). Additionally, we observed that the overexpression of these four HuR isoforms resulted in the disruption of the association between Trn2 and endogenous HuR (end-HuR) in all cases except for HuR-CP2 (Figure 2.11A, compare end-HuR in all four panels). These results demonstrated that wt-HuR associates with Trn2 via a domain located in HuR-CP1. Therefore, the inhibitory effect of HuR-CP1 and HuRD226A (Figures 2.5 and 2.7) could be mediated via their strong interaction with Trn2.

Figure 2.10: All GFP-tagged HuR mutants express at high levels in C2C12 cells.

C2C12 myoblasts were transfected with GFP, GFP-HuR, GFP-HuR-CP1, GFP-HuR-CP2, or GFP-HuRD226A plasmids. 48h later, cells were collected and Western blot analysis was performed as described above using 15 μg of total cell extract, and the blot was probed with an antibody against GFP. Representative Western blots of three independent experiments are shown.

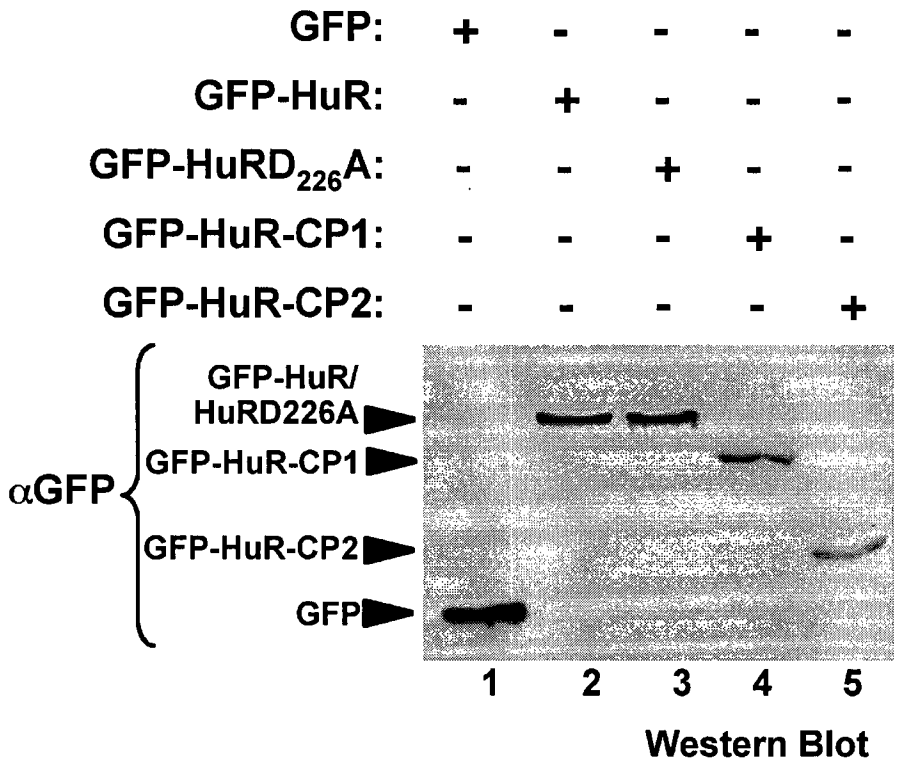
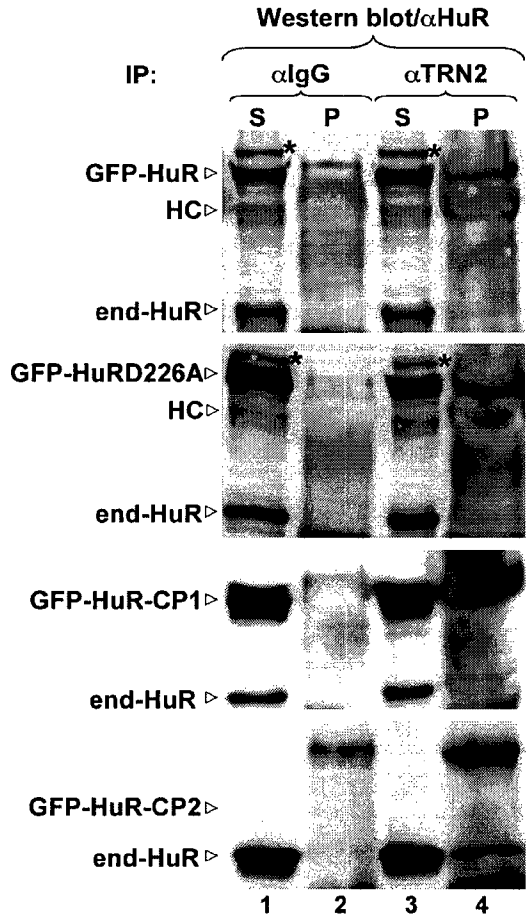

GFP:
GFP-HuR:
GFP-HuRD226A:
GFP-HuR-CP1:
GFP-HuR-CP2:
+ - - - -
- + - - -
- - + - -
- - - + -
- - - - +
αGFP
GFP-HuR/
HuRD226A
GFP-HuR-CP1
GFP-HuR-CP2
GFP
1 2 3 4 5
Western Blot

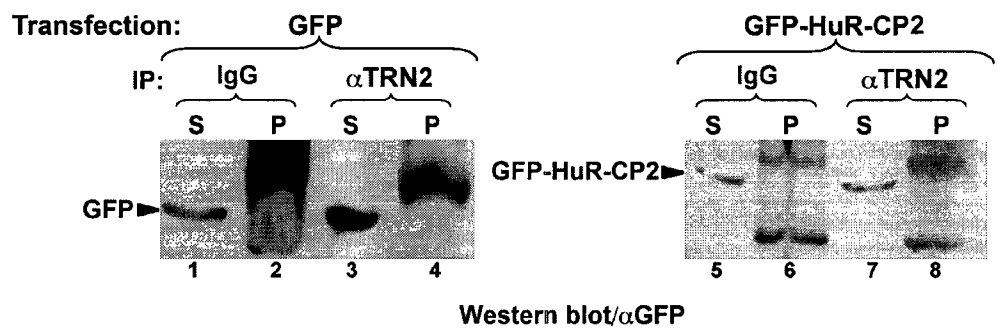
Figure 2.11: HCP1 and HuRD226A, but not HCP2, interact with the nuclear transporter TRN2

(A-B) Total extracts from transfected C2C12 cells with GFP-tagged HuR, HuR-D226A, HuR-CP1 and HuR-CP2 described in Figure 2.10 were used for an immunoprecipitation with the anti-TRN2 antibody, or IgG control, bound to protein A sepharose beads. The immunoprecipitate (P) and the supernatant (S) were analyzed by Western blot using antibodies against HuR (A) and GFP (B). (A) The bands marked with an asterisk (A – lanes 1 and 3) represent an unspecific band. HC indicates heavy chain of anti-TRN2 antibodies, and End-HuR is the endogenous HuR found in the cells. The position of GFP, GFP-HuR, GFP-HuR-CP1, GFP-HuR-CP2 and GFP-HuRD226A proteins are indicated. Representative western blots of two independent experiments are shown.

A


Western blot/αHuR
IP:
αIgG
αTRN2
S
P
S
P
GFP-HuR
HC
end-HuR
GFP-HuRD226A
HC
end-HuR
GFP-HuR-CP1
end-HuR
GFP-HuR-CP2
end-HuR
1
2
3
4

B


Transfection:
GFP
GFP-HuR-CP2
IP:
IgG
αTRN2
IgG
αTRN2
S
P
S
P
S
P
S
P
GFP
GFP-HuR-CP2
1
2
3
4
5
6
7
8
Western blot/αGFP

2.7 The HuR-CP1 modulates myogenesis in a Trn2-dependent manner

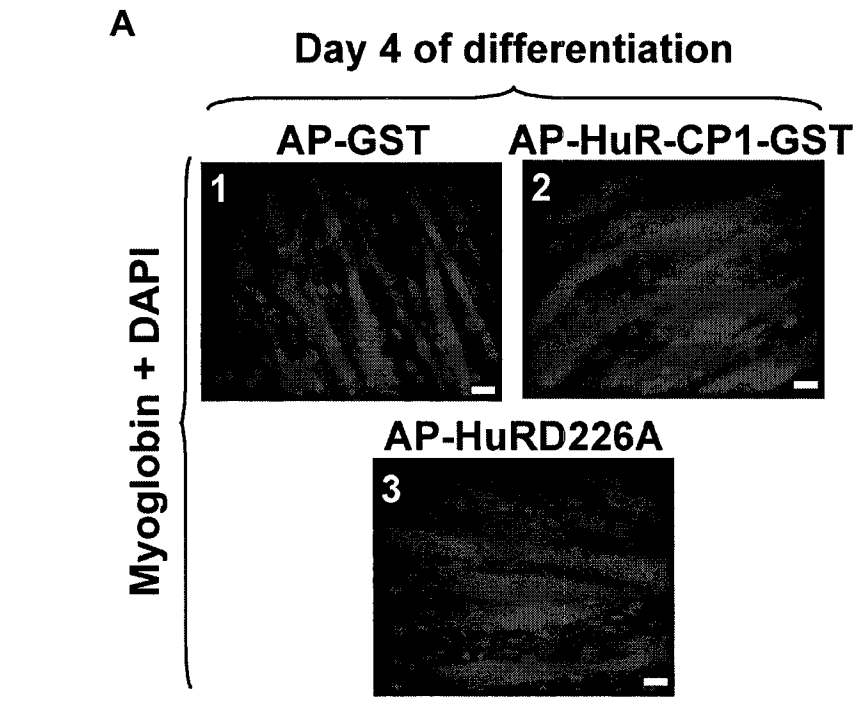
Since the amount of cleaved HuR (Figure 2.1A) corresponds to the amount of HuR that associates with Trn2 (van der Giessen and Gallouzi, 2007), it is possible that during myotube formation, HuR-CP1 remains associated to Trn2, leading to the release of HuR-CP2, which positively affects myogenesis. It is also possible that after cleavage, HuR-CP1 interacts with Trn2, competing away HuR from the complex leading to its accumulation in the cytoplasm. Nevertheless, either of these two scenarios could lead to the formation of a stable HuR-CP1/Trn2 complex, which neutralizes their respective moderatory effects on the myogenic process. This assumption was confirmed by the fact that the addition of AP-HuR-CP1 or AP-HuRD226A to fully differentiated myotubes (day 4 and after) had no effect on myogenesis (Figure 2.12).

To assess the possible effect that HuR-CP1 as well as HuR-CP2 and HuRD226A would have on HuR nucleocytoplasmic localisation, GFP-HuR was transfected into myoblasts that were subsequently treated with AP-GST, AP-HuR-CP1-GST, AP-HuR-CP2-GST, or AP-HuRD226A-GST. Following fixation, staining for DAPI was performed and by fluorescent microscopy was performed to follow the cellular distribution of GFP-HuR. We observed that GFP-HuR becomes more detectable in the cytoplasm in cells treated with AP-HuR-CP1-GST (Figure 2.13, panel 4 and 6) when compared to those treated with AP-HuR-CP2-GST, or AP-HuRD226A-GST (Figure 2.13, panel 7 and 9 and panel 10 and 12). When the cells were treated with the control AP-GST, GFP-HuR was detected only in the nucleus

(Figure 2.13, panel 1 and 3). These data suggested that AP-HuR-CP1-GST, by interacting with Trn2 (Figure 2.10), might release GFP-HuR from the Trn2 leading to its cytoplasmic accumulation. The fact that, as for GFP-HuR and –HuRD226A, GFP-HuR-CP1 was completely nuclear (Figure 2.14), confirmed this assumption and strongly suggested that HuR-CP1 is also imported to the nucleus in a Trn2-dependent manner. GFP-HuR-CP2, however, was found in both compartments but it was more evenly distributed (Figure 2.14, Panel 10 and 12). These observations supported the idea that HuR-CP1 could compete away wt-HuR from the Trn2 complex, leading to its cytoplasmic accumulation during muscle cell fusion and myotube formation.

Figure 12: The inhibitory effect of HuR-CP1 and HuRD226A is no longer observed when the cells are treated late during myogenesis.

(A) C2C12 cells induced for differentiation during 3 days, then treated with AP-conjugated GST, HuR-CP1 or HuRD226A. The next day these cells were used for immunofluorescence experiments with DAPI staining and the anti-myoglobin antibodies. A single representative field for each cell treatment is shown. Bars, 20 μm. **(B)** The fusion index is shown, calculated as described in Figure 1. Error bars represent the SEM of two independent experiments.


A
Day 4 of differentiation
AP-GST
AP-HuR-CP1-GST
AP-HuRD226A
Myoglobin + DAPI
1
2
3

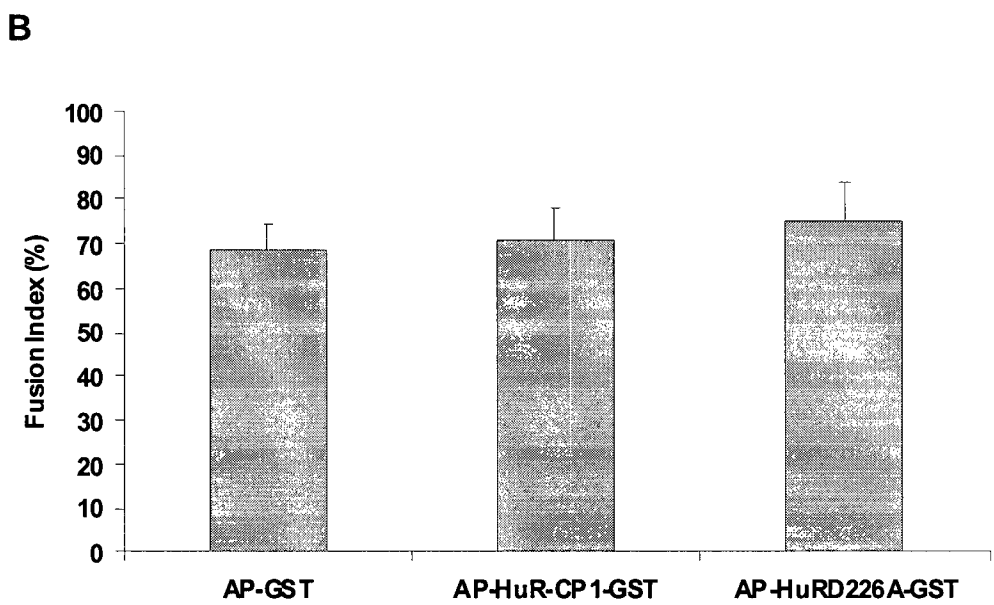

B
Fusion Index (%)
100
90
80
70
60
50
40
30
20
10
0
AP-GST
AP-HuR-CP1-GST
AP-HuRD226A-GST

Figure 2.13: GFP-HuR relocalizes to myoblast cytoplasm upon treatment with AP-HuR-CP1-GST.

C2C12 myoblasts were transfected with GFP-HuR and 24 hrs later when the cells were at 60% confluency, they were treated with AP-conjugated GST, HuR-CP1-GST, HuR-CP2-GST, or HuRD226A-GST. Then, 8 hrs later, the cells were fixed and stained for DAPI. A single representative field for each cell treatment is shown. This result is representative of two independent experiments. Bars, 20 μm.

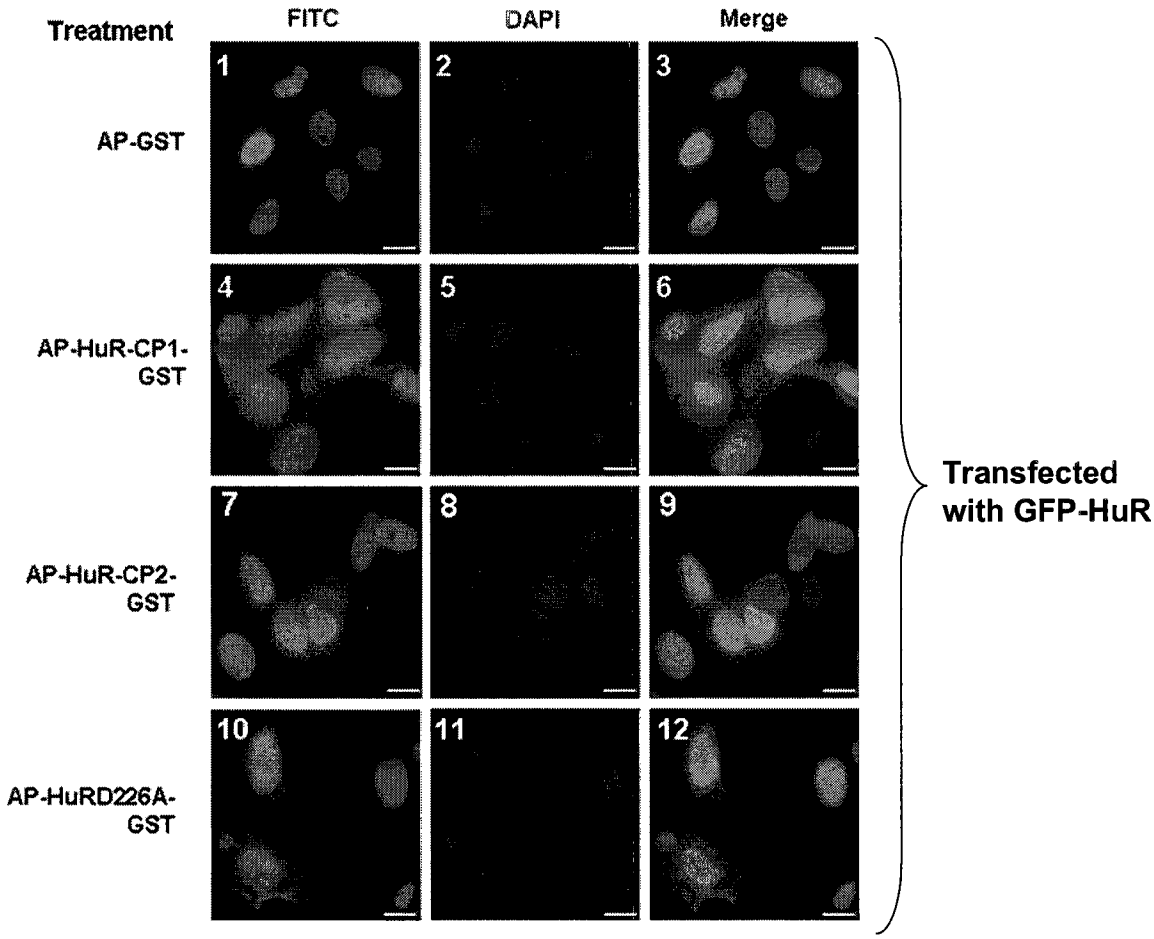
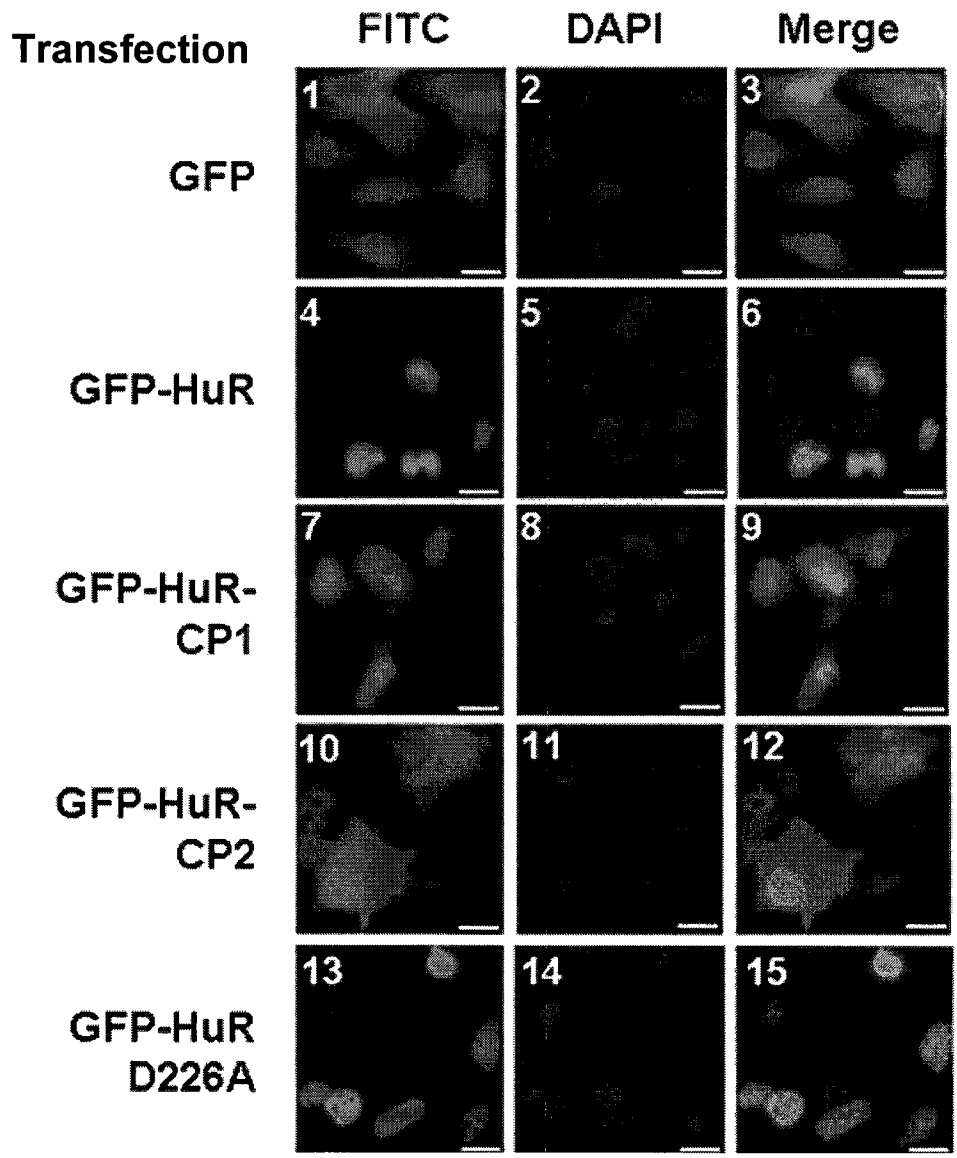

Treatment
FITC
DAPI
Merge
1
2
3
AP-GST
4
5
6
AP-HuR-CP1-GST
7
8
9
AP-HuR-CP2-GST
10
11
12
AP-HuRD226A-GST
Transfected with GFP-HuR

Figure 2.14: Nucleo-cytoplasmic localization of HuR isoforms.

C2C12 myoblasts were transfected with GFP, GFP-HuR, GFP-HCP1, GFP-HCP2, or GFP-HuRD226A. 24 hrs later, the cells were fixed and stained for DAPI. A single representative field for each cell treatment is shown. This result is representative of two independent experiments. Bars, 20 μm.

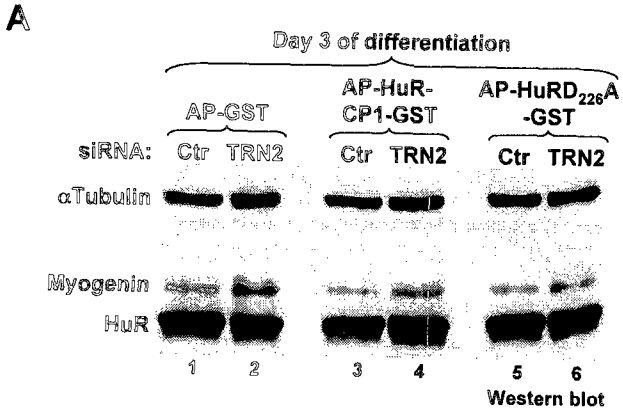

Transfection
FITC
DAPI
Merge
GFP
GFP-HuR
GFP-HuR-CP1
GFP-HuR-CP2
GFP-HuR D226A
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

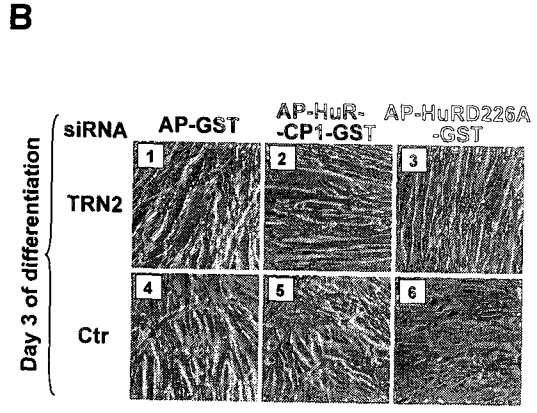
These results together with the fact that when HuR-CP1 is associated to Trn2, less wt-HuR is seen to form a complex with the transporter (Figure 2.11) argued that upon formation, the endogenous HuR-CP1-Trn2 complex remained stable and could not be displaced by the addition of exogenous Trn2 ligands. This could also provide an explanation for the inhibitory effect of both AP-HuR-CP1 and -HuRD226 at the early stages of myogenesis. Indeed, the absence of endogenous HuR-CP1 and the lower affinity with which wt-HuR associates to Trn2 could indicate that AP-HuR-CP1 or AP-HuRD226A replaces HuR in the HuR-Trn2 complex leading to the inhibition of myogenesis. If this is true, the inhibitory effects of these two isoforms should depend on the presence of Trn2 in the cell. To test this possibility, C2C12 cells were treated with control (Ctr) or Trn2 siRNA (van der Giessen and Gallouzi, 2007) and then stimulated for differentiation in the presence of AP-conjugated HuR-CP1 or HuRD226A. As expected (van der Giessen and Gallouzi, 2007), the knockdown of Trn2 generally caused an increase in the efficiency of myogenesis (Figures 2.15A-B). Additionally, we observed that in Trn2-depleted cells, AP-HuR-CP1 and AP-HuRD226A were no longer able to prevent muscle differentiation (Figures 2.15B-C). Western blotting analysis on total cell extracts from these samples using the anti-myogenin antibodies confirmed these results, and revealed that Trn2-knockdown caused an increase in myogenin protein expression, regardless of whether HuR-CP1 or HuRD226A was present or not (Figure 2.15A). These observations clearly demonstrated that the HuR-CP1- and HuRD226A-mediated inhibition of myogenesis requires their association with Trn2. Therefore, it is possible that one of the main

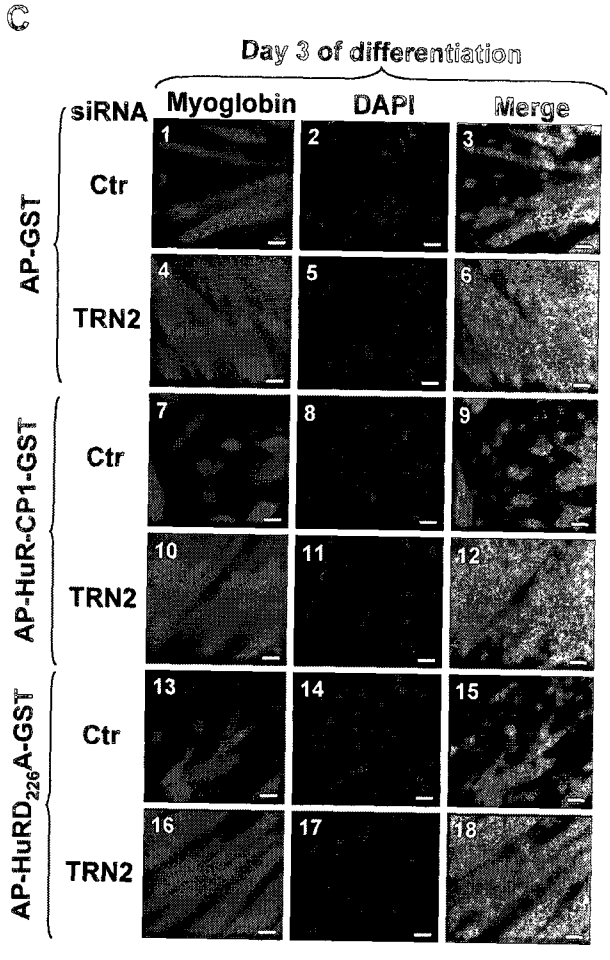
functions of HuR-CP1 is to help the cytoplasmic accumulation of HuR during muscle fiber assembly by interfering with Trn2-mediated nuclear import of HuR.

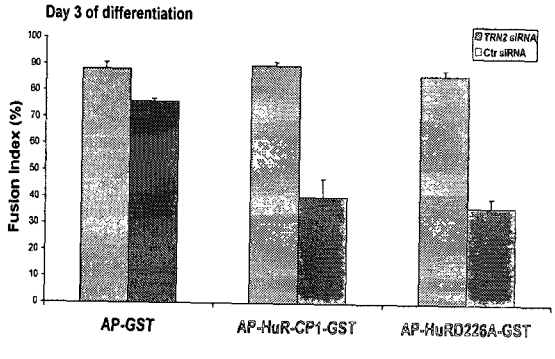
Figure 2.15: HuR-CP1 and HuRD226A affect and prevent myogenesis in a TRN2-dependent manner.

(A-D) C2C12 myoblasts were treated with siRNA duplexes against TRN2 as described in (Figure 2.9A). The cells were treated twice with AP-GST, -HuR-CP1-GST or -HuRD226A-GST 24 hrs prior to the initiation of differentiation and again at the initiation of differentiation. AP-GST was used as a control. **(A)** Total cell extracts from cells treated with HuR isoform conjugated with AP as described in figure 2.4 were prepared at Day 3 of differentiation and analysis by western blot was done using antibodies against αTubulin, HuR and myogenin on 30 μg of protein. Representative western blots of three independent experiments are shown. **(B)** Phase-contrast pictures of a single representative field of view for each cell treatment on Day 3 of differentiation are shown. **(C)** TRN2- or control-siRNA-treated cells were fixed and immunofluorescence staining was performed with DAPI and against myoglobin on the C2C12 cells at Day 3 of differentiation. A single representative field for each cell treatment is shown. Bars, 20 μm. **(D)** The fusion index is shown, calculated as described in Figure 2.1. Error bars represent the SEM of three independent experiments.


A
Day 3 of differentiation
AP-GST
AP-HuR-CP1-GST
AP-HuRD226A-GST
siRNA: Ctr TRN2 Ctr TRN2 Ctr TRN2
αTubulin
Myogenin
HuR
1 2 3 4 5 6
Western blot


B
siRNA
AP-GST
AP-HuR-CP1-GST
AP-HuRD226A-GST
Day 3 of differentiation
TRN2
Ctr
1 2 3 4 5 6


C
Day 3 of differentiation
siRNA
Myoglobin
DAPI
Merge
AP-GST
Ctr
TRN2
AP-HuR-CP1-GST
Ctr
TRN2
AP-HuRD226A-GST
Ctr
TRN2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18


D
Day 3 of differentiation
TRN2 siRNA
Ctr siRNA
Fusion Index (%)
0 10 20 30 40 50 60 70 80 90 100
AP-GST
AP-HuR-CP1-GST
AP-HuRD226A-GST

3. Discussion

Collectively, our experiments have established that the caspase-mediated cleavage of HuR in the cytoplasm is one of the main regulatory mechanisms required for myogenesis. In previous studies, HuR was shown to control the expression of myogenic messages such as *MyoD* and *myogenin* following the induction of myogenesis (Figueroa et al., 2003; van der Giessen et al., 2003). Furthermore, HuR was demonstrated to accumulate in the cytoplasm later during myogenesis at the same time as when disruption of the HuR-Trn2 complex was observed. Here we show that when myoblasts fuse to form myotubes, the accumulation of HuR in the cytoplasm leads to its cleavage by caspases. In the absence of endogenous HuR, the cleavage product HuR-CP2 was sufficient to reestablish myogenesis even in the presence of HuR-CP1. On the other hand, HuR-CP1 was able to interfere with myotube formation in a Trn2-dependent manner only if introduced at early steps of the myogenic process. We also showed that HuR-CP2, but not HuR-CP1, was able to increase the half-lives of *MyoD* and *myogenin* mRNAs to the same levels as wt-HuR. Therefore, we suggest a model whereby at the early steps of myogenesis, HuR is imported to the nucleus by Trn2 where it affects the fate of muscle cells by a yet unknown mechanism (Figure 3.1, blue arrows). Then, as soon as the formation of myotubes begins, HuR is cleaved, generating two CPs (HuR-CP1 and -CP2) which participate in the pro-myogenic function of HuR. Indeed, while HuR-CP1 forms a stable complex with Trn2 that correlates with the cytoplasmic accumulation of HuR, HuR-CP2 helps the remaining non-cleaved HuR to stabilize *MyoD* and *myogenin* mRNAs (Figure 3.1, red arrows).

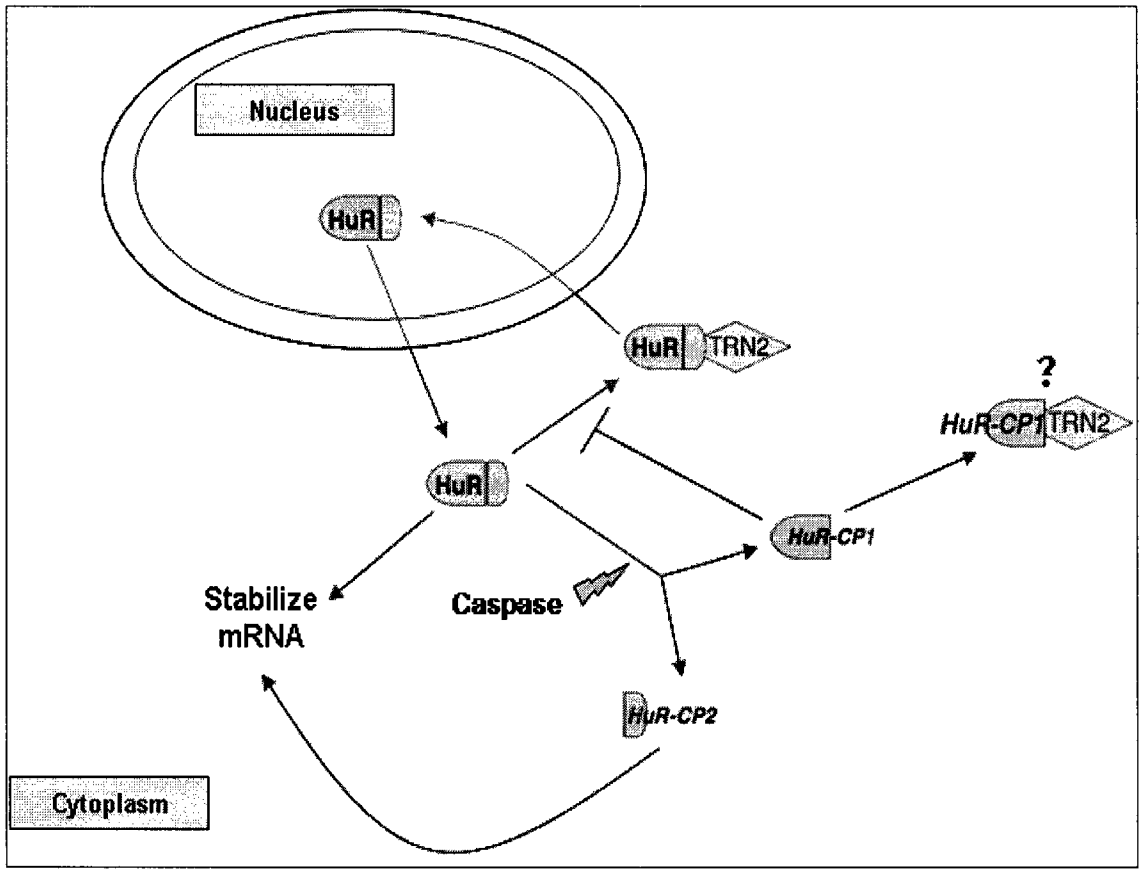


Figure 3.1: Model for the role of HuR and its cleavage products during myogenesis. (Blue arrows) Before and during the early steps of myogenesis, HuR is imported to the nucleus by the nuclear import factor Trn2. **(Red arrows)** At the beginning of the formation of myotubes, HuR is cleaved by caspases producing two cleavage products: HuR-CP1 and HuR-CP2. HuR-CP1 associates with Trn2, which reduces the amount of HuR that associates with Trn2, leading to an increase in the amount of HuR found in the cytoplasm. The function of HuR-CP1-Trn2 complex is still elusive. In the case of HuR-CP2, it helps HuR in its stabilizing function of *MyoD* and *myogenin* mRNA.

We showed that only HuR-CP2 was able to stabilize *MyoD* and *myogenin* mRNA at the same level as wt-HuR (Figure 2.8). We were surprised, however, to observe that despite the clear inhibitory effect that HuR-CP1 overexpression in myoblasts has on myotube formation, this CP was able to stabilize *MyoD* and *myogenin* mRNA, albeit at lower levels than HuR-CP2 and wt-HuR. The high expression levels of exogenous AP-HuR-CP1 in these experiments, as well as the fact that it harbors two RNA-binding motifs (RRMs 1 and 2), could explain in part the observed stabilizing effect.

Interestingly, we have seen that the same amount of total HuR that associates with Trn2 (5%) (van der Giessen and Gallouzi, 2007) corresponds to the amount of cleaved HuR in the cytoplasm during myogenesis (Figure 2.1), suggesting the possibility that HuR-CP1 blocks Trn2 activity, and initiates the cytoplasmic accumulation of non-cleaved HuR. Additionally, since the knockdown of Trn2 expression, which is known to activate the myogenic process (van der Giessen and Gallouzi, 2007), leads to a significant increase in the level of endogenous HuR-CP1, it may be argued that this cleavage is Trn2-independent (Figure 2.9B). Collectively, these observations further support the idea that one of the reasons for which HuR dissociates from Trn2 during myotube formation is to expose cytoplasmic HuR to caspases.

However, the HuR-CP1-mediated inhibition of myogenesis raised the possibility that this CP could affect other cellular mechanisms known to regulate the promyogenic function of HuR. In fact, since HuR-CP1 is able to associate with Trn2, it might be able to bind to MRF mRNAs, bringing them inside the nucleus to prevent

their translation. This possibility was further supported by the fact that in the absence of Trn2, HuR-CP1 and HuRD226A were unable to block myogenesis (Figure 2.15). These results are consistent with previous observations, showing that during the fusion phase of myogenesis, the HuR-Trn2 complex is disrupted, allowing the cytoplasmic accumulation of HuR and the formation of muscle fibers (van der Giessen and Gallouzi, 2007).

Our data also show that the expression of HuR-CP1 in myoblasts, where endogenous HuR is not normally cleaved, results in a complete inhibition of myogenesis (Figure 2.5). This could explain in part why muscle cells trigger the caspase-mediated cleavage of HuR only later during the myogenic process, when HuR is more needed in the cytoplasm to participate in myotube formation. However, even though HuR-CP1 inhibits muscular differentiation when overexpressed alone, when it is overexpressed together with HuR-CP2, it no longer exhibits its negative effect on myogenesis, and it can complement the promyogenic role of HuR-CP2 to give normal muscular differentiation (Figure 2.7). In fact, when HuR-CP2 is overexpressed alone in HuR knockdown myoblasts, rescue of myogenesis is observed, though the fibers formed are much thinner than those observed when a rescue with wt-HuR is performed. Fiber formation to resemble HuR-rescue was only achieved when both HuR-CP1 and –CP2 were present, showing that both are needed in order to exhibit accurate HuR-like promyogenic properties. The HuR-CP1 moderating effect observed on myogenesis might be explained by the fact that equilibrium between the presence of both cleavage products (*physiological* conditions) is required to conduct the normal process.

During myogenesis, overexpression of HuR-CP1 in myoblasts resulted in the accumulation of endogenous HuR in the cytoplasm, while the majority of HuR-CP1 localized to the nucleus. Moreover, by a co-immunoprecipitation experiment where GFP-HuR-CP1-transfected C2C12 cell extracts were treated with an anti-Trn2 antibody, we demonstrated that in the presence of GFP-HuR-CP1, only a minimal amount of endogenous HuR manages to remain complexed with Trn2. (Figure 2.11). This allows us to hypothesize that during normal muscular differentiation, as HuR begins to be cleaved, the HuR-CP1 that is generated begins to associate with Trn2. Furthermore, HuR-CP1 would compete with HuR for Trn2-binding using the large portion of the HNS domain that it still possesses and would prevent the formation of HuR-Trn2 complex. These results correlate with previous observations where it was shown that at day 4 of myogenesis, the HuR-Trn2 complex is disrupted (van der Giessen and Gallouzi, 2007). Since we have seen that on day 4, endogenous HuR-CP1 is at its highest level in myotubes, it is possible that at this time HuR-CP1 have the maximum activity by competing for the association with Trn2.

While we did not look for a possible myogenic role for Trn1 in this study, it was previously shown that Trn1 expression is quite low during early myogenesis, and is only minimal up to day 2, where we performed most of our experiments (van der Giessen and Gallouzi, 2007). However, in future experiments on the regulation of myogenesis during later stages, it might be interesting to determine if Trn1 could play a role by associating with HuR or HuR-CP1 (van der Giessen and Gallouzi, 2007).

By identifying the caspase-dependent cleavage of HuR as part of an important regulatory event of myogenesis, our group has now seen two systems (apoptotic-cell

death and myogenesis), where the cleavage of HuR leads to specific downstream effects. During apoptotic cell death, HuR-CP1, but not HuR-CP2, associate with full length HuR (Mazroui et al., 2008) (data not shown). This may also occur during myogenesis, but has yet to be clearly demonstrated. We do know at this time, however, that HuR can homodimerize, as recently demonstrated using the technique of Fluorescent Resonance Energy Transfer (FRET) as well as using low-molecular-weight inhibitors of HuR dimmers formation (David et al., 2007; Meisner et al., 2007). Though no functional consequences of this homodimerization have been determined yet, cleavage may show to be required for HuR to exercise its normal functions, or that to be cleaved by caspases, HuR must be specifically in a dimerized form. Interestingly, we observed that during myogenesis, HuR-CP1 and HuRD226A have negative effects on myogenesis. Similar inhibitory effects were also observed during apoptosis for these proteins (Mazroui et al., 2008). These data support the possibility that the presence of the dominant-negative non-cleavable mutant of HuR in HuR dimers could cause inhibition of myogenesis. The possibility for HuRD226A or HuR-CP1 to interfere with the formation of HuR homodimers may also provide insight on how the addition of either of these proteins to undifferentiated myoblasts interferes with the function(s) of HuR by blocking its nucleocytoplasmic movement (Fan and Steitz, 1998; van der Giessen and Gallouzi, 2007). Likewise, it may be the case that HuRD226A or HuR-CP1 heterodimerization with wt-HuR may modify its association with mRNA and/or protein partners during myogenesis.

Since a role for caspases and other proapoptotic factors in myogenesis has already been suggested (Fernando et al., 2002; Huh et al., 2004; Porrello et al., 2000),

we were not surprised to observe that HuR is cleaved in a caspase-dependent manner in this process. In a stress-response system, we previously observed that one role of HuR involves the association between HuR-CP2 and the well-known HuR ligand, pp32 (Mazroui et al., 2008). Intriguingly, pp32 was found to be a promoter of apoptosis, and will determine if this HuR-CP2/pp32 interaction is needed for the proapoptotic role of pp32. In this current study, we have observed that while HuR-CP2 may not associate with Trn2, HuR-CP1 does interfere with myogenesis in a Trn2-dependent manner. These data raise the possibility that the effects on cell fate exercised by HuR CPs occurs dependent on the nature of the interactions they have with their ligands.

Even though we have furthered understanding of the promyogenic role of HuR, as mediated by its cleavage during myogenesis, these data leave some questions unanswered, while also raising several new questions. For example, while we know that the cleavage of HuR occurring during myogenesis is performed by a caspase, we have not confirmed which exact caspase(s) this is. Though caspase 3 was reported to be necessary for myogenesis(Fernando et al., 2002; Mukasa et al., 1999), we previously saw that in apoptosis, both caspases 3 and 7 are involved in HuR cleavage (Mazroui et al., 2008). It will be interesting to see if these results correlate to the myogenic caspase-mediated cleavage seen in my thesis work. Likewise, the mechanistic and temporal regulations of how the caspase(s) responsible becomes active, and then generate the HuR-CPs, would increase our understanding of how myogenesis occurs.

Moreover, the roles and functions of each cleavage product during myogenesis must be better characterized. What is the role of the complex formed by HuR-CP1 and Trn2? Does it sequester Trn2 from associating with HuR, thus solely acting to promote cytoplasmic accumulation of HuR, or does it act through alternative mechanisms simultaneously? With regards to HuR-CP2, although we observed that it stabilizes *MyoD* and *myogenin* mRNAs to the same extent as does HuR, it will be important to perform experiments to determine if HuR-CP2 directly associates with these mRNAs to mediate this effect, or if it acts indirectly via other proteins. Such a study would demonstrate which domains of HuR are required to bind to mRNAs and stabilize them. While these results have further validated the value of posttranscriptional and posttranslational processes as a means of regulating myogenesis, the more specific details of such regulations leave much to be clarified.

To conclude, our observations show that the caspase-mediated cleavage of HuR represents a posttranslation modification capable of affecting HuR as other posttranslational modifications such as phosphorylation (Abdelmohsen et al., 2007) and methylation (Li et al., 2002), and that this regulation has physiological significance. Establishing a link between posttranscriptional regulators such as HuR, and key myogenic effectors such as caspases, increases our understanding of how the skeletal muscle tissues can deal with different extracellular assaults to maintain its integrity. Furthermore, by continuing to investigate the roles of the cleavage products of HuR, and the regulation of their generation, we will gain a greater understanding of how the ubiquitously-expressed HuR protein mediates stabilization, translocation, and

translation-based effect on large family of messages. Equally, if not more importantly, uncovering novel mechanisms of myogenic regulation may significantly advance our understanding of muscular dystrophies and muscle related diseases, which may in consequence lead to better therapies to treat these ailments.

4. Materials and methods

4.1 Plasmid construction and protein purification

AP-HuR-CP1-GST and AP-HuR-CP2-GST were generated by cloning HuR-CP1 and HuR-CP2 fragments into the AP/GST-vector as described (van der Giessen et al., 2003). The PCR amplification of HuR-CP1 and HuR-CP2 was performed using the GST-HuR plasmid as template (Brennan and Steitz, 2001) with the primers HuR-CP1-GST-For (5'- GGC GCG GCC GCA TCT AAT GGT TAT GAA GAC CAC -3') and AP-HuR-CP1-Rev (5'- GGC CTC GAGGTGATC GAC GCC CAT GGG -3') and HuR-CP2-GST-For (5'- GGC GCG GCC GCA CAC ATG AGC GGG CTC TCT -3') and AP-HuR-CP2-Rev (5'- GGC CTC GAG TTT GTG GGA CTT GTT GGT TTT -3') respectively. The Not1/Xho1 fragment of HuR-CP1 PCR product and the Not1/Xho1 fragment of HuR-CP2 PCR product were inserted in the Not1/Xho1 sites of the Gateway AP-GST vector to produce AP-HuR-CP1-GST and AP-HuR-CP2-GST. AP-HuR-GST and AP-HuRD226A were generated and used as described (Mazroui et al., 2008).

The proteins were expressed and produced as previously described (van der Giessen et al., 2003) except for the following modifications: the proteins were eluted in a buffer containing 300 mM Tris pH 8.8 and 120 mM NaCl, with 10 mM glutathione for the first elution and 20 mM gluthatione for further elutions.

The GFP-tagged HuR isforms, GFP-HuR, -HuRD226A, -HuRD254A and –HuRD256A, were generated and used as described in (Mazroui et al., 2008). The GFP-HuR-CP1 and –CP2 were generated as follow. The first 226 amino acids of HuR (1-226) (HuR-CP1) or the last 100 amino acids (227-326) (HuR-CP2) were amplified by polymerase chain reaction (PCR), using wild-type HuR as the template. A BglII site was created at the 3'-end and a EcoRI was generated at the 5'-end of the PCR product for both HuR-CP1 and HuR-CP2. GFP-HuR-CP1 was generated using the following primers, forward: 5'-GGC AGA TCT AAT GGT TAT GAA GAC CAC A-3', and reverse: 5'-GGC GAA TTC TTA ATC GAC GCC CAT GGG-3'. We generated GFP-HuR-CP2 using; forward: 5'-GGC AGA TCT CAC ATG AGC GGG CTC TCT-3 ', and reverse: 5'-GGC GAA TTC TTA GTA AGC TGC GAG AGG AG-3'. Those PCR fragments were cloned within the BglII/EcoRI sites of pAcGFP1-C1 vector (BD Bioscience) and both fragments were inserted in frame of the GFP cDNA.

The YFP and YFP-Trn2 were generated as follow. YFP was inserted in HindIII/KpnI site of pcDNA3 vector (Invitrogen). Trn2 was inserted in the pcDNA3 vector containing YFP in the BglII/NotI site. Trn2 PCR fragment was cloned within this site using the following primers, forward: 5'- CGC GGA TCC GCG GAC TGG CAG CCA GAC GAG -3', and reverse: 5'- GGC GCG GCC GCG CCC TAG ACC CCA TAG AAA GCC G -3'

4.2 Cell Culture and Transfection, zVAD and cell-permeable protein treatments

C2C12 cells (ATCC) were grown and induced for differentiation when they reached ~100% confluency using low serum media (2% horse serum (Gibco), 98%

DMEM (high glucose) (Sigma-Aldrich), 10 ug/uL transferrin (Gibco), 10 ug/uL insulin (Sigma-Aldrich), 100X Penicillin-Streptomycin (Sigma-Aldrich), 50 mM HEPES, pH 7,4 (Sigma-Aldrich)), along with the transfections of small interfering RNA (siRNA) into these cells (van der Giessen et al., 2003). The control, HuR and TRN2 siRNAs used were previously described (van der Giessen et al., 2003; van der Giessen and Gallouzi, 2007). Transfections were done with two knockdowns on two consecutive days; the first when cells were at 25% confluency and the second when at 50%. 8h after the second transfection, two identically-treated wells were pooled together. 24h after the second siRNA transfection, differentiation was induced, as previously described (van der Giessen et al., 2003). The transfection of GFP-constructs was performed using 7 μg of plasmid per 10 cm plate and transfection of YFP and YFP-Trn2 required 1,5 μg of plasmid per well in a 6-well plate.

To look at caspase-dependent cleavage of HuR by western blotting, zVAD (Sigma-Aldrich) was added directly to myotubes on Day 3 of differentiation for 16h, at a final concentration of 10 μM, after which they were supplemented with another 10 μM zVAD for 12 hours before harvesting the cells on Day 4 as described below. To assess the localization of HuR by immunofluorescence, zVAD was added on day 1 of differentiation for 16h, at a final concentration of 10 μM, then supplemented with another 10 μM zVAD for 12 hours before fixing the cells on Day 2.

For cell-permeable fusion protein experiments, all proteins were produced as described earlier and were added to C2C12 myoblasts in a final concentration of 50nM the day before, and the day of, induction of differentiation. For each day of

treatment, the proteins were mixed with C2C12 growth or differentiation media, and then 1 ml of the media/protein solution was added to each well of a six-well plate containing C2C12 myoblasts. The media was changed 24 h after protein treatment, and cells were allowed to differentiate according to standard protocol.

4.3 Immunoblotting, Immunofluorescence, and Preparation of Cell Extracts

Total cell extracts were prepared by incubating cells on ice for 15 min. with lysis buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 10% glycerol, 1% Triton, 10 mM pyrophosphate sodium, 100 mM NaF, 1 mM EGTA, 1,5 mM $MgCl_2$, 1X protease inhibitor (Roche), 0,1 M orthovanadate, 0,2 M PMSF), then centrifuge at 12 000 rpm at 4°C and the supernatant was kept (van der Giessen et al., 2003). Western blotting was performed as previously described (Gallouzi et al., 2000), probing with antibodies against HuR (3A2) (Gallouzi et al., 2000), myogenin (F5D), MyHC (MF-20) (Developmental studies Hybridoma Bank, USA), α-tubulin (Sigma-Aldrich), GFP (Clonetech, USA), and hnRNP A1 (Abcam, USA). Immunofluorescence was performed as previously described (van der Giessen et al., 2003) using antibodies against HuR, myoglobin (DAKO) and MyHC and staining with 4',6-diamidino-2-phenylindole (DAPI). The cells were observed with a Zeiss Axiovision 3.1 microscope using 40X or 63X oil objective, and immunofluorescence pictures were taken with an Axiocam HR (Carl Zeiss) digital camera.

4.4 Northern (RNA) Blot Analysis and Actinomycin D Pulse-Chase Experiments

Northern blot analysis was performed using 12 μg of total RNA and as previously described (van der Giessen and Gallouzi, 2007). Briefly, after transferring the RNA onto a Hybond-N membrane (GE Healthcare), the RNA was UV-cross-linked and the blots were hybridized with *MyoD*, *myogenin*, and *GAPDH* cDNA probes (van der Giessen et al., 2003). The stability of *MyoD* and *myogenin* messages was assessed by first treating C2C12 cells, after 16h of differentiation, with 50 nM of AP-GST, AP-HuR-GST, AP-HuR-CP1-GST or AP-HuR-CP2-GST. 1 hour after this, cells were treated with the RNA pol II inhibitor actinomycin D (ActD) (Sigma-Aldrich) at a concentration of 5 μg/ml. Total RNA was isolated from the cells after 0, 1, 2, 3, and 6 hours following ActD treatment using TRIzol reagent (Invitrogen), and analyzed by Northern blotting.

4.5 Immunoprecipitation Analysis

Total cell lysates for immunoprecipitation were prepared from C2C12 cells that were transfected with GFP, GFP-HuR, GFP-HuR-CP1, GFP-HuR-CP2, and GFP-HuRD226A DNA constructs, prior differentiation initiation. C2C12 at different stages of the differentiation process were scraped in phosphate-buffered saline (PBS) and centrifuged at 2000 rpm for 5 min at 4°C. Total cell extracts were prepared by lysing the cells with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS) containing complete protease inhibitors (Roche). Lysates were incubated on ice for 15 min, with mixing by vortex every 5 min, followed by centrifugation at 14,000 rpm for 5 min. The supernatant represented the total cell extract. Protein

concentration of the total cell lysate was determined using the Bradford protein assay (Bio-Rad). The total cell lysate was added to protein A beads (GE Healthcare) for pre-clearing. Then, equal amounts of total protein (2 mg) were added to protein A beads previously bound (overnight) to anti-TRN2 (Open Biosystems) or IgG control (BioCan, Jackson ImmunoResearch Laboratories) antibodies. The cell lysate- protein A-antibody mixtures were incubated for 4 h at 4°C, and then washed three times for 3 min with RIPA buffer. The immunoprecipitate was resuspended in 2x Laemmli dye, boiled for 5 min, and then vortexed twice for 30 s. Samples were then analyzed by Western blotting.

5. References

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