The HuR/YB1 Complex Stabilizes Pro-myogenic mRNAs by Interacting with a Consensus U-rich Motif in the 3'UTR

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ABSTRACT

Post-transcriptional regulation of gene expression enables cells to fine-tune the expression of a protein in a spatial and temporal manner. This regulation is mediated by the binding of trans-acting factors, such as RNA-binding proteins, to *cis*-elements in their mRNA targets. RNA-binding proteins modulate the post-transcriptional regulation of mRNAs by regulating their splicing, localization, turnover, and translation. One of the most extensively studied trans-acting factors, the RNA-binding protein Human Antigen R (HuR), has been shown to play a key role in modulating the outcome of cellular processes such as muscle cell differentiation or the onset of diseases such as cancer-cachexia induced muscle wasting. HuR has been shown to modulate the splicing, polyadenylation, turnover, and translation of its mRNA targets. Although the mechanisms through which HuR regulates its mRNA targets at these different post-transcriptional levels are not fully understood, our laboratory and others have shown that they are mediated by the interaction of HuR with other trans-acting factors such as RNA-binding proteins and miRNAs. Previous work from our laboratory established the Y-Box binding protein 1 (YB-1) as a binding partner of HuR during muscle cell differentiation. YB-1 is a well-characterized DNA and RNA binding protein that has been shown to regulate the stability of its mRNA targets. Our data demonstrated that HuR and YB-1 interact in an RNA-independent manner and collaborate to regulate the stability of the myogenin mRNA. To identify mechanistically the function of the HuR/YB-1 complex during the myogenic process we performed RNA-immunoprecipitation coupled to sequencing (RIP-seq) experiments for HuR and YB-1 to determine the complete network of their common targets in a cell culture model of muscle cell differentiation. We identified that HuR and YB-1 associate to 409 common mRNA targets. We validated the interaction to two of these mRNA targets, MyoD and Gata4, which have been previously shown to play a prominent role in regulating the myogenic process. Additionally, we demonstrated that HuR regulates the expression of these mRNAs since the knockdown of HuR or YB-1 affected both the level and the stability of these mRNAs. Our results show that HuR and YB-1 cooperate in regulating these mRNAs post-transcriptionally since knocking down either one decreased the binding of the other to these targets. Having established the

importance of this complex, we sought to determine the mechanism by which it mediates the stability of these mRNAs. Using bioinformatical analysis of the HuR and YB-1 common targets from the RIP-seq data, we identified a 40-nucleotide long GU-rich consensus motif in the 3'UTR of some of the common targets. Interestingly, this motif overlaps with the previously identified HuR/YB-1 binding site in the myogenin mRNA 3'untranslated region. This consensus site is also found in the 3'UTR of the MyoD mRNA. Taken together, our data establish that HuR stabilizes pro-myogenic mRNAs by interacting with YB-1 to mediate the binding of this complex to a consensus motif in the 3'untranslated region of these targets. Our results, therefore, provide new insights into the central role of this post-transcriptional regulatory network in modulating muscle fiber formation.

RÉSUMÉ

La régulation post-traductionelle de l'expression des gènes permet aux cellules de contrôler précisément l'expression d'une protéine. Cette régulation est médiée par la liaison de facteurs de traduction, tels que les protéines de liaison à l'ARN, et aux éléments cis de leurs ARN messagers. Les protéines de liaison à l'ARN sont connues pour médier la régulation post-traductionelle des ARNm en régulant l'épissage, la localisation, la stabilité et la traduction de ces ARNm. L'un des facteurs de traduction les plus étudiés est la protéine de liaison à l'ARN Human Antigen R (HuR). HuR s'est avéré jouer un rôle important dans plusieurs processus cellulaires tels que la différenciation des cellules musculaires ou l'apparition de maladies telles que la perte musculaire induite par le cancer et la cachexie. Il a été démontré qu'HuR module l'épissage, la polyadénylation, la stabilité et la traduction de ses ARNm cibles. Bien que les mécanismes par lequels HuR régule ses cibles ARNm à ces différents niveaux post-traductionnels ne soient pas entièrement compris, notre laboratoire et d'autres ont démontré qu'ils sont médiés par l'intéraction d'HuR avec d'autres facteurs trans-actifs, tels que d'autres protéines liant l'ARN et les microARN. Nous avons préalablement établi que la protéine de liaison Y-Box Binding Protein 1 (YB-1) est un partenaire de liaison d'HuR au moment de la différenciation des cellules musculaires. YB-1 est une protéine de liaison à l'ADN et à l'ARN bien caractérisée qui régule, entre autres, la stabilité de ses cibles ARNm. Nos données démontrent qu'HuR et YB-1 interagissent de manière indépendante de l'ARN et collaborent pour réguler la stabilité de l'ARNm myogenin. Pour identifier mécanistiquement la fonction du complexe HuR/YB-1 au cours du processus myogénique, nous avons réalisé des expériences d'immunoprécipitation d'ARN couplée à un séquençage de l'ARN (RIP-seq) pour HuR et YB-1 afin de déterminer le réseau complet de cibles communes dans un modèle de culture cellulaire de différenciation des cellules musculaires. Nous avons identifié qu'HuR et YB-1 s'associent à 409 ARNm communs. Nous avons validé l'interaction de deux de ces ARNm, MyoD et Gata4, qui jouent un rôle important dans la régulation du processus myogénique. De plus, nous avons démontré qu'HuR régule l'expression de ces ARNm puisque la déplétion d'HuR ou YB-1 affecte à la fois les niveaux et la stabilité de ces ARNm. Nos résultats démontrent

qu’HuR et YB-1 coopèrent dans la régulation post-traductionnelle de ces ARNm puisque la déplétion de l'un ou de l'autre diminue la liaison de l'autre aux ARN messagers. Après avoir établi l'importance de ce complexe, nous avons cherché à déterminer le mécanisme par lequel ils interviennent dans la stabilité de ces ARNm. En utilisant une analyse bio-informatique des cibles communes d’HuR et YB-1 à partir des données du RIP-seq, nous avons identifié un séquence riche en guanines et en uridines. Cette séquence, de 40 nucléotides, est située dans la région 3’ non codante de certaines des cibles communes. Il est intéressant de noter que ce motif chevauche le site de liaison du complex HuR/YB-1 précédemment identifié dans la région 3' non codante de l'ARNm Myog. Cette séquence consensus se trouve également dans la région 3’ non codante de l’ARNm MyoD. Nos données établissent que HuR stabilise les ARNm pro-myogéniques en interagissant avec YB-1 pour médier la liaison de ce complexe à un motif consensus dans la région 3' non codante de ces cibles. Nos résultats fournissent donc des nouvelles informations sur le rôle central de ce réseau de régulation post-traductionelle dans la modulation de la formation des fibres musculaires.

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CONTRIBUTION OF AUTHORS

I declare that Dr. Brenda J. Sanchez performed the RIP-seq experiment. (Figure 2.2b) I performed the complete Bioinformatical analysis of the RIP targets (Figures 2.2, 2.3, 2.10, 2.11, 2.12, 2.13). Phillipe Carle contributed to the coding required for the extraction of the sequence of the 3'UTRs for this analysis (Figure 2.10). Secondly, I declare that I performed the experimental analyses under the guidance of Souad Mubaid (a current Ph.D. student in the Gallouzi Laboratory) (Figures 2.1, 2.2, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9) Dr. Brenda J. Sanchez and Souad Mubaid treated and collected C2C12 for some of the replicates of the experiments. Dr. Sergio Di Marco assisted in conceptualizing experiments and reviewed the thesis. Dr. Imed Gallouzi conceptualized, reviewed and edited the thesis.

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1. INTRODUCTION

1.1. Post-transcriptional Regulation

Gene expression is regulated at multiple levels to allow cellular processes to function properly. The transcription of a gene into a messenger RNA (mRNA) is primarily regulated by the accessibility of the gene to the transcription machinery. [1] This process is modulated by multiple factors including the binding of transcription factors to the promoter region, the chromatin state and epigenetic modifications.[1] Once the transcription machinery is assembled and transcription is initiated, the elongation and termination processes can also be regulated.[1] Post-transcriptional regulation is the control of the newly synthesized mRNA until it is translated into a protein.[2] This control is exerted by a regulatory network composed of RNA-binding proteins (RBPs) that bind mRNA to form messenger ribonucleoproteins (mRNPs) as well as non-coding RNAs (ncRNAs), such as microRNAs (miRNAs), that interact with these RBPs and/or the mRNAs.[3–5] All of these factors contribute to a coordinated effort to maintain proper cellular functions by expressing the necessary proteins.[6] This network controls the splicing, poly-adenylation, export, turnover and translation of mRNAs.[6] The interactions required for this regulation are bipartite. They require both a *cis*-element, a sequence or structure in the RNA, and a trans-acting factor, a protein or ncRNA, that recognizes this sequence and binds to affect the fate of the RNA target. [3,7,8]

1.1.1. Types of *Cis*-elements

The regulatory elements found in the mRNA sequence are known as *cis*-elements. (Figure 1.1) These are found mainly in the non-protein-coding regions of the mRNA, such as the 5' and 3' untranslated regions, as well as in intronic regions.[8] Splicing regulatory signals, for example, are the sites required for the proper binding and function of the spliceosome.[8] These include the 5' and 3' splice sites, the branch site and a polypyrimidine tract.[8] (Figure 1.1a) Other sites in proximity to exon-intron boundaries can also recruit enhancers or silencers of mRNA splicing to produce alternative isoforms of an mRNA.[8]

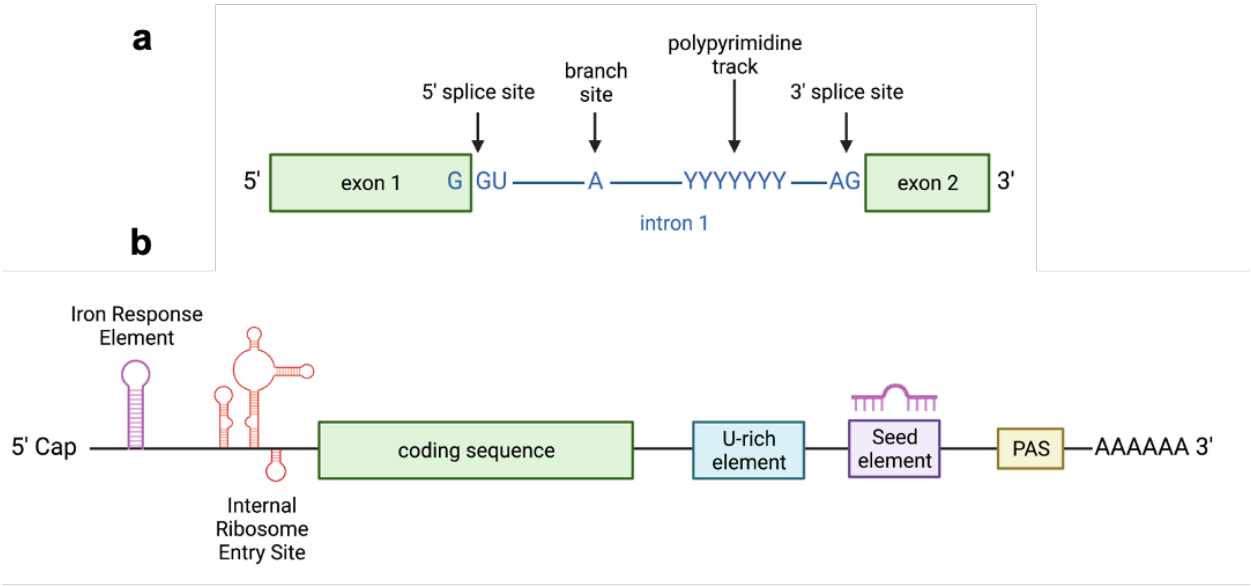


Figure 1.1 Common Types of *Cis*-elements. Common types of *cis*-elements found in mRNAs. a) *Cis*-elements modulating splicing: 5' and 3' splice sites, branch site and the polypyrimidine track **b)** Examples of cis-elements: Iron Response Elements, Internal Ribosome Entry Sites, U-rich elements, Seed elements and Polyadenylation sites. These are represented as a schematic on a model mRNA. (Adapted from Zhao et al., 2011)[8]

Another *cis*-element that modulates the formation of a specific mRNA isoform is the polyadenylation site (PAS).[8] (Figure 1.1b) The PAS consists of a repetition of six AAUAAA sequences that directs the binding of the polyadenylation factors to the mRNA resulting in the addition of a poly(A) tail.[8,9] One mRNA can contain multiple PAS and the differential use of these sites is referred to as alternative polyadenylation (APA).[9] The preferential binding of the polyadenylation machinery to one site over the other can be modulated by the binding of other trans-acting factors in the vicinity of the PAS to increase or decrease its availability.[8,9] The creation of different mRNA isoforms by APA can result in the inclusion or exclusion of other *cis*-elements from the final mRNA based on the location of the preferred PAS.[9] For example, a PAS located near the end of the coding sequence will create a short 3'UTR, but a distal PAS creates a longer 3'UTR which may include *cis*-elements such as Uridine-rich (U-rich) sequences.[9]

One of the most commonly known types of U-rich sequences is the adenine and uridine rich sequences (AREs) that contain an AUUUA pentamer and are found in the 3'UTR of mRNAs.[10] (Figure 1.1b) They are classified into five classes based on the number of pentamer motifs.[11] Theses classes regroup respectively mRNAs with AREs containing five, four, three, two or one iteration(s) of the AUUUA pentamer.[11] Different trans-acting factors seem to have a preference for different classes of AREs. [11] For example, the HuR protein seems to bind more commonly to class 1 AREs, and the KH-type splicing regulatory protein (KSRP) protein seems to bind to class 3 AREs preferentially.[11] AREs were initially discovered to modulate the turnover of mRNAs encoding proteins that need to be tightly regulated, such as cytokines, oncogenes, and growth factors.[11] These sequences and their trans-acting factors were extensively studied and found to be implicated in a wide range of regulatory pathways.[11,12] They promote the turnover of mRNAs through a mechanism called ARE-mediated decay (AMD).[8] The mechanism was discovered to consist of RNA-binding proteins binding to AREs and recruiting deadenylation and/or decapping factors that allow exonucleases, such as the exosome or Xrn1 to degrade the mRNA.[8] In contrast, some RNA-binding proteins bind to AREs to promote their stabilization by preventing AMD.[8] Since the discovery of AREs, other U-rich sequences have been discovered to play a similar role.[13] Guanine and uridine rich (GREs) sequences were found to be preferentially bound by certain RNA-binding proteins and to modulate protein expression.[13] Similarly to AREs, GREs can be classified based on the number of the GUUUG pentamer present in the sequence.[13]

The UTRs of mRNAs can also contain sites named "seed" elements that have partial complementarity to miRNAs. [8] (Figure 1.1b) These sites recruit miRNAs and the RNA-induced silencing complex (RISC) to prevent the translation of the mRNA.[8] They can overlap or be located near RBP binding sites, allowing for multi-factor regulation.[8]

Additionally, structural elements in the 5'UTR called Internal ribosomal entry sites can modulate mRNA translation.[8] (Figure 1.1b) These sites, which bind the 40S ribosomal subunit, enable the initiation of cap-independent translation.[8] Another example of a structural *cis*-element are Iron-response elements.[8] (Figure 1.1b)These hairpin structures

are found in the UTRs of mRNAs related to iron metabolism.[8] These sites enable the expression of these mRNAs to be regulated by cellular iron levels.[8] Mechanistically, these *cis*-elements are bound by iron regulatory proteins (IRP) that sense cellular iron levels.[8,14] Depending on the mRNA target and its role in iron metabolism, the binding of the IRPs has been demonstrated to prevent translation or promote mRNA stability.[8,14] These are just a few examples of possible sequences found in mRNAs that can allow them to be targeted for binding by trans-acting factors and impact their expression.

1.1.2. Trans-acting Factors

1.1.2.1 Non-coding RNAs

Partial complementarity of sequences allows ncRNAs to recognize, bind and impact mRNAs. The field of miRNA-mediated post-transcriptional regulation is well-established. miRNAs are small non-coding RNAs around 21 nucleotides in length that can modulate the turnover and translation of mRNAs.[15] In mammalian cells, miRNAs form a ribonucleoprotein (RNP) complex with the Ago2 protein and the GW182 scaffolding protein.[15] The binding of this complex, named the RNA-induced silencing complex (RISC), to a seed element in the 3'UTR of an mRNA target can promote mRNA turnover or translation inhibition.[16,17] The current proposed turnover model requires the interaction between the scaffolding protein GW182 and the PolyA-binding protein bound to the polyA tail of the mRNA.[16,17] The RISC complex recruits the CCR4-NOT1 deadenylase complex, which removes the polyA tail.[16,17] The next step is the recruitment of the decapping enzymes DCP1 and DCP2, which remove the m^7G cap and make the mRNA vulnerable to 5' to 3' degradation by the exonuclease Xrn1.[16,17] The mechanisms of miRNA-mediated translation regulation has been reported by multiple groups.[16,17] Recently, the most accepted model for this process is the inhibition of translation initiation, as mentioned above, which proposes that the miRISC complex inhibits cap-recognition and/or assembly of the ribosome to prevent translation initiation.[16,17]

Furthermore, long noncoding RNAs (lncRNAs) have emerged as post-transcriptional regulators of gene expression. Many studies have implicated lncRNAs in post-transcriptional regulation as binding partners of RNA-binding proteins.[4] However, it was

also discovered that lncRNAs could themselves bind to *cis*-elements in the RNA and recruit other trans-acting factors to mediate mRNA turnover.[4]

1.1.2.2 RNA-Binding Proteins (RBPs)

RNA-binding proteins modulate every step of mRNA metabolism from the processing the mRNA in the nucleus to its translation in the cytoplasm.[18] As soon as an mRNA is synthesized, it is bound by RBPs to form messenger ribonucleoproteins (mRNPs).[3] The RBP composition of mRNPs changes during the lifespan of an mRNA depending on its subcellular localization, the *cis*-elements it contains, the state of the cell, the availability of certain RBPs and many other additional factors.[3] RNA-binding proteins are composed of RNA-binding domains that confer their ability to recognize RNA. [19] The combination of different domains has been proposed to partially explain the specificity and activity of RNA-binding proteins.[19] Some of the most well-known examples of RNA binding domains include the RNA-recognition domain (RRM), the K-homology domain (KH), the double-stranded RNA-binding domain (dsRBD), zinc finger domains, the DEAD/DEAH box domains and the PAZ domains.[6,19] The functions of these different domains have been determined by establishing their physical structure and the interactions they form with RNA.[19]

In addition to the composition of their binding domains, the function of RBPs is also heavily influenced by their interactions with other RBPs.[19] These interactions include binding interactions, which can be RNA-independent (if the complex can form without the context of their RNA targets) or RNA-dependent (using the RNA as a scaffold), and competition interactions where the binding of one factor prevents the binding of the other.[7,19] An example of an RNA-independent binding interaction is the formation of a complex between the TTP, BRF1 and hnRNP F proteins. [20] This complex forms and then associates with its mRNA targets to promote their decay much more strongly than the individual proteins.[20] On the other hand, the formation of the AUF1/HuR complex has been shown to be RNA-dependent in vitro because the proteins did not interact in the presence of RNase.[21] This indicates that unless they are bound to one of their mRNA targets, such as p21, the AUF1/HuR complex could not form.[21] In some cases, the binding

of one protein to an mRNA impacts the ability of other proteins to bind or exert their function on this common target without directly binding to each other.[7] These interactions can be competitive or collaborative.[7] For instance, the CELF1 protein, also known as CUGBP1, competes for binding to the 3'UTR of the OCLN mRNA with HuR.[22] Since their binding sites overlap, only one of the two proteins can bind to and affect the mRNA.[22] This interaction controls the fate of the OCLN mRNA since the CELF1 protein promotes translation inhibition while HuR promotes its stabilization.[22] Lastly, two proteins bound to the same mRNA without binding each other can also have a cooperative effect.[7] The PTBP1 protein can cooperate with HuR to promote the translation of the HID-1 alpha mRNA even if they bind to different regions of the mRNA and do not interact with each other.[23]

1.2. Human Antigen R (HuR)

1.2.1. Overview

The most extensively studied RNA-binding protein is Human Antigen R (HuR), first characterized in 1996.[24] It is a member of the Hu/ELAV family, the vertebrate homologs of the drosophila Embryonic lethal abnormal vision (ELAV) family.[24] This family consists of four members HuA/HuR, HuB, HuC and HuD. All members function as RNA-binding proteins and are expressed in neuronal cell types.[24] HuR protein, however, is ubiquitously expressed.[24] The HuR gene is located on chromosome 19 at position 19p13.2 and encodes for a 32kDa protein.[24,25]

1.2.2. Structure and RNA-binding

The HuR protein is made up of three RNA-recognition motifs (RRM1-3) and a hinge region (Figure 1.2).[24,26] Each RRM is composed of the canonical βαββαβ fold.[27] The RRM1 and RRM2 domains, which modulate HuR's RNA-binding activity, are located at the N-terminal of the protein.[24] Although the RRM1 and RRM2 domains are required for HuR's RNA-binding activity; recent affinity studies have shown the importance of RRM3 for the maximal affinity of HuR binding to its mRNA targets.[28] Additionally, RRM3 has been suggested to play a role in HuR dimerization, RNA binding and protein-protein interactions.[28,29] In its RNA-free conformation, also known as Apo, the HuR protein does

not display its complete affinity for RNA.[30] However, when HuR is bound to RNA, its conformation changes to create a binding cleft between RRM1 and RRM2 that binds the RNA with high affinity.[30] The RRM1-2 domains are separated from RRM3 by the hinge region containing the HNS motif, which mediates nucleocytoplasmic shuttling of HuR.[26]

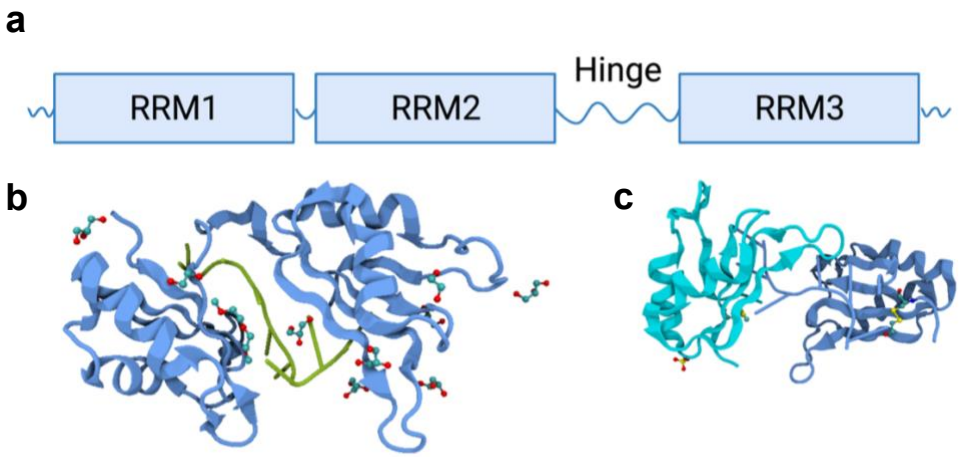


Figure 1.2 Structure of HuR a) The HuR protein is composed of three RRMs and a hinge region that separates RRM1 and RRM2 from RRM3. b) The crystal structure of RRM1/2 domains bound to RNA[30] c)The crystal structure of a duplex of the RRM3 domain bound to RNA. The full structure has not yet been determined due to the high flexibility of the hinge region.[28,31]

1.2.3. HuR Protein Expression

The expression of the HuR protein is regulated at multiple levels. Transcriptionally, the expression of the HuR is controlled by its "TATA-less" promoter, which allows for numerous transcriptional start sites leading to different lengths of its 5'UTR.[32,33] The shorter 5'UTR promotes translation of the HuR transcript while the longer 5'UTR inhibits translation due to a high GC content leading to poor scanning by the translational initiation machinery.[32,33] Although transcriptional regulation by transcriptional factors has not been extensively studied, aberrant transcription in gastric tumours is dependent on the NF-kB transcription factor, suggesting that NF-kB may play a role in the basal transcription of HuR.[34] The HuR mRNA is also regulated post-transcriptionally. Indeed, the alternative polyadenylation of the HuR mRNA has also been shown to play a role in regulating its

expression.[35] The main polyadenylation site of the HuR mRNA allows for the formation of a short 3'UTR, but the alternative polyadenylation site leads to the formation of a longer unstable isoform containing an ARE.[35] A feedback loop allows the HuR protein to stabilize its mRNA by binding to this ARE.[35] However, this ARE is also a binding site of the TTP protein, promoting the decay of the long mRNA isoform.[35] Additionally, other trans-acting factors affect the protein expression by controlling the stability and translation of the HuR mRNA. The RNPC1 protein, a stabilizing RBP, has been shown to stabilize the HuR mRNA by binding to an AU-rich element in its 3'UTR.[36] In a cancer model, the increased expression of HuR modulated by RNPC1 binding caused decreased expression of the c-Myc mRNA and repression of cell growth.[36] MiRNAs, including miR-16, however, have been reported to inhibit the translation of the HuR mRNA.[37–40] For example, in breast cancer, reduced expression of miR-16 allows for overexpression of the HuR protein.[38] In normal cells, miR-16 hinders translation by binding to the 3'UTR of the HuR mRNA.[38]

1.2.4. Functions

The RNA-binding protein HuR mediates cellular function by regulating various post-transcriptional events, including mRNA splicing, polyadenylation, turnover and translation. HuR mediates these functions by preferentially binding to U-rich sequences, including AU-rich sequences[41] and GU-rich sequences.[35,42,43] Most binding sites are located in the 3'UTR of its targets, but occurrences of binding in the 5'UTR and the intronic regions have also been studied.[43] These functions and some examples of the mRNAs regulated by HuR are detailed below (Figure 1.3).

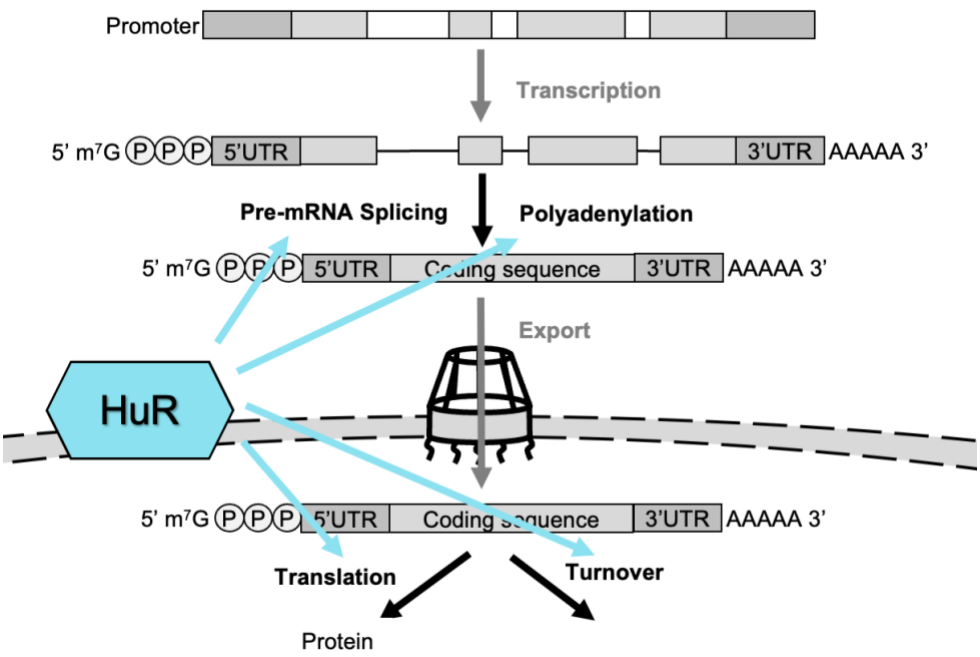


Figure 1.3 Summary of HuR functions The HuR protein plays multiple roles in the mRNA metabolism. It regulates mRNA splicing, polyadenylation, translation and turnover.

1.2.4.1 Splicing

HuR is a modulator of pre-mRNA splicing. Binding studies have identified HuR binding sites in intronic regions of mRNAs, suggesting a possible role as a splicing factor.[43] In-dept analysis of some of these sites has confirmed a role in promoting, modulating or inhibiting splicing of mRNA targets.[44,45] For example, HuR can modulate alternative splicing of the Fas mRNA by binding to URE6, an exon splicing silencer element.[45] The binding of HuR to this element stabilizes the binding of the U2AF65 protein, therefore preventing the correct 3' splice site definition.[45] The effect of HuR is specific to the sequence close to the URE6 HuR binding site. As a result, HuR does not interfere with the downstream 5' splice site definition by the TIA-1/TIAR and U1 snRNP proteins.[45] Proper 5' definition and improper 3' definition causes exon 6 skipping.[45] Furthermore, the Hu protein family was demonstrated to modulate the splicing of the neurofibromin (NF1) mRNA.[46] The binding of Hu proteins to AREs upstream located downstream of exon 24 prevented the recruitment of the splicing factors required for proper boundary definition and led to the exclusion of exon24.[46] In this study, HuR was shown to compete with TIA-1/TIAR, which promotes the inclusion of exon 24 for binding to these sites.[46]

1.2.4.2 Polyadenylation

The HuR protein has been shown to modulate the alternative polyadenylation (APA) of its own mRNA.[47] Mechanistically, this has been described to be caused by the binding of the HuR protein to a GU-rich element in the 3'UTR of its mRNA. In doing so, HuR masks the main polyadenylation site from cleavage stimulation factors required for polyadenylation of the mRNA.[47] Because the main polyadenylation site is hidden, an alternative site downstream is used instead, which leads to the expression of a longer isoform of the HuR mRNA, which is less stable than the canonical HuR mRNA because it contains an additional ARE sequence which is usually not included in the mRNA due to its location further downstream than the main polyadenylation site.[47] This ARE can be bound by TTP, leading to the destabilization of the long isoform or by HuR, leading to its stabilization.[47] Hence, HuR modulates its expression by promoting APA and forming a less stable mRNA isoform. The Hu protein family has been demonstrated to play a role in alternative polyadenylation of other mRNAs, such as the calcitonin and SVL mRNAs, by preventing the binding of cleavage factors to their normal binding sites.[48] This role was proposed to be performed in two complementary ways. First, the binding of the Hu proteins to U-rich sequences near the binding sites of the CstF and CPSF cleavage factors abrogates their binding to the mRNA.[48] Secondly, the interaction of the Hu proteins with the cleavage factor proteins blocks their ability to interact with the mRNA target.[48]

1.2.4.3 Translation

HuR has been shown to promote the translation of mRNA targets by binding to *cis*-elements in the 3' UTR (as is the case for the p53 mRNA) or 5'UTR of messages (such as HIF-1alpha). However, the mechanism through which HuR promotes the translation of its mRNA targets remains elusive.[23,49] Some mechanistic studies by our lab and others have shown that HuR binding can promote translation of these mRNAs by inhibiting the recruitment of the miRNA/RISC complex, which would otherwise repress translation by inhibiting eIF4F binding to the cap structure and the recruitment of the 40S ribosomal subunit.[50–54] Some studies proposed that HuR also mediates the translation of mRNAs by decreasing the binding of miRNAs to seed elements that are located in close proximity to the HuR binding site.[51] HuR, interestingly, has opposing roles in the translation of

some of its mRNA targets. Indeed, HuR was shown to repress the translation of some of its mRNA targets, such as the IGF-IR mRNA, by inhibiting cap-dependent translation (by inhering the 43S translation pre-initiation complex scanning).[55] HuR also represses cap-independent translation of certain mRNA targets, including the p27 mRNA and viral mRNAs, such as HIV-1 and HCV, by blocking the association of ribosome to internal ribosome entry sites (IRES) in the 5'UTR.[55-56] Lastly, HuR can repress translation by promoting miRNA-mediated translation inhibition. For example, HuR promotes the interaction of the let-7 miRNA with the c-Myc 3'UTR leading to translational inhibition.[57]

1.2.4.4 mRNA Turnover

HuR was first discovered as an AU-rich element (ARE) binding protein which stabilizes mRNAs by preventing their decay.[58,59] AREs are mostly found in the 3'UTR of messages with important cellular functions such as protooncogenes, cytokines and growth factors.[60] The AREs tightly regulate the expression of these mRNAs by targeting them for rapid decay by the AU-rich mediated decay mechanism(s).[61] The binding of HuR to an ARE protects the mRNA from the decay machinery by preventing the binding of pro-decay factors such as AUF1 or TTP.[58,62,63] In the absence of HuR, these pro-decay factors bind to the AREs. Consequently, deadenylases are recruited to remove the poly(A) tail. Furthermore, the exosome (promotes 3' to 5' decay) or decapping enzymes (which allow 5' to 3' decay by exoribonucleases such as Xrn1) are recruited to mediate the decay of the mRNA.[62–64] HuR has been shown to interact and stabilize messages containing AREs including b-actin, GM-CSF, VEGEF, c-fos, IL-3, p21, MyoD and myogenin. [24,65–68] Another mechanism through which HuR stabilizes mRNAs is by competing with miRNAs for binding to target mRNAs. These miRNAs promote decay, as indicated above, by recruiting deadenylating factors, decapping factors and eventual degradation by Xrn1.[15] Indeed, the binding of HuR to its mRNA targets can prevent the decay of mRNAs by negating the association of miRNAs to seed elements in these messages. For example, the seed element of miR-181 and the binding site of HuR in the TNF alpha mRNA overlap.[69] The binding of HuR thus prevents miR-181 from triggering the decay of the message.[69] In some instances, this mechanism can exist in parallel to the classical view of HuR stabilization involving its binding to an AU-rich element in its mRNA targets, as is

the case for the COX-2 mRNA.[70] HuR is known to mediate the stability of COX-2 mRNA by associating with AU-rich elements in its 3'UTR and, additionally, by downregulating miR-16 expression, which would otherwise target COX-2 mRNA for degradation.[70]

Although it was first discovered as a stabilizing protein, studies have shown that HuR can destabilize some mRNA targets. For instance, it can promote mRNA decay by cooperating with the KH-type splicing regulatory protein (KSRP) to recruit ribonucleases, such as PARN and the exosome, to the mRNA.[71] Our laboratory has shown that this complex promotes the decay of the nucleophosmin (NPM) mRNA to promote muscle fiber formation.[71] Additional studies showed that this complex also promotes the decay of the PGC-1α mRNA to promote and sustain glycolytic muscle fibers (type II).[72] Lastly, the HuR protein can also promote mRNA decay by collaborating with the AUF1 protein in an RNA-dependent manner.[73] The interaction of both proteins with the 3'UTR of the p16 mRNA was proposed to lead to the destabilization of the mRNA.[73] Interestingly, this study showed that their combined effect results in the recruitment of the RISC complex in a miRNA-independent manner and the subsequent decay of the mRNA.[73]

1.2.5. Importance of HuR in Cellular Processes

The HuR protein is ubiquitously expressed and modulates a wide range of mRNA targets. The importance of its role has therefore been extensively studied in multiple cellular processes and diseases. HuR knockout mice (referred to as ELAVL1$^{-/-}$ mice) are embryonic lethal due to defects in placenta formation, which causes a lack of vascularization, apoptosis and nutrient deficiencies.[74] This suggests that HuR plays a role in labyrinthine vascularization and establishes a role of HuR in angiogenesis.[74] Post-natal studies of the role of HuR in angiogenesis have shown that it promotes the splicing of the shorter isoform of the Eif4enif1 mRNA.[75] This isoform indirectly promotes chemotactic migration and sprouting behaviour.[75] HuR has also been shown to stabilize pro-angiogenic factors such as VEGF, HIF1-alpha and COX-2.[67,76–79] This stabilization can be beneficial in the context of tissue ischemia and wound healing [78], or detrimental when this occurs in response to hypoxia in solid tumours to promote their survival.[67,76,77,79,80]

Furthermore, studies in macrophages have shown a role for HuR in suppressing the pro-inflammatory phenotype in response to immune stimulation.[81] Oppositely, HuR is also known to promote an inflammatory phenotype by other immune cells such as monocytes, B-cells and T-cells.[82–84] Upon induction of an immune response, HuR translocates to the cytoplasm of monocytes and stabilizes pro-inflammatory genes, including interferon-stimulated genes and their transcriptional regulators.[82,85] In B-cells, HuR regulates the splicing of mRNAs encoding factors that mediate mitochondrial metabolism, cell survival and normal antibody production.[83,86] Cytokine production, an important driver of the immune response, is a tightly regulated process.[87] HuR is known to play an important role in this process by modulating the expression of multiple cytokines such as IL-6, IL-8, TNF-α, TNF-β and IFN-γ.[88] Unsurprisingly, HuR is a key player in inflammatory diseases due to its multi-level regulation of inflammation.[88] For example, HuR is involved in asthma because it stabilizes the TNF-α and GM-CSF mRNAs in eosinophils.[89] HuR's regulation of the TNF-α mRNA also implicates it in rheumatoid arthritis.[90]

In brown adipose tissue, HuR has been demonstrated to promote a pro-myogenic program resulting in the inhibition of adipogenesis.[91] This effect may be partially due to the HuR-mediated stabilization of the Insig1 mRNA, which has been shown to repress adipogenesis in white and beige adipocytes.[91] Further aspects of HuR's role in adipocytes are its positive regulation of lipolysis by stabilizing the ATGL mRNA and its indirect regulation of the glucose and lipid metabolism by stabilizing and promoting the translation of the PTEN mRNA.[44,92] HuR's multi-level roles in adipocytes have led to investigations and speculations of its function in multiple liver diseases, extensively reviewed by Liu et al. in 2020.[93]

Our laboratory and others have studied the complex role of HuR in apoptosis. Although it has an anti-apoptotic role under mild-stress conditions, HuR switches function to become pro-apoptotic when cells are exposed to severe stress.[94] This functional shift is partially controlled by its interaction with its binding partners, pp32 and APRIL proteins, which are promoters of HuR localization to the cytoplasm and apoptosome activation.[95] Under severe stress, HuR cleavage by caspases 3 and 7 leads to its accumulation in the

cytoplasm and the increased stabilization of pro-apoptotic mRNAs, including caspase 9, p53, p27, c-Myc and cytochrome c mRNAs.[96] HuR's accumulation in the cytoplasm and interaction with pp32/PHAP-I has also been proposed to play a pro-apoptotic role independent of the RNA-binding activity of HuR.[95]

Studies have identified increased HuR expression in a wide range of human malignancies. [88,97–99] In most cancers, this increase was associated with poor prognosis or treatment-resistant tumours, while in others, it was associated with a favourable outcome.[97] For example, increased cytoplasmic levels of HuR have been associated with worst survival outcomes in a wide range of cancers, including breast and prostate cancers.[88,97–99] Tumour cells were shown to misuse HuR mechanisms to promote multiple hallmarks of cancer.[98,99] For example, the pre-existing mechanisms through which HuR promotes angiogenesis, such as promoting HIF1-alpha translation or COX-2, and VEGF stability, can be exploited to allow tumour survival.[100] This misuse of the HuR protein can affect each of the cellular processes described above in countless ways and has been extensively studied and reviewed.[88,100–103] However, in pancreatic ductal adenocarcinoma (PDA) patients, it was associated with better outcomes as it was proposed to improve response to gemcitabine treatment.[104]

Over the past two decades, our laboratory and others have shed light on the role of HuR in post-natal myogenesis. This process is defined by the maintenance and regeneration of muscle fibers through the activation of Satellite Cells (SCs) and their subsequent differentiation into myocytes, which fuse into myotubes upon terminal differentiation.[105] These myotubes form myofibers which assemble into highly oriented bundles to form skeletal muscle.[106] Satellite cells are myogenic precursor cells that rest on the surface of the muscle fiber between the plasmalemma and the basement membrane.[105] They are quiescent and do not proliferate until activated by external stimuli, such as tissue injury caused by exercise or trauma.[105] Upon activation, a sub-population of SCs starts expressing the Myf5 protein, a member of the Myogenic Regulatory Factor family (MRF), containing MyoD, Myogenin and MRF4.[107] All members of the MRF family are transcription factors that bind E-box motifs in the promoter of genes through their basic

Helix-loop-Helix (bHLH) domain.[107] Their concerted action is what allows the myogenic program to unfold.[107] In the early phases of myogenesis, Myf5 is expressed in some SCs, which can divide to form a Myf5+ population.[107] These cells will start to express MyoD, which, in conjunction with Myf5, will support cell cycle progression and cellular proliferation to increase the number of activated SCs.[107,108] To form myotubes, these cells must exit the cell cycle and differentiate.[105,107] This is modulated by the Myogenin and MyoD proteins, promoting the expression of genes causing cell cycle exit, such as p21.[107,109,110] In the terminal stages of differentiation, the main factors involved are Myogenin and MRF4, which activate the muscle-specific proteins myosin and actin. However, studies have shown that MyoD is still involved even at these later stages.[111] The expression of myogenin is transcriptionally regulated by the transcription factor GATA4.[112] The MRFs are tightly regulated transcriptionally and post-transcriptionally. This regulation allows their expression to be modulated in response to stimuli such as exercise and diseases.[107]

HuR is one of the proteins that modulate the post-transcriptional regulation of MRFs, such as Myogenin and MyoD, during myogenesis. Under normal conditions, HuR promotes the differentiation of muscle fibers by simultaneously regulating multiple mRNA targets. In undifferentiated cells, this includes promoting the translation of the HMGB1 mRNA and destabilizing the Nucleophosmin (NPM) mRNA.[50,71] The HMGB1 protein has been demonstrated to be a part of a signalling cascade that activates pro-myogenic factors; therefore, HMGB1's increased translation promotes myogenesis.[113] On the other hand, the NPM protein has been implicated in the inhibition of differentiation in multiple cells (including myogenic cell lines), and the destabilization of its mRNA by HuR was shown to decrease its expression and promote the early steps of myogenesis.[71] During the formation of myotubes, HuR stabilizes the promyogenic factors myogenin, myoD and p21.[68] The mechanisms through which it does so, however, remain elusive and are the basis of this thesis. Lastly, our laboratory has demonstrated that HuR is involved in forming and maintaining glycolytic type II myofibers.[72] Indeed, phenotypically, muscle-specific HuR KO mice were shown to have increased oxidative type I fibers and endurance.[72] We showed that HuR forms a complex with KSRP to destabilize the PGC-

1α mRNA, an established modulator of fiber type specification, therefore promoting type II fiber formation and maintenance.[72]

Under inflammatory conditions, however, HuR switches from regulating pro-myogenic mRNAs to regulating pro-cachectic messages encoding factors that trigger muscle wasting.[114] Indeed, our lab has shown that HuR stabilizes the inducible nitric oxide synthase (iNOS) mRNA leading to the activation of the iNOS/NO pathway and contributing to muscle wasting.[114] In addition, HuR promotes the translation of the Signal transducer and activator of transcription 3 (STAT3) mRNA, a driver of pro-cachectic gene expression, by hindering the binding and preventing the translation inhibition caused by miR-330.[51]

Furthermore, HuR plays a role in senescence, stemness, erythropoiesis, heat-shock response, and DNA-damage response.[115–119] As discussed above, the HuR protein plays an important role in various cellular processes and disease phenotypes due to its multitude of functions and ubiquitous expression. Therefore, it is crucial to increase our knowledge of the mechanisms of HuR function to improve our understanding of all of these processes.

1.2.6. Mechanisms of Regulation of HuR Function

1.2.6.1 Localization

The functionality of the HuR protein depends, among other things, on its subcellular localization to the nucleus and/or to the cytoplasm, where its availability dictates its function in post-transcriptionally regulating mRNAs such as splicing in the nucleus, their transport to the cytoplasm or their translation by polysomes.[68,120] A stretch of basic amino acids found in the hinge region mediates the nucleocytoplasmic shuttling of HuR protein.[26] Although this sequence does not correspond to the classical nuclear localization or export sequence; it has been identified as the sequence responsible for HuR import and export from the nucleus.[26] The HuR Nucleocytoplasmic Shuttling (HNS) sequence mediates the interaction of HuR with Transportin (TRN) 1 and 2, facilitating its import in and out of the nucleus.[121–124] HuR is also known to translocate to the cytoplasm using CRM-1

dependent pathways. Under these conditions, HuR interacts with co-factors containing nuclear export signals, such as pp32/PHAPI and APRIL/PHAPII, which are recognized by the CRM-1 export protein for export to the cytoplasm.[124,125,126]

1.2.6.2 Post-Translational Modifications of HuR

The function of the HuR protein is, in part, regulated by post-translational modifications. Studies have shown that HuR can be methylated, phosphorylated, ubiquitinated, Parylated and neddylated, with each modification having different effects on its localization and or RNA-binding activity. For example, methylation by CARM1, a member of the PRMT family, on R217 affects the stabilizing function and subcellular localization of HuR in various cellular processes, including muscle cell differentiation.[127,128] Phosphorylation by kinases, such as PKCa, CDK1 and MAPK, impacts the localization of HuR to the nucleus or the cytoplasm.[129,130,131] For example, the Cdk1 kinase is known to phosphorylate HuR at the S202 residue resulting in its nuclear retention.[132] However, studies have shown that during the DNA Damage Response (DDR), the Chk1 kinase phosphorylates and inactivates CDK1 leading to the inhibition of CDK1-mediated HuR phosphorylation.[119,132] Therefore, during the DDR, HuR is unphosphorylated and accumulates in the cytoplasm, where it readily binds pro-survival mRNA targets.[132] Phosphorylation by Chk2 has also been suggested to modulate the affinity of HuR for certain targets such as SIRT1.[133] Hence, the binding of HuR and its ability to stabilize the SIRT1 mRNA was controlled by the phosphorylation of HuR at S100 by Chk2.[133]

Traditionally, ubiquitination of the HuR protein targets it for degradation by the proteasome and is a regulator of the abundance of HuR protein.[134] This mechanism has been shown to be modulated by external factors, which promote the ubiquitin-proteasome pathway. For example, HuR protein levels decrease during heat shock due to increased HuR ubiquitination and subsequent degradation by the proteasome.[134] Another modulator is the tumour suppressor Esophageal cancer-related gene 2 (ECRG2), which has been shown to promote the ubiquitination of HuR by an unknown E3 ligase and through an unknown mechanism to prevent the stabilization and translation of the XIAP mRNA.[135]

Furthermore, atypical ubiquitination of HuR has been shown to regulate its RNA-binding properties, although the mechanism remains unknown.[136]

Poly(ADP-ribosyl)ation, also known as PARylation, by PARP1 promotes the formation of HuR oligomers and increases its ability to displace the miRNA/RISC complex to stabilize pro-inflammatory mRNAs.[137] Neddylation of the HuR protein has been shown to promote its nuclear localization and, in turn, protect it from the degradation machinery in the cytoplasm.[138] The neddylation modification involves the linkage of NEDD8, a small molecule comparable to ubiquitin, to HuR on a lysine residue in its RRM3.[138,139] This process occurs through the passing off of the modification between E1, E2 and E3 enzymes before being added to HuR by the Mdm2 E3 ligase.[138,139]

In addition, the function and localization of the HuR protein are controlled by caspase cleavage.[95] Under stress conditions, the HuR protein can be cleaved into two products, HuR-CP1 (24kDa) and HuR-CP2 (8kDa), by caspase 3 and caspase 7 at aspartate (D) 226 located in the hinge region of the protein.[95] These cleavage products impact HuR targets and on HuR itself. The HuR-CP2 can promote apoptosis by binding to the pp32/PHAP-I apoptosome activator, while the HuR-CP1 can bind to TRN2 and sequester it from the full-length HuR protein leading to cytoplasmic accumulation of the HuR.[95,140] The HuR cleavage products are also RNA-binding proteins and can stabilize targets such as the caspase-9 mRNA to promote apoptosis.[94] Similarly, during myogenesis, HuR is cleaved by caspase 3 and HuR-CP1 sequesters TRN2 leading to the accumulation of HuR in the cytoplasm.[121,125] Our laboratory has demonstrated that this mechanism is essential for muscle fiber formation as the accumulation of HuR in the cytoplasm stabilizes promyogenic mRNAs including myogenin.[121]

1.2.6.3 Binding Partners

The RNA-binding function of HuR, in addition to its cellular localization under various cellular conditions, is known to be mediated by its interaction with protein partners.[50,71] The HuR protein, by interacting with trans-acting factors, has been shown to differentially regulate its mRNA targets at multiple post-transcriptional levels. For example, although

HuR is primarily known to be a positive regulator of mRNA stability, it has also been shown to mediate the decay of mRNAs.[141] This differential function of HuR is mediated by its interaction with different trans-acting factors such as microRNAs and other RNA-binding proteins. For example, our laboratory has shown that HuR promotes the translation of the HMGB1 mRNA by inhering the binding of miR-1192 and the subsequent inhibition of translation due to the recruitment of Ago2 and the RISC complex.[50] We also determined that HuR promotes the translation of the STAT3 mRNA, in cachexia-induced muscle wasting, by inhibiting miR-330 through a similar mechanism.[51] HuR has also been shown to mediate the translation of the CAT-1 mRNA by inhibiting the action of miR-122 and preventing the recruitment of the RISC complex (consequently resulting in the decreased localization of the mRNA to P-bodies).[52,142] Furthermore, HuR was shown to mediate the inhibition of miRNA-induced localization of the Stim1 mRNA to P-bodies.[143] However, HuR does not universally repel the interaction/action of miRNAs with/on their mRNA targets. Indeed, studies have shown that HuR can collaborate with the let-7 miRNA to promote the recruitment of Ago2 and repress translation of the c-Myc mRNA.[144,145]

The localization of HuR is modulated by its association with other proteins. The SETα, SETβ, pp32, APRIL and TRN2 proteins all modulate the localization of HuR as previously discussed.[29,122] Additionally, the Hzf protein can also bind to HuR to promote its nuclear export and the export of the p53 mRNA leading to an increase in its translation.[146] Another important role of HuR is its ability to promote or repress translation. Interactions with other proteins can modulate this role through cooperative or competitive binding. For example, the hnRNPA0 protein binds to HuR to inhibit the translation of upstream open reading frames of the AXIIR mRNA.[147] The ability of HuR to regulate mRNAs has been shown also to involve direct competition with other RBPs for binding to common motifs within the message. For example, the translation of the occludin and COX-2 mRNAs is mediated by the direct competition of HuR binding to these messages with, respectively, the CUGBP1 and CUGBP2 proteins.[22,148]

As discussed above, our laboratory has demonstrated that the interaction of HuR with the KH-type splicing regulatory protein (KSRP) recruits the ribonuclease PARN and the exosome to promote the turnover of messages .[71] The AUF1 protein can also collaborate with the HuR protein to destabilize targets, but this interaction is RNA-dependent and does not require direct binding of AUF1 and HuR.[21,73] In contrast, HuR also forms a complex with the RNPC1 protein to stabilize the p21 mRNA.[149]

Lastly, our laboratory has recently determined a novel protein-ligand of HuR required to stabilize pro-myogenic mRNAs during muscle fiber formation.[150] We found that HuR can form a complex with YB-1 in an RNA-independent manner and collaborate to bind to these mRNA targets.[150] As a proof of concept, we studied the role of this complex on the myogenin mRNA, one of its targets known to play an important role in myogenesis.[150] We demonstrated that two days after induction of differentiation of myocytes into myotubes, the HuR/YB-1 complex binds to the 3'UTR of the myogenin mRNA to stabilize it.[150] Although this study elucidated a novel mechanism of control of HuR function during myogenesis through its interaction with the YB-1 protein and the subsequent stabilization of these targets, the identity of the *cis*-elements in these mRNAs involved remains elusive.[150]

1.3. Y-box Binding factor 1 (YB-1)

1.3.1. Overview

YB-1 is a DNA and RNA binding protein of the Cold Shock Domain (CSD) family.[151] It was initially characterized in 1988 as a DNA binding protein that could bind Y-box motifs in the promoter of MHC class II genes.[152] Since then, YB-1 has also been demonstrated to be an RNA-binding protein.[153] The YB-1 protein is expressed in all somatic cells.[153] The YB-1 gene is located on chromosome 1p34 and encodes for a 43 kDa protein.[151]

1.3.2. Structure and Binding

The YB-1 protein contains an N-terminal alanine/proline-rich domain, followed by a CSD and a C-terminal domain with alternating clusters of basic and acidic amino acids(Figure 1.4a).[154] Although the full structure of the YB-1 protein remains to be elucidated,

numerous studies have inferred on its properties and established that it is a compact protein prone to multimerization.[155] The CSD comprises five anti-parallel β-strands that form a β-barrel closed with a long flexible loop.[156] (Figure 1.4b) The CSD confers the RNA-binding property of the YB-1 protein due to the presence of two RNA-recognition motifs, RNP1 and RNP2. These RNPs contain hydrophobic residues located on the outside of the β-barrel, on the second and third β-strands, that allow YB-1 to form π-π stacking interactions with four nucleotides in a ssDNA or RNA sequence.[156–158] Other residues in the β-barrel, such as the Arg69 and Lys118, also contribute to binding.[156] Overall, the CSD forms hydrogen bonds, π-π stacking and hydrophobic interactions with its targets.[157] Studies have shown that the CSD can bind RNA in a sequence-independent or dependent manner.[157] The binding site for the YB-1 protein was initially discovered to be a Y-box in DNA.[152] Various consensus sequences in RNA have been suggested to direct YB-1 binding including CA(U/C)C[157], UC/UAuC (UYAUC)[159] and UCCAG/ACAA[160]. Other studies have also demonstrated an increased affinity for GC-rich sequences.[160,161] The C-terminal domain also has nucleic acid binding affinity, although it doesn't seem to have an affinity for specific sequences.[162] Binding of the C-terminal domain increases the affinity of the YB-1 protein for its targets.[163] The C-terminal domain also plays a role in protein-protein interactions including YB-1 dimerization, oligomerization and binding to other proteins.[162,164,165]

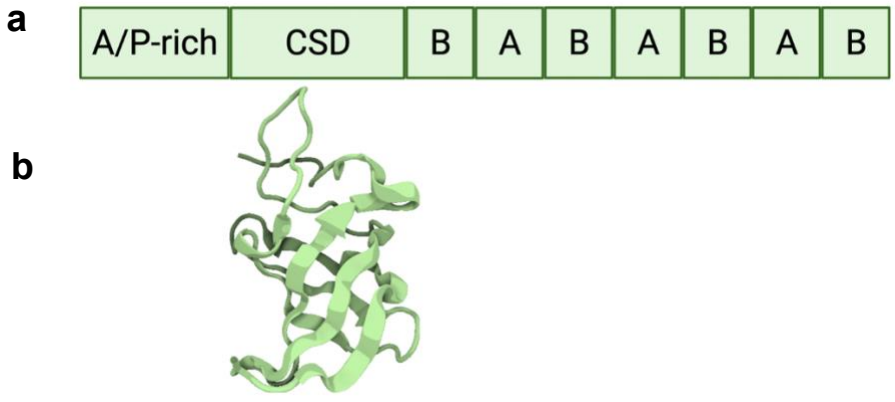


Figure 1.4 Structure of YB-1 a) The YB-1 protein is composed of an Alanine-Proline rich N-terminal domain, a cold-shock domain and a C-terminal with alternating regions of acidic and basic amino acids. b) The crystal structure of the cold-shock domain has been

determined. The full structure has not yet been determined due to the unstructured nature of the N- and C-terminal domains.[31,166]

1.3.3. YB-1 Expression

The expression of the YB-1 protein is regulated at the transcriptional and post-transcriptional levels. Instead of a TATA-box, transcription of the YB-1 gene is regulated by three enhancer boxes (E-boxes) and GATA motifs.[162,167] The E-boxes can be bound by a plethora of transcription factors to activate the transcription of the YB-1 gene.[162,167] In myocytes, upon induction of differentiation, these motifs are bound by E2F1 and Sp1.[168] As myotubes start to form, these factors are replaced by both MyoD and Myogenin proteins.[168] A study in human cancer cell lines showed that the p73 protein interacts with the c-Myc protein to promote the transcription of the YB-1 gene by increasing the recruitment of c-Myc to an E-box.[169] In other cell types, other transcription factors, such as Twist and Math2, have also been shown to promote the transcription of the YB-1 gene.[170,171] The translation of the YB-1 mRNA, on the other hand, is facilitated by the binding of the PolyA binding protein (PABP) when the mRNA is not polyadenylated.[172–174] The PABP protein competes for binding with the YB-1 protein, which binds to a sequence in the 3'UTR to inhibit the translation of its mRNA.[172] It also competes for binding with the hnRNPQ protein, a translational inhibitor.[175] The YB-1 protein also autoregulates its translation by binding its 5'UTR to repress translation initiation.[176] Lastly, YB-1 is a part of a self-regulatory loop with the miR-548ac and the Snail protein.[177] YB-1 promotes the transcription of the Snail protein, which in turn stimulates the transcription of the miR-548ac, which binds and inhibits the translation of the YB-1 mRNA.[177]

1.3.4. Functions

The YB-1 protein is a DNA (DBP) and RNA (RBP) binding protein that allows it to perform a wide range of functions in the nucleus and in the cytoplasm. As a DBP, it acts as a transcription factor and has a role in DNA repair.[151,162] As an RBP, it has been shown to regulate both pre-mRNA splicing and RNA sorting to subcellular vesicles as well as mRNA stability and mRNA translation.[151,162] These functions are detailed below. (Figure 1.5)

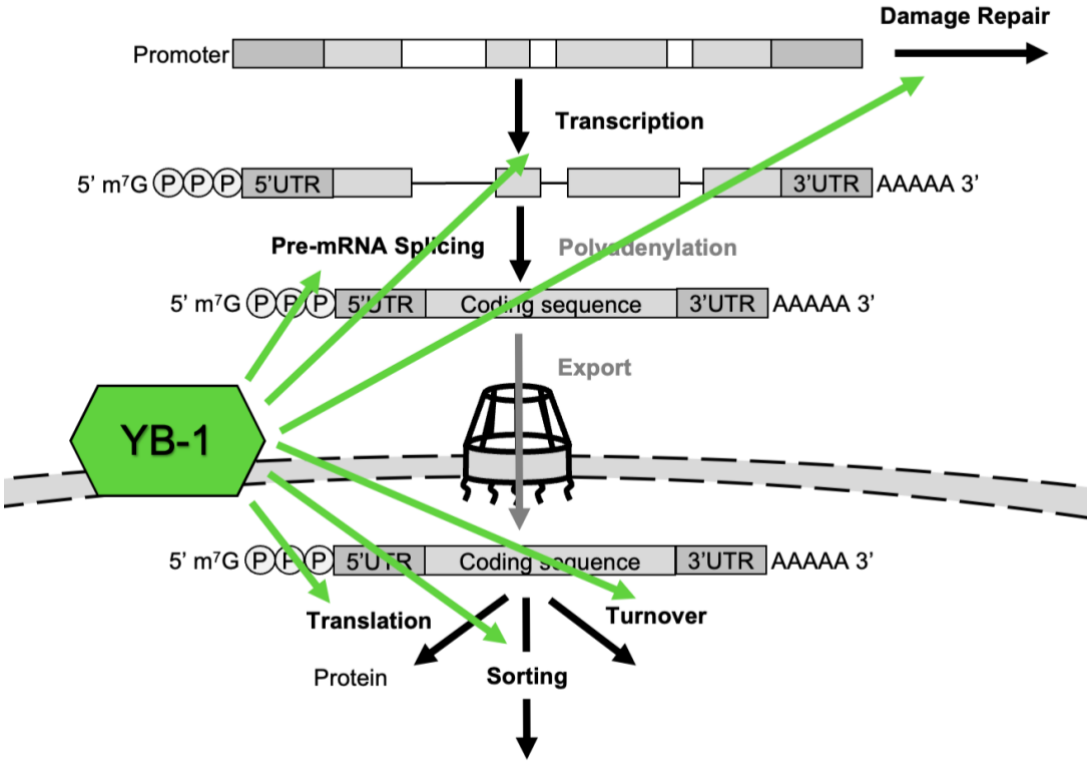


Figure 1.5 Summary of YB-1 functions

The YB-1 protein plays multiple roles as a DNA and RNA binding protein. As a DNA-binding protein, it modulates transcription and plays a role in DNA repair. As a RNA-binding protein, it regulates mRNA translation, splicing, sorting into subcellular vesicles and turnover.

1.3.4.1 Transcription

Initial studies on the functionality of the YB-1 protein focused on its role as a transcription factor through its ability to bind to Y-box sequences 5'-CTGATTGGC/TC/TAA-3'.[152] Further studies suggest that its ability to stabilize ssDNA and its affinity for GC-rich regions are also characteristics that allow it to mediate transcription.[161,178] YB-1 has been shown to activate or repress transcription by impacting the binding of other factors to gene promoters.[178]

1.3.4.2 DNA Damage Repair

YB-1 has been suggested to play a role in DNA repair based on its ability to bind damaged DNA, including cisplatin-modified sequences, abasic sites and ssDNA.[179–181] YB-1 was, furthermore, demonstrated to bind to the PCNA protein, which plays a prominent role in nucleotide excision and the mismatch repair pathways and possesses 3' to 5' exonuclease activity.[180,181] This suggests that it may play a role in DNA damage recognition and in the opening of the DNA helix to allow for nucleotide excision repair (NER) to occur.[180,181] Additional studies showed that YB-1 could separate DNA duplexes at sites of damage, had endonucleolytic activity and bound to proteins in the base excision repair and mismatch repair pathways.[182,183] Furthermore, in base excision repair, YB-1 was suggested to act as a co-factor of PARP1 due to their binding and reciprocal regulation.[183,184] YB-1's ability to form multimers allows it to bind to DNA crosses and infers a possible role in homologous recombination.[165] Taken together, these studies delineate a role for YB-1 as a positive regulator of repair pathways. However, YB-1 binding to PCNA has also been shown to hinder the mismatch repair pathway by preventing the formation of the MutSα/PCNA complex.[185] Therefore, YB-1 is a member of the DNA damage response, but the exact mechanistic functions of YB-1 in these various DNA repair pathways remain unclear.

1.3.4.3 Splicing

YB-1's role in splicing was studied after it was identified as a member of the pre-spliceosome.[186,187] It was recently discovered that YB-1 plays a vital role in the Maternal to Zygotic transition of the embryo development by modulating the alternative splicing of key genes such as the EIF3I, EED and hnRNPm mRNAs.[188] For example, YB-1 was shown to promote the exclusion of EIF3I exon 9 to form the Eukaryotic Translation Initiation Factor 3 Subunit I mRNA.[188] However, the precise mechanisms responsible for YB-1's impact remain undiscovered.[188]

1.3.4.4 Sorting of RNAs into Subcellular Bodies

The ability of YB-1 to bind RNAs has led to the investigation of its role in the recruitment of RNA to subcellular bodies. YB-1 was identified as a component and marker of stress

granules and P-bodies.[189] These RNA granules are membrane-less and contain translationally-inactive mRNPs.[190,191] Briefly, stress granules contain stalled translation initiation factors while P-bodies contain translational repressors and decay factors.[191] Although YB-1 is essential to the formation of stress granules, the mechanism of its role in SG assembly remains unclear.[192] A recent study demonstrated that YB-1 could form P-bodies by liquid-liquid phase separation and recruits specific miRNAs to these entities.[193] Moreover, YB-1 is known to bind to ncRNAs and modulate their packaging into exosomes.[194] These novel roles of the YB-1 protein are currently being investigated.[192]

1.3.4.5 mRNA Turnover

The YB-1 protein acts as a general mRNA stabilizing factor when the ratio of YB-1 to mRNA is high.[195] The same mechanism that prevents translation also allows YB-1 to protect mRNAs from exoribonucleases.[195] This protection is sequence-independent as it only requires the binding of the CSD of YB-1 to the mRNA 5'cap. [195] In some cases, YB-1 has also been shown to stabilize specific mRNAs in complex with other proteins. For example, YB-1 can stabilize the IL-2 mRNA by binding to a *cis*-element in its 5'UTR and forming an RNA-dependent complex with the Nucleolin protein bound to the 3'UTR.[196] YB-1 has also been shown to stabilize the GM-CSF mRNA by binding to its 3'UTR.[89,197] A proposed mechanism for this stabilization is by forming a complex with HuR and hnRNP.[89,197] Additionally, YB-1 can scan mRNA sequences for 5-methylcytosine (m^5C) modifications and bind to these modified nucleotides in the 3'UTR of specific messages such as the heparin-binding growth factor mRNA.[198] In doing so, YB-1 stabilizes these mRNAs by recruiting HuR.[198]

1.3.4.6 Translation

YB-1 was shown to play a prominent role in regulating the general translation of mRNAs.[151] However, the ability to do so is dependent on its levels in cells.[199–202] Elevated levels of YB-1 protein result in the inhibition of translation initiation since YB-1 competes with eIF4G for binding to the cap structure.[203] However, when the levels of YB-1 protein are low, eIF4G binds to the cap to promote translation.[203] Elevated levels of YB-1 can also inhibit translation due to the formation of tightly packed messenger

Ribonucleoproteins (mRNP) complex, which occurs due to YB-1 multimerization.[155,158,165,204] These compact mRNP repress general translation by sequestering mRNAs away from polysomes.[155,158,165,204,205] Interestingly, when YB-1 is a part of mRNPs, it interacts with proteins of the cytoskeleton, such as actin and tubulin.[206–208] The interaction between YB-1 and actin was shown to occur in loosely packed mRNPs and to modulate their location at the time of translation; hence, YB-1 affects the site of protein synthesis.[206] The interaction between YB-1 and tubulin was suggested to impact mRNP formation or mRNP localization.[207,208]. Interestingly, YB-1 has also been shown to regulate the translation of specific mRNA through multiple mechanisms. For example, it was shown to differentially modulate the translation of messages, including the ferritin, G3BP1 and c-Myc mRNAs, by binding to *cis*-elements (such as an IRES motif in the case of c-Myc) in their 5'UTR[209].[210] [211]

1.3.5. Importance of YB-1 in Cellular Processes

YB-1 has been studied in many cellular processes due to its various roles and its expression in all somatic cells. The importance of YB-1 during embryonic development is underscored by the fact that the genetic ablation of YB-1 is embryonically lethal.[212] Knocking out YB-1 in mice, indeed, affected the later stages of embryogenesis by causing growth retardation leading to mortality.[212,213]

The role of YB-1 in the stress response is two-fold. First, a truncated isoform of YB-1 is retained in the nucleus conferring adaptability to genotoxic stress such as the DNA damage stress caused by chemotherapy.[151,214,215] This YB-1 cleavage product promotes cell survival by participating in DNA repair pathways and promoting the transcription of pro-survival genes such as MDR1.[214,216] Second, the YB-1 protein can confer resistance to oxidative stress by promoting the translation of the G3BP1 mRNA and hence the formation of stress granules.[217]

The role of YB-1 in cell proliferation has been suggested to be due to its role as a transcriptional activator of growth-associated genes such as the cyclin A1, cyclin B2 and CDC6 genes, which promote the transition of G1 into the S phase of the cell cycle.[218–220]

YB-1 was also proposed to promote the transition from the G2 phase to the M phase by binding to key mRNAs such as Cdc25b, but its functional role on these mRNAs was not elucidated.[221] A recent study demonstrated that YB-1 controls cytokinesis, thus regulating cell proliferation by binding and organizing microtubules, actin, and other cytoskeletal proteins to define the cleavage plane.[222]

The YB-1 protein has been most extensively studied for its implication in the oncogenic process. It has been identified as a player in breast, ovarian, bladder and lung cancers, among others.[151] For example, Bargou et al. established that YB-1 is a transcriptional activator of the MDR1 gene in a subset of breast tumours associated with poor prognosis.[223] Further studies found that high YB-1 protein levels were a marker of breast cancer aggressiveness and resistance to therapy.[151] The role of YB-1 as an oncogene has been extensively studied, specifically its ability to promote proliferation, protect cells against apoptosis, promote metastasis and allow epithelial to mesenchymal transitions (EMT).[151,224–226]

YB-1 modulates inflammation in a cell-specific manner by regulating the transcription and stability of inflammatory mediators. For example, YB-1 translocates to the nucleus of monocytes to promote the expression of the chemokine CCL5 at the early stages of inflammation. [227] When these monocytes differentiate into macrophages, YB-1 protein levels are downregulated, resulting in the reduced expression of CCL5.[227] In macrophages, YB-1 binds to the IL-6 mRNA and promotes its translation and subsequent secretion, which lowers intracellular levels of the pro-inflammatory cytokine.[228] However, in dendritic cells, YB-1 acts as a stabilizer of the IL-6 mRNA and promotes the expression of IL-6 protein.[228] In T-lymphocytes, YB-1 inhibits immune activation by binding to the fourth intron of the IL-10 gene and promoting its transcription.[229] Lastly, YB-1 stabilizes the GM-CSF mRNA by binding in its 3'UTR in a complex with HuR and hnRNP. [89,197] This increases the levels of GM-CSF protein, which promotes eosinophil survival and inflammatory diseases such as asthma or allergic reactions.[197]

YB-1 has been shown to play a prominent role in regulating myogenesis. Specifically, YB-1 was shown to repress the translation of the AChRα mRNA, an important factor that needs to be highly regulated for proper neuromuscular junction innervation.[230] YB-1 was also shown to repress muscle cell differentiation by promoting the translation of the ROCK1 mRNA, a known inhibitor of the formation of myotubes.[231] Furthermore, a cleaved isoform of YB-1, YB-1/p32, was shown to be retained in the nucleus and to cooperate with the Msx1 homeoprotein to repress the transcription of the MyoD gene and, in turn inhibit myoblast differentiation.[232]

As mentioned above, our laboratory recently showed that YB-1 is required for myocyte differentiation into myotubes.[150] We demonstrated that YB-1, under these conditions, forms a complex with HuR in an RNA-independent manner. The HuR/YB1 complex promotes myogenesis by regulating the expression of the myogenin mRNA. Indeed, depletion of YB1, similarly to HuR, destabilizes the myogenin mRNA, decreases Myogenin protein levels, and inhibits myogenesis.[150] These results led us to investigate the generality of this regulatory complex and the mechanism by which it binds and stabilizes messages.

2. RATIONALE AND OBJECTIVES

The diversity of roles played by the HuR protein has been attributed to its localization, post-translational modification, and interactions with other RNA-binding proteins and ncRNAs. Previous work by our laboratory elucidated the mechanisms through which HuR promotes the turnover and translation of mRNAs that modulate muscle homeostasis.[50,51,71] We showed that HuR fosters the translation of the HMGB1 mRNA by inhibiting miR-1192 during myogenesis and, similarly, the translation of the STAT3 mRNA (by inhibiting the action of miR-330) during inflammation-induced muscle wasting.[50,51] We also established that the HuR protein could form a complex with the KSRP protein to regulate the turnover of the NPM mRNA during muscle differentiation.[71] The HuR/KSRP complex mediates the turnover of this mRNA by recruiting deadenylases and the exosome. [71]

Despite our advances in elucidating the function of HuR during myogenesis and muscle-related diseases, the mechanisms through which HuR functions to stabilize pro-myogenic mRNAs, such as myogenin and MyoD, during myogenesis remains elusive. Our laboratory's interest in identifying mechanistically how HuR promotes the stability of pro-myogenic mRNAs during muscle fiber formation binding partners led us to identify the Y-box binding protein 1 (YB-1) as a binding partner of HuR in C2C12 myoblasts.[150] We determined that the YB-1 protein forms an RNA-independent complex with the HuR protein in C2C12 upon induction of muscle differentiation.[150] The functionality of this complex was studied using the myogenin mRNA as a proof of concept. [150] Our laboratory established that the complex binds to a GU-rich region in the 3'UTR of the myogenin mRNA, increasing its stability.[150] Overall, these results established YB-1 as a binding partner of HuR during myogenesis.

Based on this work and previous publications, we aimed to understand mechanistically the importance of the HuR/YB-1 complex on the fate of its mRNA targets during muscle cell differentiation.[150,197,198] To do so, we aimed to investigate the ability of HuR and YB-1 to regulate the stability of their common pro-myogenic mRNA targets and, additionally, if they do so by associating to a consensus *cis*-elements in the 3'UTR of these messages.

In addition to the MyoG mRNA, we demonstrate that the HuR and YB-1 proteins cooperate to regulate the stability of other mRNAs, including MyoD and Gata4, which were previously shown to regulate the myogenic process.[107,112] Our MEME analysis uncovered that the HuR/YB-1 complex might associate to a consensus U-rich consensus motif found in the 3'UTR of these common pro-myogenic mRNAs. Our findings, therefore, clearly establish the cooperation between both HuR and YB1 as a novel regulatory mechanism in muscle cells regulating mRNA targets containing a consensus U-rich motif that is necessary for the formation of muscle fibers. The work presented in this complex thus furthers our knowledge of the interactions that regulate the key post-transcriptional regulator HuR during the myogenic process.

3. RESULTS

3.1. RNP-Immunoprecipitation of HuR and YB-1

HuR and YB-1 have been shown to form an RNA-independent complex two days after induction of differentiation of myocytes into myotubes.[150] We have recently established that, during this process, this complex mediates the stability of the myogenin mRNA, an important myogenic regulatory factor.[150] To further our understanding of the molecular mechanisms through which the HuR/YB-1 complex promotes myogenesis, we aimed to determine the complete network of pro-myogenic messages regulated by this complex. To this end, we performed RNP-immunoprecipitation (RIP) experiments followed by RNA-sequencing (RIP-seq) using muscle cell lysates obtained two days after the induction of differentiation. These experiments were performed using the C2C12 mouse model of muscle cell differentiation. In this cellular model, myoblasts initially proliferate exponentially (Exp) until they reach confluency (D0). (Figure 2.1a,b). Once a monolayer of myoblasts is formed, the media is changed to a low-serum media to induce differentiation of these myoblasts into myotubes. (Figure 2.1a,b). In using this system, we demonstrate that the differentiation of myotubes is not correlated with changes in the expression of these RBPs. Indeed, we demonstrate that the expression of the HuR and YB-1 proteins remain unchanged throughout the differentiation process. (Figure 2.1c).

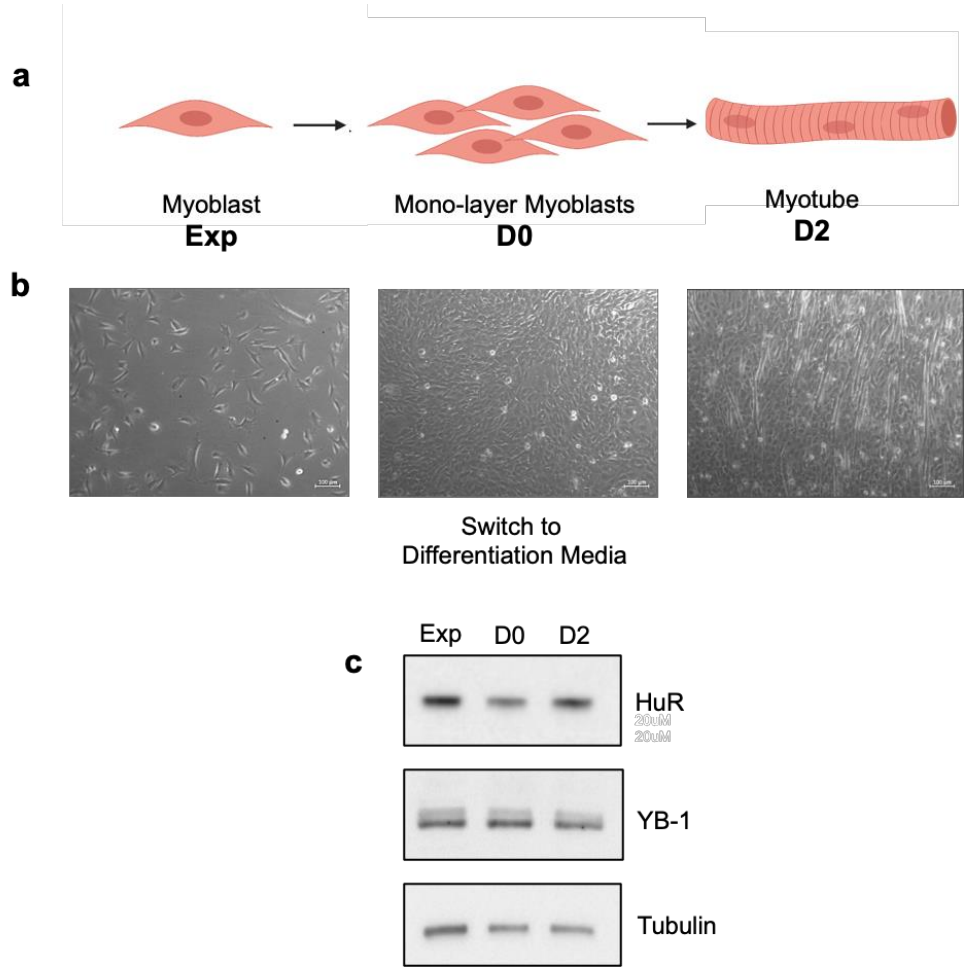


Figure 2.1 C2C12 Cellular Model mRNA targets bound by HuR and YB-1 were identified at D2 of differentiation in C2C12. **a)** Schematic of the differentiation process[31] **b)** Phase Contrast Images of myoblasts in the exponential phase (Exp), myoblasts at confluency (D0), and myotubes two days after induction of differentiation (D2) **c)** Western Blot showing the expression of HuR and YB-1 at Exp, D0 and D2 post-induction of differentiation.

The RIP-seq experiments were performed using anti-HuR or anti-YB-1 antibodies to immunoprecipitate HuR or YB-1, respectively (Figure 2.2a) from lysates collected at D2 of muscle cell differentiation (where HuR and YB-1 were shown to interact with each other[150]). mRNAs associating with these RBPs were identified as targets of these proteins if the levels bound to the immunoprecipitated HuR or YB-1 proteins were two-

fold or more when compared with an IgG negative control.[150,233] By comparing the list of mRNAs bound to HuR or YB-1, we were able to identify 409 common mRNAs between these two proteins.[150] (Figure 2.2b, Supplementary Table 8.1). These common targets were analyzed using the PANTHER Gene Ontology software and found to encode for a wide range of protein classes which were further characterized according to their molecular functions and cellular processes they are involved in. (Figure 2.3). The most prominent protein class represented in the targets was the "defense/immunity protein" class. (Figure 2.3a) The most common molecular function was "binding" representing 44% of the targets. (Figure 2.3b) Lastly, the targets spanned 36 types of cellular processes without an apparent specificity for one process. (Figure 2.3c)

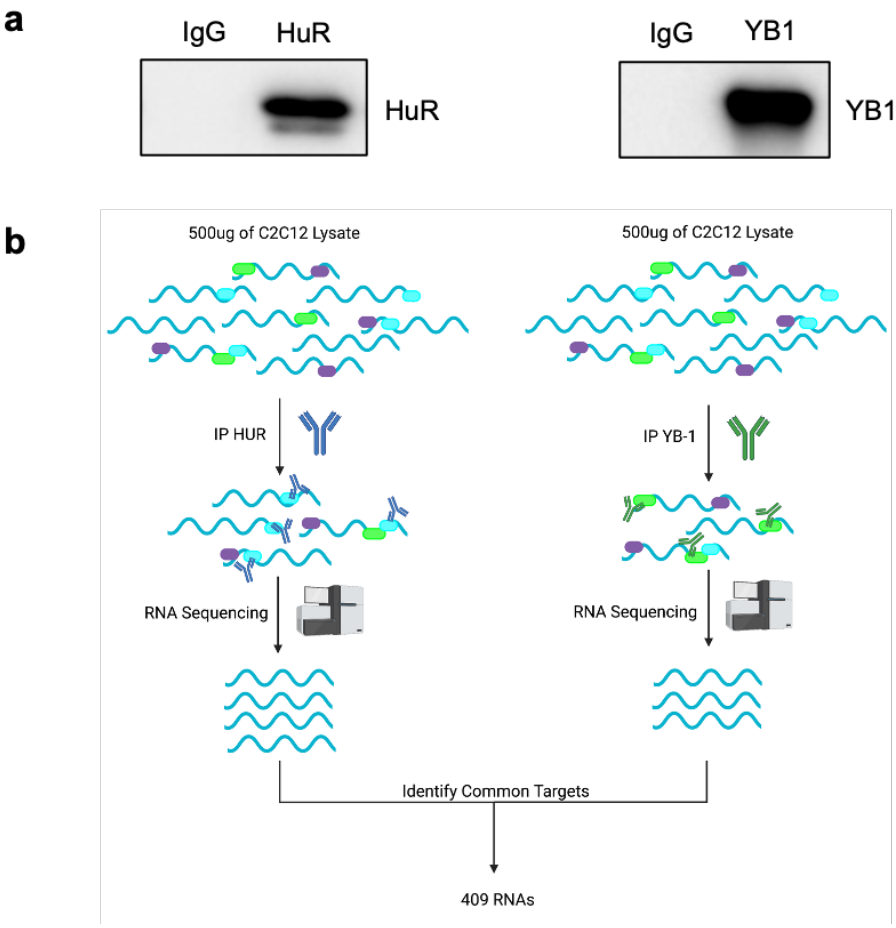


Figure 2.2 Identification of Common Binding Targets of HuR and YB-1 in C2C12 **a)** Western blot showing the immunoprecipitation of HuR (left) and YB-1 (right) from C2C12 cell extracts at D2. **b)** Schematic of the protocol.[31] RNA-immunoprecipitation experiments were performed using 500ug of C2C12 lysate for each protein followed by

RNA-sequencing by Illumina. Targets were identified for each protein and compared to obtain a list of 409 targets.

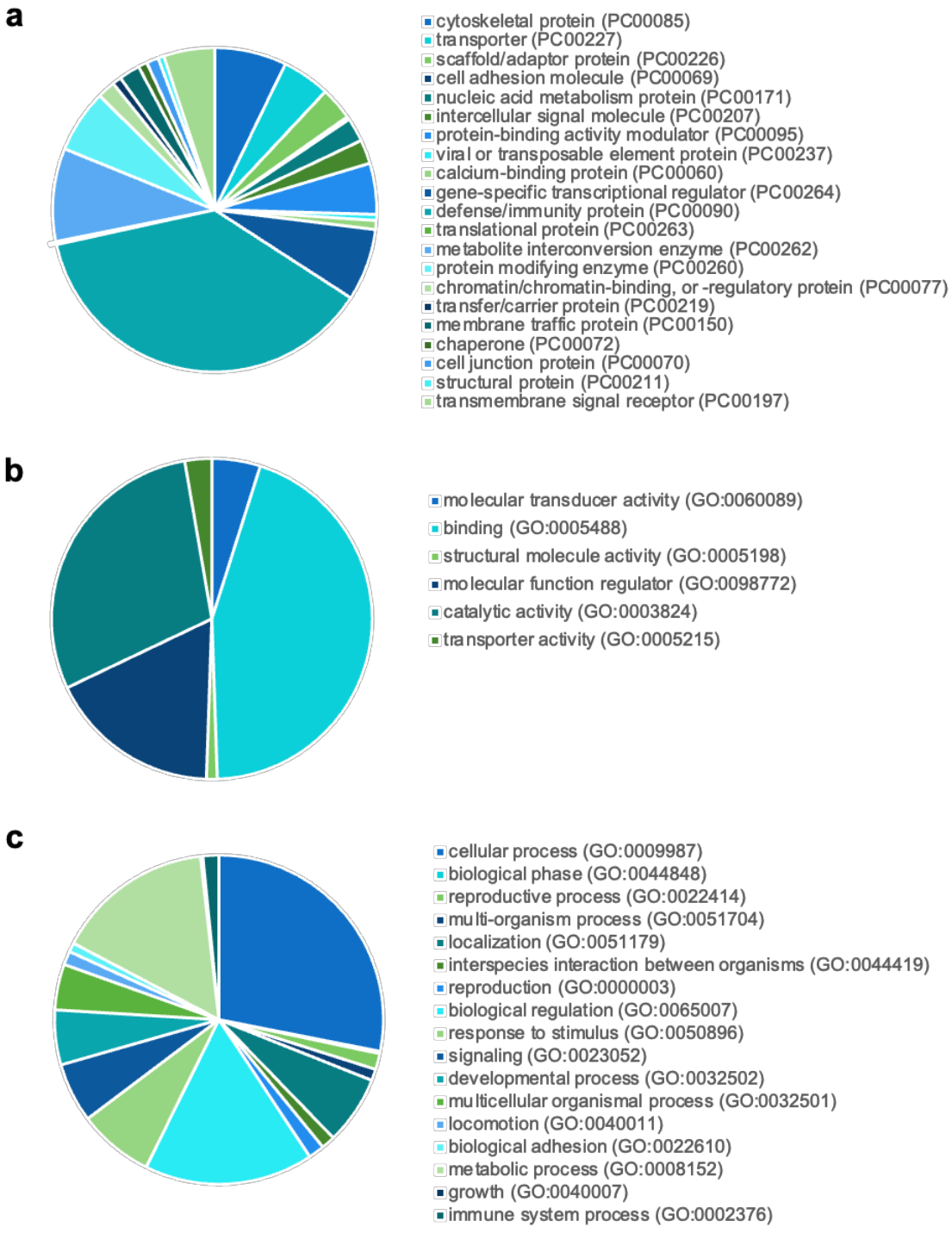


Figure 2.3 Classification of mRNA Targets Bound by HuR and YB-1. Gene Ontology analysis of the 409 common targets performed using PANTHER version 16.0 **a)** Protein Class **b)** Molecular Function **c)** Biological Process

To validate the RIP-seq experiments, we assessed the binding of HuR and YB-1 to the myogenin mRNA as previous data from our laboratory established it as a target of the HuR/YB-1 complex.[150] Our data from the RIP-seq indicated that the association of the myogenin mRNA with HuR, or YB-1, using the anti-HuR or anti-YB-1 antibodies, was substantially greater (15.71 and 2.32 fold, respectively) than that was observed using IgG (which was included as a negative control) (Figure 2.4a). We next performed RIP-coupled to RT-qPCR experiments with primers specific for the myogenin mRNA to further confirm the validity of the RIP-seq results. As expected, both HuR and YB-1 associate strongly with the Myog mRNAs compared to IgG. (Figure 2.4b).

a

	Fold Change	
	HuR vs IgG	YB-1 vs IgG
Myog	15.71	2.32

b

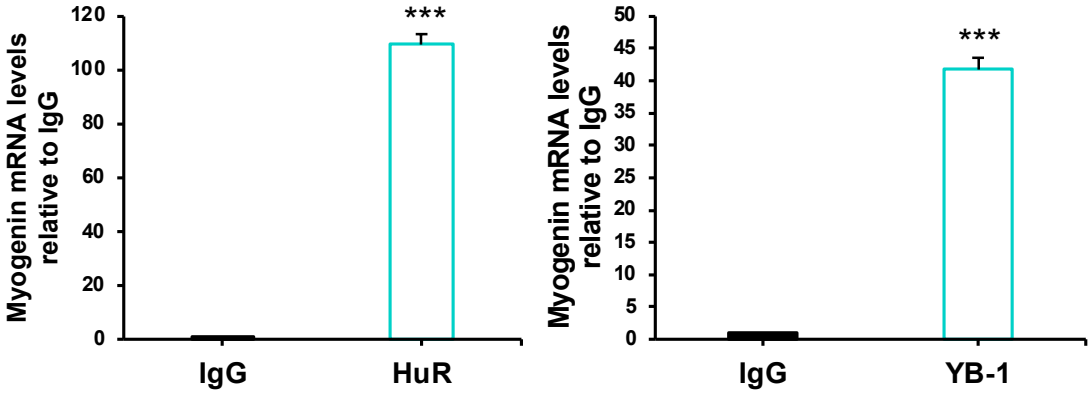


Figure 2.4 HuR and YB-1 Bind the Myog mRNA. a) Fold change of the association of the Myogenin mRNA with HuR (left) or YB-1 (right). **b)** Validation of the myogenin mRNA association to HuR (left) and YB-1 (right). The RNA-immunoprecipitation experiments were performed using IgG and anti-HuR or anti-YB-1 antibodies. Isolated RNA was processed by RT-qPCR with primers for the myogenin and GAPDH mRNAs. Myogenin

mRNA levels were standardized against GAPDH mRNA levels and plotted relative to IgG. The error bars represent the S.E.M. of N=3 ***P<0.001

3.2. Validation of Additional targets of the HuR/YB-1 complex

We next aimed to determine if the HuR/YB-1 complex was involved in regulating the expression of additional common mRNA targets that, in addition to Myogenin, encode factors that regulate the myogenic process. Among the 409 common mRNA targets, we identified that the MyoD and Gata4 mRNAs encode factors that participate in regulating muscle fiber formation[107,112] The RIP-seq data indicated that both targets (MyoD and Gata4) were strongly associated with HuR and YB-1 when compared to the IgG negative control. (Figure 2.5a) HuR's association with the MyoD and Gata4 mRNAs (which were pulled down using an anti-HuR antibody) was approximately three-fold and twelve-fold higher, respectively, than their association with IgG. (Figure 2.5a) We demonstrate, additionally, that the interaction of YB-1 with these mRNAs (MyoD and Gata4) in muscle fibers is also approximately three-fold and four-fold higher respectively than the IgG control. (Figure 2.5a) We next validated RIP-seq data by performing, as described above, RIP coupled to RT-qPCR experiments. By performing these experiments, we confirmed that both HuR and YB-1 associated strongly with the MyoD and Gata4 mRNAs. (Figure 2.5b-c),

a

	Fold Change	
	HuR vs IgG	YB-1 vs IgG
MyoD	3.13	3.02
Gata4	11.59	4.20

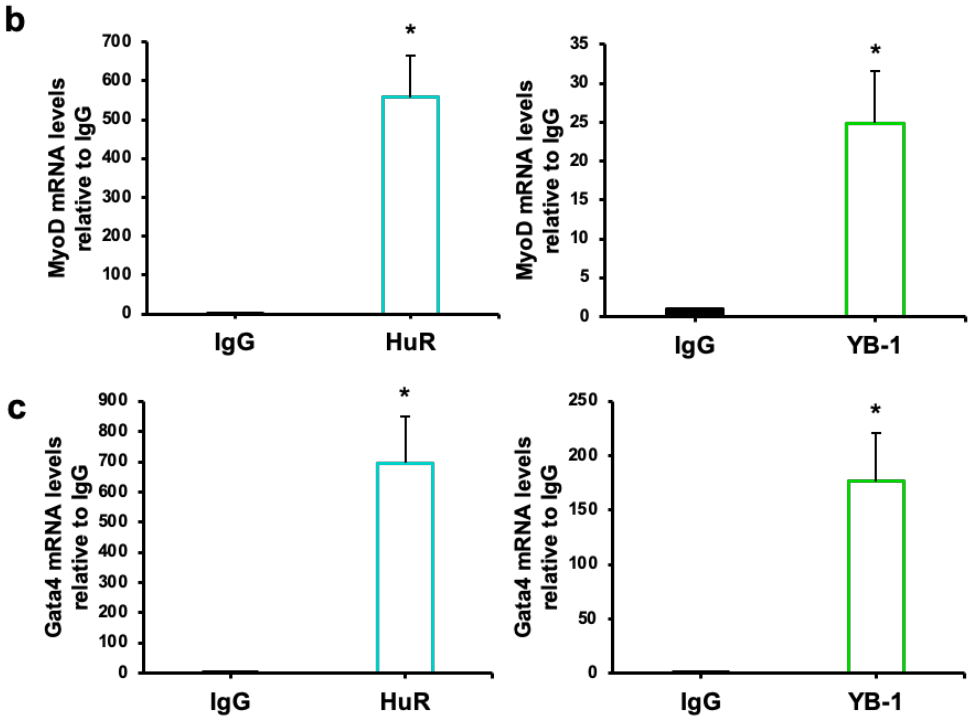


Figure 2.5 HuR and YB-1 Bind the MyoD and Gata4 mRNAs. **a)** Fold Change of the association of the MyoD and Gata4 mRNAs with anti-HuR vs IgG (left) and anti-YB-1 vs IgG (right) from the RIP-Seq experiment **b)** MyoD mRNA association to HuR (left) and YB-1 (right). **c)** Gata4 mRNA association to HuR (left) and YB-1 (right). RNA-immunoprecipitation experiments were performed using IgG and anti-HuR or anti-YB-1 antibodies. The isolated RNA was processed by RT-qPCR with primers for the MyoD, Gata4, and GAPDH mRNAs. Target mRNA levels were standardized against GAPDH mRNA levels and plotted relative to IgG. The error bars represent the S.E.M. of N=3 *P<0.05

Having shown that both HuR and YB-1 associate to these messages, we next assessed the functional importance of these proteins on the regulation of the MyoD and Gata4 mRNAs. As a first step, we assessed the impact of depleting either HuR or YB-1 on the

expression of these mRNAs (Figure 2.6a). Our results indicate that the depletion of either HuR or the YB-1 significantly decreased the levels of the MyoD and Gata4 mRNAs (by ~60% or more) when compared to siCTL treated cells. (Figure 2.6b-c). Overall, these results indicate that both HuR and YB-1 regulate the levels of the MyoD and Gata4 mRNAs.

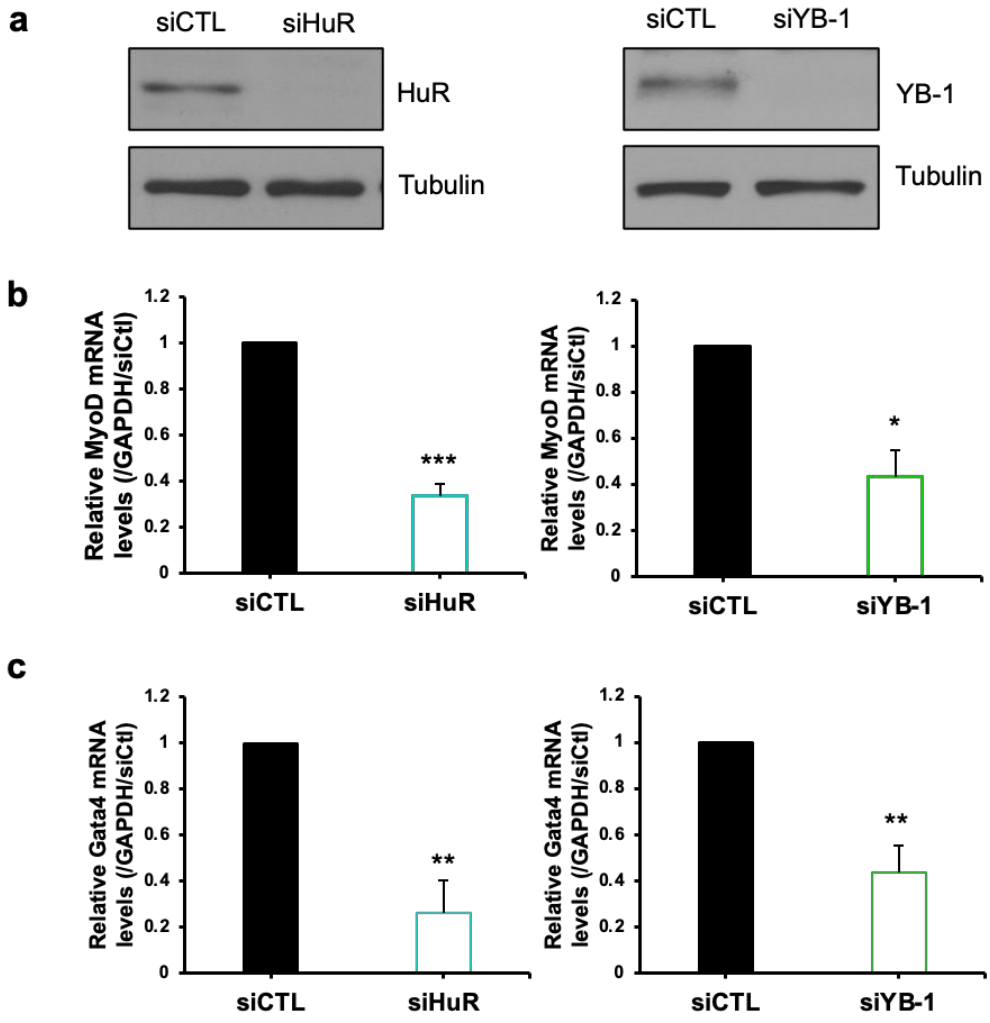


Figure 2.6 Depletion of HuR or YB-1 Decreases MyoD and Gata4 mRNA Levels. C2C12 cells were treated with siCTL, siHuR (left) or siYB-1(right). **a)** Western Blot showing the depletion of HuR (left) and YB-1 (right) by siRNA. **b-c)** RNA was isolated at D2 post-induction of differentiation. RT-qPCR was performed with primers specific to **b)** MyoD and GAPDH and to **c)** Gata4 and GAPDH. mRNA levels were standardized against

GAPDH mRNA levels and plotted relative to siCTL. The error bars represent the S.E.M for N=3. *p<0.05, **p<0.01 ***p<0.001

Our previous work demonstrated that the HuR/YB-1 complex regulates the stability of the myogenin mRNA. As a next step, we aimed to determine if the HuR and YB-1 proteins also regulated the stability of these other common targets. We, therefore, performed an Actinomycin D pulse-chase experiment to determine the rate of decay of the MyoD and Gata4 mRNAs in the presence or absence of either protein. By performing these experiments, we demonstrate that the depletion of HuR decreased the half-life of the MyoD mRNA by ~30min. (Figure 2.7a) while the depletion of YB-1, similarly, decreased the half-life of this message by ~15min. (Figure 2.7b) Additionally, we show that knockdown of HuR or YB-1 decreased the half-life of the Gata4 mRNA by more than 50min and 30min, respectively (Figure 2.7c-d). Our results, therefore, demonstrate that both HuR and YB-1 regulate the stability of the MyoD and Gata4 mRNAs during muscle cell differentiation.

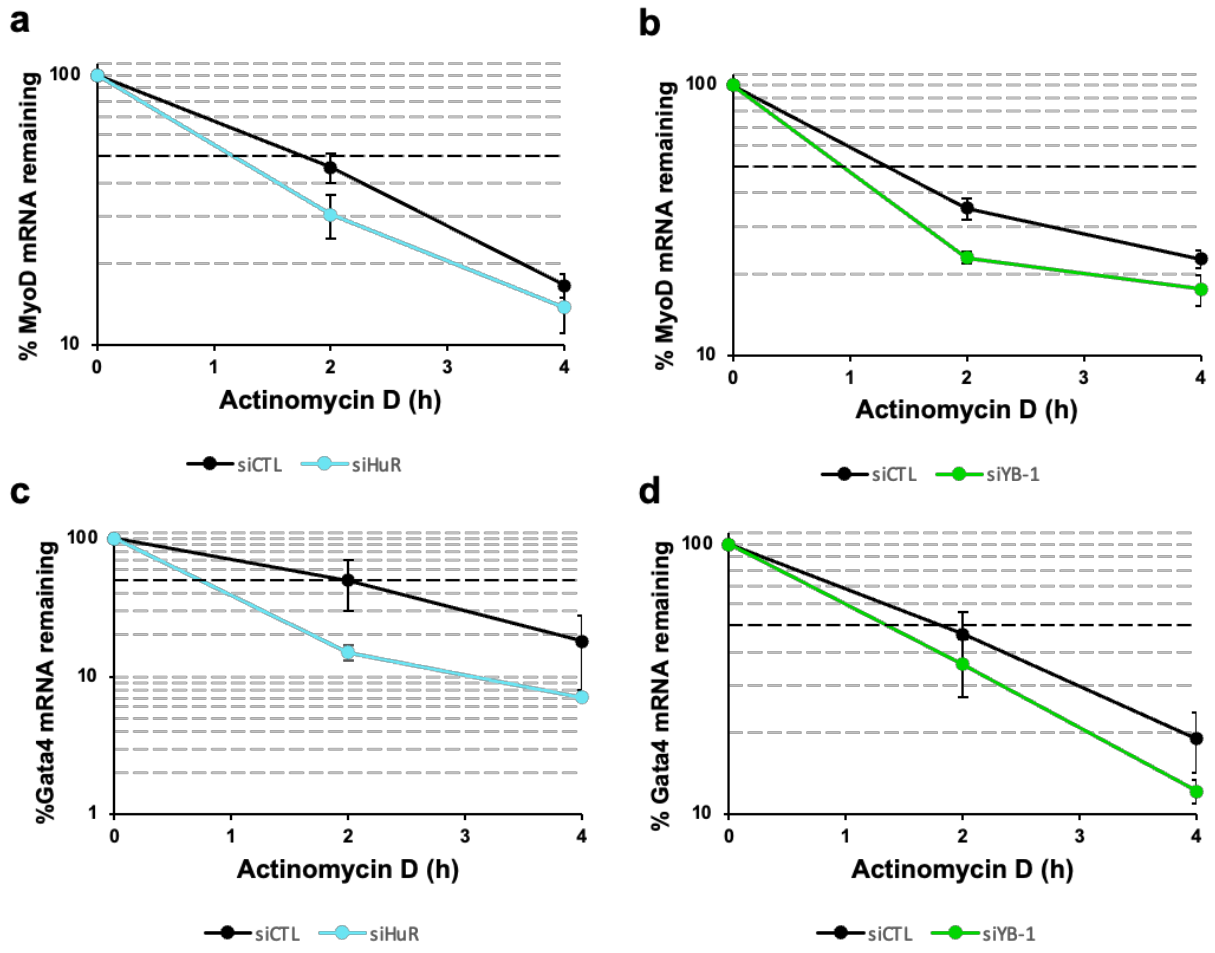


Figure 2.7 HuR and YB-1 Regulate the Stability of the MyoD and Gata4 mRNAs. The decay of the mRNAs was determined by performing Actinomycin D (ActD) pulse-chase experiments using C2C12 cells treated with siCTL, siHuR or siYB-1. These cells were treated with ActD for 0,2,4h two days post-induction of muscle cell differentiation. Total RNA was collected at each time point and analyzed by RT-qPCR with primers specific to **a-b)** MyoD, **c-d)** Gata4 or GADPH (using as a control). The expression level of each mRNA was standardized to GAPDH mRNA levels and plotted logarithmically relative to the expression at 0h, which was plotted as 100%. The data are presented +/- S.E.M of two independent experiments.

Next, we sought to determine if HuR and YB-1 could cooperate to bind to these mRNAs. We, therefore, assessed if the binding of HuR to these mRNAs is dependent on YB-1

and, vis versa, if the binding of YB-1 is dependent on HuR (Figure 2.8a). To this end, we depleted either HuR or YB-1 by siRNA (Figure 2.8b) and performed RNP-immunoprecipitation experiments (Figure 2.8c) with antibodies for the other protein to assess the association of the IPed RBP to these mRNAs (Figure 2.9).

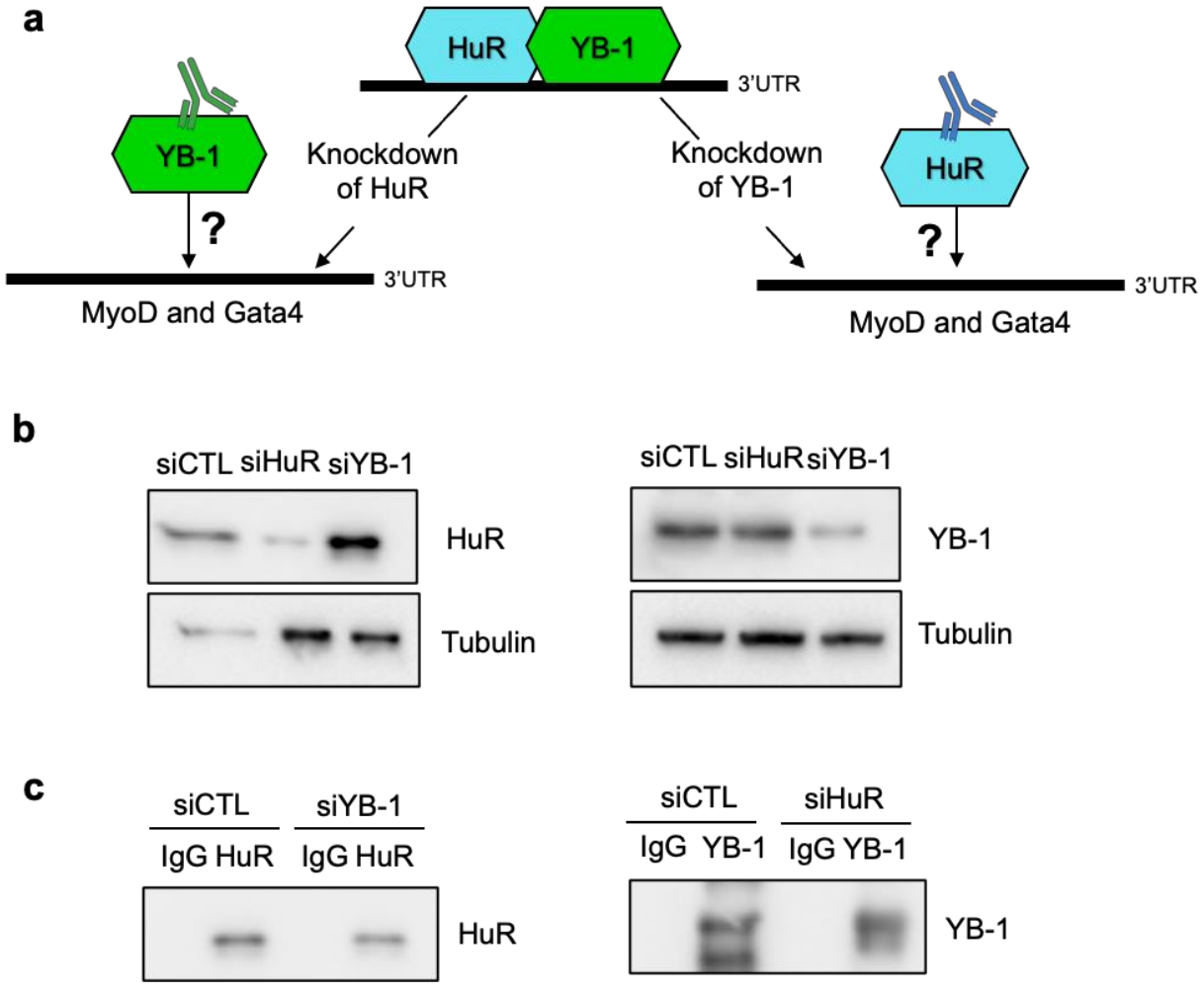


Figure 2.8 Cooperativity of HuR and YB-1 binding to the MyoD and Gata4 mRNA a) Schematic of the experiment. (b) Western Blots showing the knockdown of HuR (left) and YB-1 (right) in C2C12 cell two days post-induction of differentiation c) Western Blot showing the immunoprecipitation of HuR in siYB1 treated C2C12 cells (left) and, vis versa, the immunoprecipitation of YB-1 in siHuR treated C2C12 cells (right).

We demonstrate, by performing these experiments that the association of YB-1 with the MyoD and Gata4 mRNAs is substantially decreased when HuR was depleted from these cells (Figure 2.9a-b). Likely, the depletion of YB-1 significantly reduced the binding of HuR to these mRNAs. (Figure 2.9a-b) Together, these results validate that the MyoD and Gata4 mRNAs are targets of HuR and YB-1 and suggest that the HuR and YB-1 proteins cooperate to modulate the stability of their targets similarly to the myogenin mRNA.

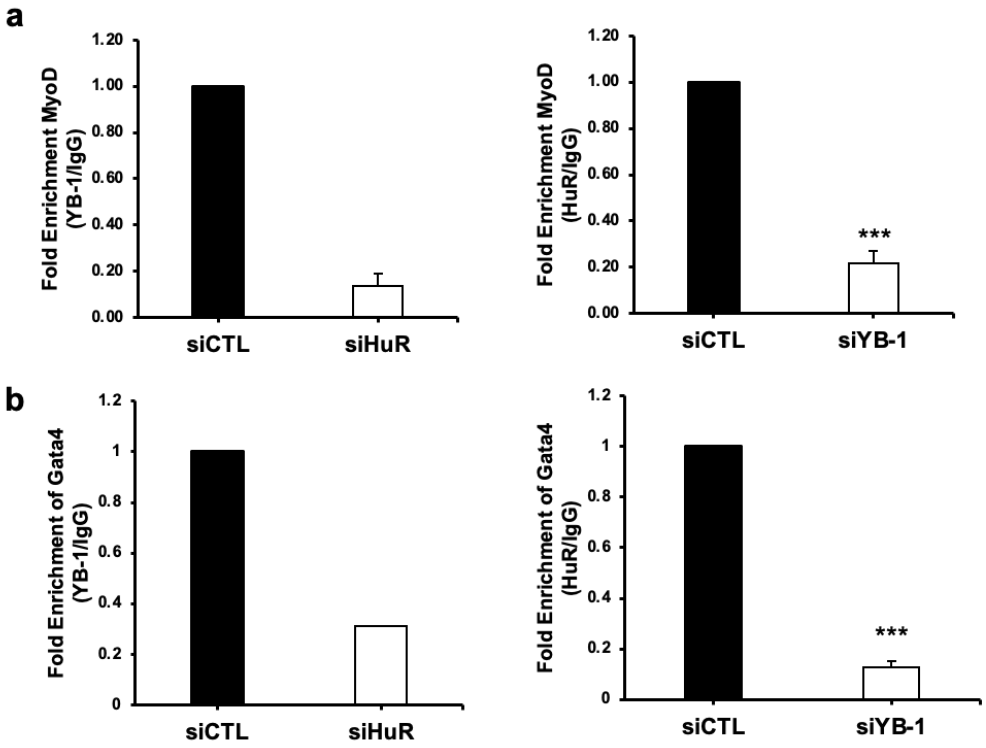


Figure 2.9 HuR and YB-1 Cooperate to Bind the MyoD and Gata4 mRNAs. a-b) RNA-immunoprecipitation experiments for YB-1 and IgG were performed in C2C12 treated with siCTL or siHuR and for HuR and IgG in C2C12 treated with siCTL or siYB-1. The isolated RNA was processed by RT-qPCR with primers for the **a)** MyoD mRNA and **b)** Gata4 mRNA. mRNA levels were standardized against IgG and plotted relative to the siCTL expression level. The data are shown +/- S.E.M of N=2 (left) and N=5 (right) experiments, respectively. ***p<0.001

3.3. Identification of a Consensus Motif

Our previous data established the formation of an HuR/YB-1 complex in myotubes. In addition, the work presented above indicates that the HuR and YB-1 proteins can cooperate to stabilize messages, which may occur through the formation of this complex. Hence, we sought to determine if the common mRNA targets of HuR and YB-1 contained a *cis*-element motif guiding the binding of the cooperating proteins. In this manner, we aimed to uncover the mechanism by which the HuR/YB-1 complex mediates the stability of its common targets. We, therefore, performed a bioinformatical analysis of the target sequences identified in the RIP-seq data. Further analyses were performed strictly on the 3'UTR of these target sequences as previous studies by our laboratory and others have shown that YB-1 possesses a sequence-specific stabilizing ability when bound in the 3'UTR. [89,150,197] We obtained the sequence of the 3'UTR of the identified common targets using the sequence of the total mRNA and subtracting the sequence up to the end of the coding region. (Figure 2.10) These 3'UTRs were compiled into a sequence library and inputted for motif discovery to the MEME analysis software (Figure 2.10). The program searched for the top three statistically significant motifs (Figure 2.10, Figure 2.11a). The most common motif was identified in 147 of these common mRNA targets (Figure 2.11a, Table 2.1). Several of these mRNAs were previously described as targets of HuR and/or YB-1 in the literature. (Table 2.1). Interestingly, the motif is present in the MyoD but not the Gata4 mRNA. The other two motifs identified were present in a much smaller subset of these common messages (14 and 13, respectively). (Figure 2.11a) Hence we focused on the most common motif for further analyses. The sequence of the most common motif was NWNTKTKTKTKTKTKTTTKTTTKTTTTTKTTTTTKTTT (Figure 2.11b). Since the K letter stands in for a position that may be taken by a Guanine or a Uracil, and the Ts represent Uracils in the mRNA, the most common motif in these HuR/YB-1 targets is thus a 40 nucleotide long GU-rich sequence.

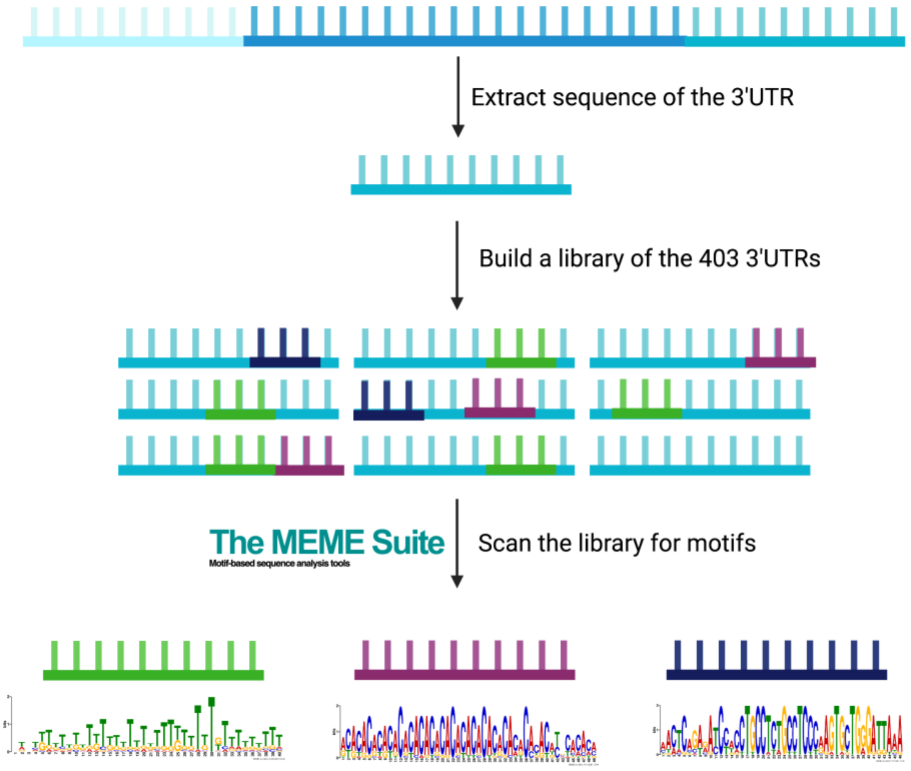
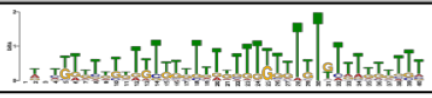
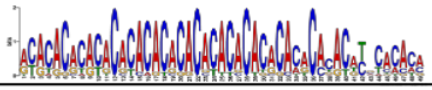
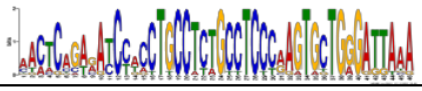


Figure 2.10 Motif Discovery Pipeline in the Common Targets. The 3'UTR of the mRNA targets were obtained from the subtraction of the 5'UTR and CDS from the full-length sequence of each mRNA target. The library of targets was submitted to the MEME program for motif discovery (only the given strand was scanned). The program identified three motifs.[31]

a

	Motif	E-value	Sites	Width (nt)
1		1.90E-81	147	40
2		1.50E-69	14	49
3		5.40E-28	13	46

b

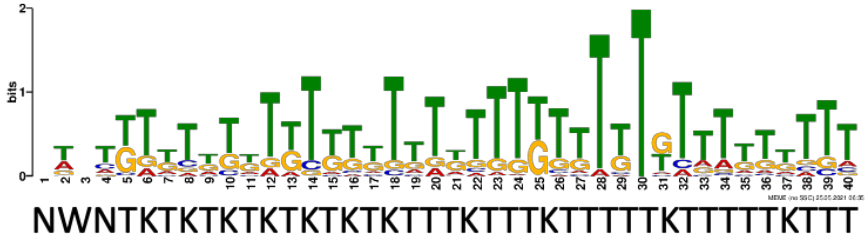


Figure 2.11 Consensus GU-rich Motif in the 3'UTR. The output of the MEME analysis. a) The top three motifs with the lowest E-values. The E-value represents the likelihood of this motif (width, site, letter frequency) occurring in a same-sized set of random sequences as the input messages. The number of sites indicates the number of mRNAs containing this motif, and the length is the number of nucleotides in each consensus motif. b) The most common motif is GU rich. Data represented in MEME format where W=A or T, K= G or U, T=U

The MEME analysis also allowed us to pinpoint the location of this consensus motif in the mRNA targets. Interestingly, the most common identified motif overlaps with the previously identified HuR/YB-1 binding site in the myogenin mRNA 3'UTR, which was determined by gel shift.[150] (Figure 2.12). Similarly to Myogenin, the MEME software also allowed us to demonstrate that the consensus motif is present in the 3'UTR of the MyoD mRNA (Figure 2.13). Based on these results, we propose that this GU-rich consensus sequence may constitute the binding site of the HuR/YB-1 complex in a subset of targets in muscle cells.

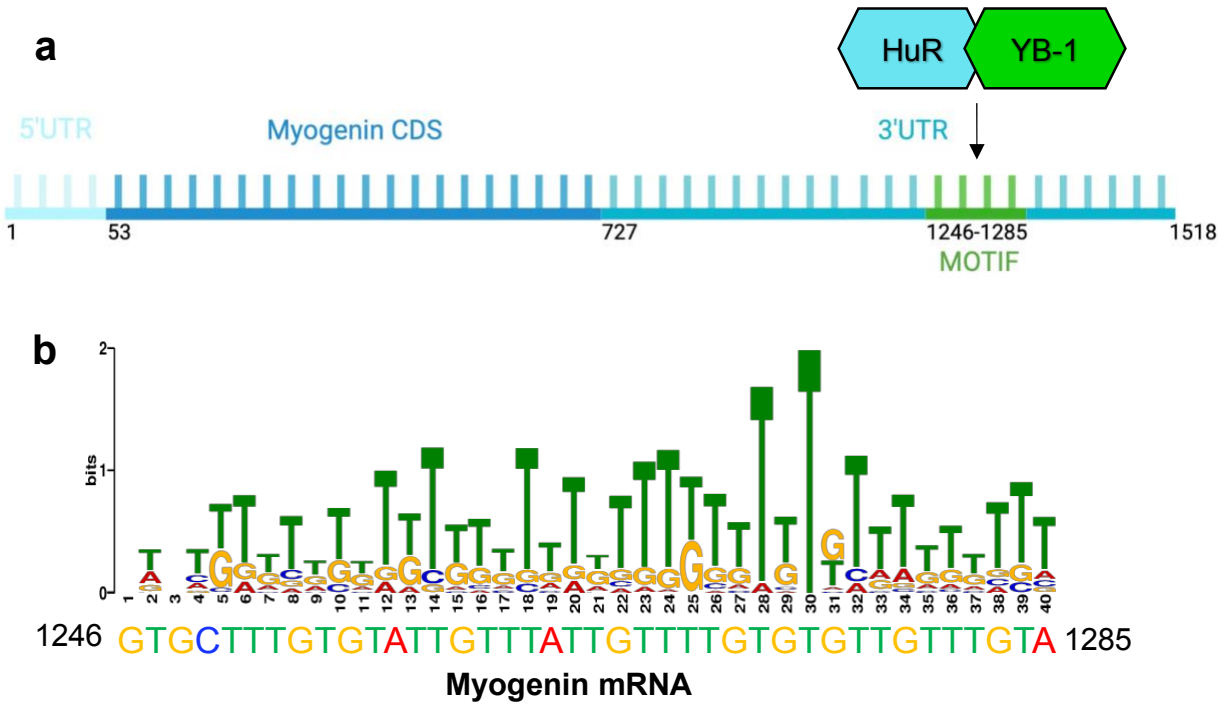


Figure 2.12 The Consensus Motif Aligns with the HuR/YB-1 Binding Site. The most common consensus motif maps to the 1249-1285 position in the 3'UTR of the myogenin mRNA, which was shown by gel shift assays to correspond to the HuR/YB-1 binding site. a) Schematic of the myogenin mRNA, including the location of the HuR/YB-1 binding site.[31] b) Alignment between the previously identified motif in the myogenin mRNA 3'UTR and the sequence identified by the MEME software.

Table 2.1 List of mRNA Targets of HuR and YB-1 that Contain the Consensus Motif Identified by MEME Blue (established targets of HuR), Green (established targets of YB-1), Pink (established targets of both proteins)

4933412E12Rik	Cntrob	Gpr63	**Myod1**	Sfxn3
Actb	Cpne2	Hmox1	**Myog**	Shisal1
Adgrg6	Cxcr4	Hmx2	Nek2	Sla
Aim2	Dclk1	Igf2bp2	Nemp2	Slc6a17
Arl14epl	Depdc1b	Igsf11	Nhlrc4	Slc7a5
AU018091	Dhcr7	Il12a	Nmnat2	Soat1
Aurkb	Dlg4	Il1rn	Npas3	Specc1
Bcl3	Dlx3	Katnal2	Nppb	Speer4a
Birc5	Dmbx1	Kctd7	Olfml2b	Spidr
Bsn	Dnase1l3	Kif14	Onecut2	Spock1
Btbd11	Dynll1	Kif24	Orai2	St3gal4
Calr	Dzip1	Kifc5b	P4ha3	Stambpl1
Calr4	Eno2	Lef1	Palb2	Syt13
Car5b	Epb4.1l3	Letmd1	Parpbp	Tcf7
Casp8	Fads2	Lif	Pced1b	Tgif1
Ccdc92	Faim	Limk1	Pcgf2	Tigd3
Cd44	Fgfbp1	Lpar1	Pcyox1l	Tnfrsf22
Cdc25b	Foxq1	Lpar2	Pdpn	Tpgs2
Cdca8	Fzd2	Lpcat4	Peg10	Tpx2
Cdh1	Gatm	Macroh2a2	Peg12	Tram1l1
Cenpw	Gja3	Map3k19	Polr3g	Trp53cor1
Cep164	Gjd2	Meis2	Pqlc3	Tspan31
Chn2	Glipr2	Mgam	Qrfp	Tyms
Chrna1	Glis3	Mis18bp1	Rab31	Uhrf1
Chst11	Gm10471	Mmp12	Rubcnl	Unc5c
Chsy1	Gm4371	Morrbid	Runx3	Vash2
Ckap2l	Gm6634	Mxd1	Scml4	Zic1
Cmtm3	Gm8234	Myc	Sdc1	
Cnr1	Gm8615	Mycl	Sema4b	
Cntd1	Gm9776	Myl12b	Serpinb9b	

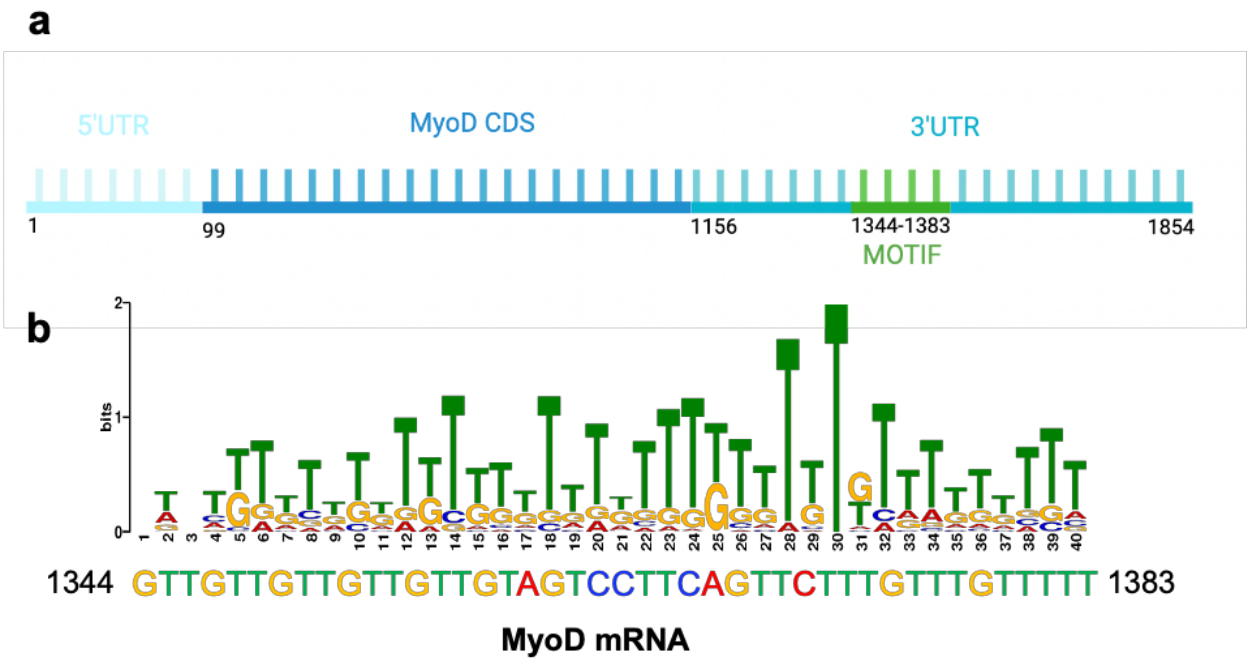


Figure 2.13 The MyoD mRNA Contains the Consensus Motif a) Alignment of the consensus motif in the 3'UTR of the MyoD mRNA.[31] **b)** Sequence identified in the 3'UTR of the MyoD mRNA that aligns with the consensus motif

The incidence of this motif in the 3'UTR of the Myogenin and MyoD mRNAs, but not the Gata4 mRNA indicates that the HuR and YB-1 proteins may regulate in different ways the stability of their targets. Overall, we identified a GU-rich consensus motif present in the 3'UTR of common targets of the HuR and YB-1 proteins. We propose that this motif is one of the mechanisms that directs the binding of the HuR/YB-1 complex to stabilize mRNAs.

4. MATERIALS AND METHODS

Cell Culture

C2C12 cells (ATCC, Manassas, VA, USA) were grown on tissue culture plates (Corning) in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 20% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin antibiotic solution (Sigma). Cells were grown to 100% confluency and differentiated in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 2% horse serum (Gibco life technologies) and 1% penicillin/streptomycin antibiotic solution (Sigma).

Western Blot

Cells were lysed in mammalian lysis buffer (50mM HEPES pH7.0, 150mM NaCl, 10% glycerol, 1% Triton X-100, 10mM sodium pyrophosphate, 100mM NaF, 1mD EGTA, 1.5mM $MgCl_2$, 1X protease inhibitor). The lysed cells were centrifuged (12 000 rpm, 4°C, 15min) to remove cellular debris. The extracts were run on 10% acrylamide SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were immunoblotted overnight with antibodies against HuR ($3A2^{118}$, 1:10 000), YB-1 (ab12148, 1:500) and tubulin (Developmental Studies Hybridoma Bank, 1: 1000).

Transfection

Cells were grown in 6-well plates and treated with the 60nM of siRNA in 1mL of media. Two wells were treated with siYB-1 to account for the cell death caused by the siRNA treatment. The cells were 40-50% confluent at the time of the initial transfection. After 4 hours, an additional 1mL of media was added. The second transfection occurred 24h later when the cells had reached 70-80% confluency. After 4 hours, an additional 1mL of media was added. 48h after the initial transfection the cells had reached 100% confluency, and the media was changed to differentiation media. Two days post-induction of differentiation, the cells were collected for subsequent analysis.

RNA Immunoprecipitation

Cells were lysed in high salt lysis buffer (50mM Tris-HCl pH 8.0, 0.5% triton 100X, 150mM NaCl, 100mM NaF, 1X protease inhibitors (Roche)). 60uL of pre-washed Protein A beads were incubated with the 15uL of anti-HuR, anti-YB-1 or anti-IgG antibodies for 4 hours at 4°C followed by three washes with low salt buffer (50 mM Tris pH 8, 0.5% Triton X-100, 150 mM NaCl, 1 X protease inhibitors). 500 ug of total cell extracts were added to the antibody-bound beads, and the samples were rotated overnight at 4°C. The samples were washed three times with low salt buffer. The co-immunoprecipitated RNA was purified, and 4 µl of the RNA was used for RT-qPCR analysis. Validation of the immunoprecipitation was performed by western blot using 2.5uL of eluate and antibodies against the protein of interest.

RNA extraction and RT-qPCR

RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's protocol.1ug of total RNA or 4uL of immunoprecipitated RNA was used for reverse transcription. The 5X iScript reagent (Bio-Rad) was used according to the manufacturer's protocol. The cDNA was diluted 20 folds to perform the qPCR. The SsoFast EvaGreen Supermix (Bio-Rad) was used to perform the qPCR. RNA levels of the genes of interest were normalized to the levels of the housekeeping gene GAPDH. The $2^{-\Delta\Delta C_T}$ method of analysis was used to determine the relative level of expression. In this method, the ΔΔCT is the difference in CT (cycle number at which the amount of amplified target reaches a fixed threshold) between the target mRNA and GAPDH.

Table 1. Sequences of qPCR primers

Myogenin (m)	F: 5'-CTA CAG GCC TTG CTC AGC TC-3' R: 5'-AGA TTG TGG GCG TCT GTA GG-3'
MyoD (m)	F: 5'-CGA CAC CGC CTA CTA CAG TG-3' R: 5'-TTC TGT GTC GCT TAG GGA TG-3'
Gata4 (m)	F: 5'- GCA GCA GCA GTG AAG AGA TG -3' R: 5'- GGG ACA GCT TCA GAG CAG AC -3'
GAPDH (m)	F: 5’-AAG GTC ATC CCA GAG CTG AA-3’ R: 5’-AGG AGA CAA CCT GGT CCT CA-3’

Actinomycin D (ActD) Pulse-chase

Cells were transfected with a siCTL, siHuR or siYB-1 as described above. The cells were treated with 5 µg/ml of actinomycin D (ActD) for 0,2h or 4h.[114,150] RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions and analyzed by RT-qPCR. The mRNA levels were plotted logarithmically relative to the mRNA abundance at 0h considered as 100% mRNA levels.

RNA sequencing and Target identification

RNA sequencing as performed as described in Sanchez, 2021.[150] Briefly, RNA was immunoprecipitated from C2C12 lysate obtained at day 2 of differentiation using Trizol LS reagent (Invitrogen) following the manufacturer's protocol. RNA samples were assessed for quantity and quality and sequenced with the Illumina NextSeq 500 platform at the Institute for Research in Immunology and Cancer (IRIC) Genomics Core Facility, University of Montreal. The reads produced were trimmed for sequencing adapters and aligned to the reference mouse genome version mm10 (GRCm38) using Tophat version 2.0.10. Gene quantification was performed on the mapped sequences using the htseq-count software version 0.6.1. Binding targets were selected if the fold change (FC) of association with the protein of interest vs IgG was above 2.00. The list of common targets was compiled by comparing the list of HuR targets and the list of YB-1 targets to identify common mRNAs.

Gene Ontology Analysis

The PANTHER version 16.0[234] was used to perform the gene ontology analysis on the targets identified by the RIP-seq as described by Mi et al., 2019.[235] The protein classification, Molecular Function and Biological Processes were analyzed.

Motif Identification

The 3'UTR of the 403 of the 409 targets were obtained by extracting the length of the complete mRNAs and the position of the end of the coding sequence. The nucleotide

sequence between the end of the coding sequence and the end of the mRNA was used as the 3'UTR sequence. Of the six targets excluded from our analysis, two were suspected artifacts from the sequencing because they were not genes present in mice. The other four were due to a lack of annotation of the end of the coding sequence or technical problems preventing their conversion into recognizable sequences by the program. The FASTA format of these sequences was compiled and submitted to the motif analysis tool *Multiple EM for Motif Elicitation (MEME)*. This program identifies recurring, ungapped motifs in the input sequences by using statistical modelling techniques as described by Bailey TL and Elkan C. 1994.[236] The web interface of the MEME suite was used to perform the analysis as described in Bailey et al. 2015 with the modification that the program was only allowed to scan the given sequence and not the complementary strand as the query pertained to RNA.[237]

Statistical Analyses. The significance of the difference between sample groups was assessed by an unpaired *t*-test for normally distributed variables. p-values equal or less than 0.05 were considered significant: 0.05-0.01 (*), 0.01-0.001 (**), and less than 0.001 (***).

5. DISCUSSION

Previous work by our laboratory established that the formation of a HuR/KSRP complex, in proliferating C2C12, is required for the turnover of the NPM and PGC-1a mRNAs.[71,72] We also determined that the function of HuR was impacted by its interaction with microRNAs, such as miR-1192 and miR-330, causing it to adopt a pro-translation role as it prevents RISC-mediated translational repression.[50,51] Although the role of HuR in cellular processes, including myogenesis, has been extensively studied, our knowledge of the modulators of its function in regulating the stability of mRNAs is still actively evolving.

Our interest in binding partners and their impact on HuR function in stabilizing mRNA targets led us to search for novel HuR ligands. We determined that in differentiating C2C12, the YB-1 protein could form an RNA-independent complex with HuR and, together, this complex could bind and stabilize the myogenin mRNA.[150] In this investigation, we also searched for other targets of the complex by performing RIP experiments for each protein and selecting common targets. We identified 409 putative common mRNA targets. [150] (Figure 2.2) These were analyzed based on protein class, molecular function and biological processes. (Figure 2.3) As expected, the myogenin mRNA was present in the list of common targets. Hence, we used the myogenin mRNA to validate the RIP-Seq experiment. (Figure 2.4) Next, we aimed to determine if the other targets identified in the RIP-seq were also targets of the HuR/YB-1 complex. We selected two targets known to play a functional role in myogenesis, MyoD and Gata4.[107,112] The binding of HuR and YB-1 to these messages was established, as well as the impact of the knockdown of either protein of the mRNA levels and stability of these messages. (Figure 2.5-2.7) The cooperativity of binding was assessed by measuring the relative binding of HuR to these mRNAs in the absence of YB-1 and, vice-versa, the binding of YB-1 in HuR depleted cells. (Figure 2.8, 2.9) Our results demonstrate that the binding of one of the proteins to the RNA tends to diminish when its partner is depleted. Additionally, we sought to understand the mechanism by which the HuR/YB-1 complex selects and confers stability to its targets. To this end, we performed bioinformatical analyses of the

targets that revealed a consensus motif in the 3'UTR of 147 of the targets. (Figure 2.10, 2.11) This motif was found to align with the previously established HuR/YB-1 binding site in the 3'UTR of the myogenin mRNA. (Figure 2.12) It was also found in the 3'UTR of MyoD, a known target of HuR and a key player in myogenesis and in many other targets. (Figure 2.13, Table 2.1) However, although Gata4 seemed to be modulated similarly to myogenin and MyoD, this motif was not identified in its 3'UTR. Based on these results, we propose the following model. (Figure 5.1). The HuR and YB-1 proteins form a complex that can recognize and bind a consensus sequence in the 3'UTR of mRNAs resulting in their stabilization. Our bioinformatical analyses suggest that this sequence is GU-rich, follows the sequence shown in Figure 5.1 and is around 40 nucleotides in length. (Figure 5.1) We propose that this motif directs the binding of the RNA-independent HuR/YB-1 complex to a majority of common targets such as the myogenin and MyoD mRNAs, but that other targets such as Gata4 are regulated by HuR/YB-1 complex in a manner that is independent of this consensus motif.

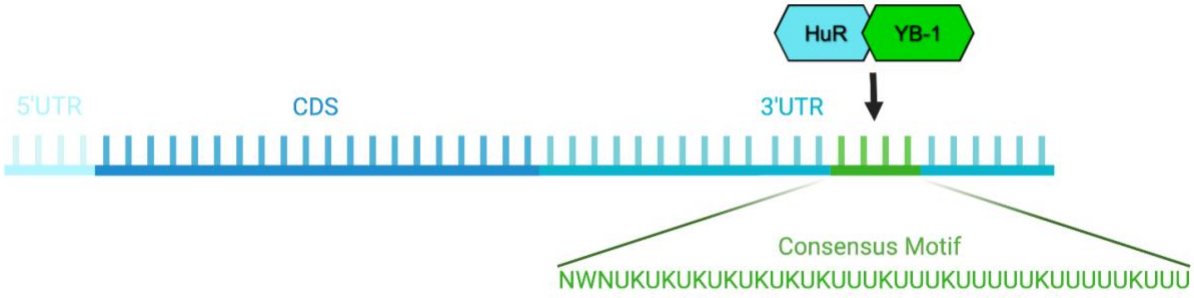


Figure 5.1 The HuR/YB-1 Complex Binds to a Consensus Motif in the 3'UTR.
The HuR and YB-1 proteins form an RNA-independent complex that binds to a GU-rich sequence in the 3'UTR of its mRNA targets increasing their stability. W=A or T, K=G or U.[31]

After establishing the binding of the targets to HuR and YB-1, we assessed the impact of their depletion on the mRNA levels of the targets. We expected the three mRNAs to bind HuR as they have been identified as targets of HuR in other studies. [24,68] We observed decreased levels of MyoD and Gata4 mRNAs when HuR or YB-1 was depleted by siRNA. (Figure 2.6) These results aligned with previous observations of the depletion of HuR,

causing decreased levels of MyoD. [68] We determined that these decreased levels were due to the reduced stability of each target in the absence of either protein. (Figure 2.7) To elucidate if the proteins worked as a complex, as in our recent study by Sanchez et al., we assessed the binding of YB-1 to MyoD and Gata4 mRNAs when HuR was depleted by siRNA and vice-versa, the binding of HuR to MyoD and Gata4 mRNAs when YB-1 was depleted. (Figure 2.8) We observed that the depletion of one protein tends to affect the ability of the other to bind to the target. This suggests that the proteins also act as a complex in a cooperative manner when binding to these mRNAs as they did on the myogenin mRNA. To further our understanding of the complex, our laboratory aims to investigate how the HuR and YB-1 proteins bind to each other. Determining this interaction will allow us to elucidate the importance of the formation of the complex, versus the presence of the individual proteins, in the stabilization of the targets.

Our MEME analysis of the 3'UTR of the common mRNA targets yielded three consensus motifs ranked according to their likelihood of being true motifs and not occurring randomly based on the number and length of provided sequences (E value).[237] The motif with the smallest E value was a poly-Uracil motif interspersed with Uracils or Guanines. (Figure 2.9, 2.10) Traditionally, HuR binding sites were thought to be AU-rich based on the binding and regulation of many mRNAs containing AREs by HuR. [238] The stabilizing role of HuR as an inhibitor of AU-rich mediated decay also supported this model. [239] A specific binding study by PAR-CLIP identified the HuR binding site as an AU-rich sequence with a preference for the UAUUUAU sequence. [240] The results of this study included the conclusion that U-rich or GU-rich content was not sufficient to attract HuR. [240] However, these results are debated due to the presence of other competing studies that propose a U-rich binding site as the preference of the HuR protein. [42,241,242] The Auer group proposed that a sequence of 9 nucleotides following the N-N-U-U-N-N-U-U-U motif was the binding site of the HuR protein. [241] Their analysis was based on a previous study that established the binding site of the HuD protein, another member of the ELAVL family, and on multiple binding analyses with different sequence fragments. [241,243] Other binding analyses, including cDNA arrays and RNAcompete, have also shown a clear preference of HuR binding for U-rich sequences. [42,242] The preferred sequence length varies between

studies, with the largest being 17-20nts and the smallest being around 7nts. [42,242] A systematic analysis of the binding sites of a wide range of RBPs found that HuR prefers U-rich sequences with can include Adenine or Guanines. [242] This same study investigated the binding site of the YB-1 protein and found a motif containing a central UG flanked by Cytosines. [242] Previous gel-shift results had demonstrated, however, that the preferred motif of YB-1 in RNA was UCCA(G/A)CAA.[172] Of note, some of these studies consider the full mRNA sequence. Hence, in some cases, YB-1 may have an mRNA site preference. However, this preference may be conditional to the cellular levels of YB-1 as high levels have been shown to stabilize mRNAs indiscriminately of sequence. [195] Indeed, YB-1 was shown to preferentially bind AC-rich exon enhancers, which may affect the protein's apparent sequence preference.[245] Lastly, the affinity of YB-1 for sequences containing different combinations of each of the nucleotides was measured in vitro, and YB-1 was determined to prefer GC-rich mRNA sequences. [160] Taking these all together, the consensus motif identified by our analysis seems to align with the previously published data since it is U-rich. However, the binding studies for each protein can hardly be directly translated into a preference of the complex. Therefore, we aim to conduct our own analyses of the binding affinity of the HuR/YB-1 complex for the consensus motif versus the individual proteins or versus other pre-identified target sequences by surface-plasmon resonance (SPR). An additional factor to consider in our future analyses will be the structure of the RNA binding site. Conflicting studies have argued that HuR has a preference for ssRNA or for hairpins. [42,240,242] The previously mentioned study by the Auer group proposed that HuR preferentially binds to ssRNA and that RNA structure plays a role in the binding site accessibility. [241] They suggest that the secondary structure of the RNA must be modified by the binding of a small RNA "opener" prior to the 3'UTR of the target. Once this small RNA binds, the structure of the RNA target changes such that the HuR binding site is now ssRNA allowing HuR binding to its target.[241] Based on our knowledge of the proximity between HuR and microRNA seed elements, it is worth investigating if a third trans-acting factor may participate in this interaction as the "opener" of the RNA sequence that allows the Hur/YB-1 complex to bind the consensus motif in its target.[246]

The most common motif aligned with a sequence in the 3'UTR of the myogenin mRNA, which our laboratory had previously established as a target.[150] (Figure 2.11) Interestingly, this sequence overlaps with the binding site identified by gel shift by Sanchez, 2021.[150] This indicates that the motif could represent the binding site of the Hur/YB-1 complex that mediates the stability of the mRNA. Indeed, we demonstrated that mutation of this site prevents the binding of HuR and YB-1 to the myogenin mRNA 3'UTR resulting in the decreased stability of the message.[150] These experiments thus directly link the functionality of the binding of the HuR/YB-1 complex to the consensus site to the stability of the message. This consensus motif was identified in the 3'UTR of 147 mRNAs (including the MyoD mRNA), representing about one-third of the total common targets. (Figure 2.12) We speculate that these targets are the ones that are bound by the RNA-independent HuR/YB-1 complex due to the alignment with the myogenin binding site. Furthermore, these 147 targets included established targets of HuR, YB-1 and of both HuR and YB-1 that play important roles in multiple cellular processes. (Table 2.1) For example, the Cd44 mRNA, an important modulator of cancer progression, has been previously demonstrated to be bound by HuR and by YB-1.[247–249] Therefore, the range of identified targets suggests that this motif may play a role beyond myogenesis. It would be interesting to assess if the mutation of this consensus site in these targets also affects the stability of these messages, as we showed for the myogenin mRNA.

We propose that the rest of the common messages that do not contain this consensus site are bound by HuR and YB-1 independently or as an RNA-dependent complex. Indeed although we demonstrate that this motif was not identified in the 3'UTR of the Gata4 mRNA, our data show that both HuR and YB-1 associate with this mRNA cooperatively to regulate its stability. This suggests that there is additional specificity that goes beyond the formation of a complex between the HuR and YB-1 proteins and their association to a consensus binding site. In the case of the Gata4 mRNA, we demonstrate that the proteins cooperate to cause the stabilizing effect, which indicates that the binding of protein affects the binding of the other to the target. The regulation of the Gata4 mRNA may occur by another established mechanism described by Chen et al. [198] In this study, performed in HeLa cells, YB-1 was shown to recognize and bind m^5C modified

nucleotides and to recruit HuR to these sites to form an RNA-dependent complex. [198] They demonstrated that this mechanism regulates the stability of the HDGF mRNA. [198] Hence, it is possible that this mechanism explains the regulation of the Gata4 mRNA in our model.

Additionally, this study measured the distance between the HuR and YB-1 protein's binding sites on their common targets and found that they were bound in very close proximity.[198] Their results suggest that HuR and YB-1 always bind together on common targets. [198] However, based on the broader literature, identifying an mRNA as a common target of two RBPs does not necessarily imply that it is bound by a complex of the two proteins. Some of the identified targets may be bound by HuR and YB-1 in an RNA-dependent manner. For example, HuR could be bound to an ARE in the 3'UTR of a target, while YB-1 is bound to the 5'cap of the same target without impacting the binding or function of the other protein. Another possibility is that both proteins can bind to these messages but not simultaneously. For example, HuR could bind mRNA X in a specific subcellular location and in another mRNA X could be bound by YB-1. However, these are only speculations and further studies of the common targets that do not display the consensus motif need to be performed.

To this end, it would be interesting to perform an eCLIP experiment to identify the binding sites of the HuR and YB-1 proteins on the common targets and determine if the targets are mostly bound by both proteins as a complex. Additionally, the m^5C study found 5199 common targets of HuR and YB-1 in HeLa cells, while our experiments only found 409 in myotubes. [198] The discrepancy between this study and our results may be due to the different cell types and the method of used to capture the common targets. Although both HuR and YB-1 are expressed in myotubes and in HeLa cells, their mRNA targets may differ due to the expression of different mRNAs in different cell types. Furthermore, we used the RNA-immunoprecipitation (RIP) technique in this study, while the study in HeLa cells used the PAR-CLIP technique. The RIP may be less sensitive and capture fewer targets, and it has two major limitations. The RIP technique causes false-positives due to possible interactions post-lysis and only characterizes kinetically stable interactions.[250,251]

To resolve these differences, we have begun the process of troubleshooting the enhanced Crosslinking Immunoprecipitation (eCLIP) technique in our cells. [252] This technique surpasses the PAR-CLIP technique in specificity due to changes in the experimental procedures.[252] We aim to use the results of the eCLIP experiment to validate our proposed consensus motif based on the true binding sites of the proteins on a subset of their common targets. Confirming our consensus motif could also predict if an mRNA may be a target of the RNA-independent complex.

In this work, we have investigated the role of the HuR/YB-1 complex on its targets. Next, it would be interesting to determine if the HuR/YB-1 complex collaborates or competes with other factors for binding. The U-rich nature of the consensus site suggests that this site may also be the target of other RBPs with a preference for U-rich sites such as the KSRP, AUF1 and TTP proteins.[60] Additionally, it would be interesting to determine if the HuR/YB-1-mediated regulation of mRNA stability through its association to this consensus site represents a general mechanism that is found in other cell types.

6. CONCLUSION AND SUMMARY

In conclusion, the interaction between the RNA-independent HuR/YB-1 complex and its target mRNAs seems to be directed by a sequence-specific interaction with its targets. We used the MyoD and Gata4 mRNAs to demonstrate that the HuR and YB-1 proteins bind their common targets and promote their stabilization cooperatively. Next, we showed that a subset of the common targets of the HuR and YB-1 proteins identified by Sanchez et al. contain a consensus motif in their 3'UTRs. We propose that the RNA-independent HuR/YB-1 protein complex, previously described, recognizes and binds to this 40nt GU-rich sequence. We show that this sequence motif is present in targets of the HuR and YB-1 that are stabilized by these interactions. Furthermore, we demonstrate that not all targets stabilized by the HuR and YB-1 proteins contain this sequence, which suggests that an additional level of specificity may be at play. The work presented in this thesis provides evidence that the RNA-independent HuR/YB-1 complex binds to a GU-rich *cis*-element motif in the 3'UTR of its messages to affect their stability.

Our work to understand the mechanism of this interaction is applicable beyond the myogenesis process. The ubiquitous expression of the HuR protein and the expression of the YB-1 protein in all somatic cells allow us to presume that the formation of an RNA-independent HuR/YB-1 complex may occur in other cell types.[24,162] Instances of HuR/YB-1 complexes were already reported in eosinophils and in HeLa cells, although their dependence on RNA was not demonstrated.[197,198] Preliminary analyses have led us to suspect that the HuR/YB-1 complex described in eosinophils may be RNA-independent and binds to a *cis*-element in the 3'UTR of the GM-CSF mRNA that aligns with the consensus motif we presented. Therefore, the consensus motif could be used to predict targets of the HuR/YB-1 complex in other cell types. In addition to their established roles in inflammation, both HuR and YB-1 have also been extensively implicated in the oncogenic process and have been reported to be overexpressed in several malignancies.[88,97,98,151,153,162] Understanding the mechanism of action of the HuR/YB-1 complex, could allow us to understand if it plays a role in these malignancies. Historically, therapeutically modulating these proteins individually has been difficult due to their wide range of

functions.[141,253] However, if we understood the role and the targets of the HuR/YB-1 complex, we may be able to affect the expression of these targets by inhibiting the formation, or the RNA-binding activity, of the complex. The results presented in this thesis demonstrate that well-known proto-oncogenes contain a sequence similar to the consensus site in their 3'UTRs. These include the Myc and cdc25b mRNAs. Based on the high expression levels of both HuR and YB-1 proteins in many cancers, it is likely that these aren't the only pro-oncogenic targets that would be affected if we could modulate this complex. The consensus motif may serve as a powerful tool to identify potential targets of the complex in these cancers.

In closing, the data delineated above provides a solid basis to answer our inquiry of the mechanism by which the HuR/YB-1 complex selects and binds to its targets. Further studies remain to be performed to validate the consensus motif we introduced and to determine how the HuR and YB-1 proteins bind to each other. Overall, our findings bring us closer to understanding how the HuR protein modulates the stability of messages.

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8. ANNEX

8.1. Supplementary material

Supplemental Table 8.1 List of Common mRNA Targets of HuR and YB-1 Identified from the RIP-Seq.

42433	Calr	Dlg4	Gm4636	Map3k19	Phlda1	Spp1
42618	Calr4	Dlgap1	Gm6634	Marcksl1	Pigf	St3gal4
1110006O24Rik	Camk2n2	Dlgap2	Gm773	Mcpt8	Pnma1	St8sia1
1110059M19Rik	Capn12	Dlgap5	Gm8234	Meis2	Polr3g	St8sia2
1700008I05Rik	Car13	Dlx2	Gm8615	Mfap3l	Pqlc3	Stac2
1700012B09Rik	Car5b	Dlx3	Gm9776	Mgam	Prc1	Stambpl1
1700013G24Rik	Casp8	Dmbx1	Gp1bb	Mgarp	Prim2	Stil
1700019L03Rik	Ccdc116	Dnaic1	Gp5	Mis18bp1	Prr11	Sult2b1
1700034F02Rik	Ccdc24	Dnase1l3	Gpr126	Mmd	Psat1	Susd3
1700055N04Rik	Ccdc73	Dynap	Gpr3	Mmp10	Ptchd1	Syn1
1700086L19Rik	Ccdc83	Dynll1	Gpr37	Mmp12	Ptgds	Synpr
1700088E04Rik	Ccdc92	Dynlrb1	Gpr63	Morn4	Qrfp	Syt13
1810041L15Rik	Ccl17	Dzip1	Gprc5a	Mov10l1	Rab31	Syt8
1810055G02Rik	Ccl2	E2f1	H2afy2	Msh5	Rad51ap1	Tcf7
2210409D07Rik	Ccnb1	E2f8	Hebp2	Mt1	Rad54b	Tes
2310008H04Rik	Ccne1	Ect2	Hephl1	Mustn1	Rad54l	Tgfb1i1
2310034O05Rik	Cd27	Eme1	Hmga2-ps1	Mxd1	Ranbp1	Tgif1
2410018L13Rik	Cd44	Eno2	Hmox1	Myc	Rapgefl1	Tigd3
2610034B18Rik	Cd63	Epb4.1l3	Hmx2	Mycl1	Rgs10	Tmem121
2610318N02Rik	Cd80	Evl	Hoxc13	Myh3	Rhov	Tmem151a
2810408A11Rik	Cdc25b	Ezr	Hs1bp3	Myl12b	Rhox10	Tmem158
2810433D01Rik	Cdca3	F2rl2	Hsd11b2	Myo1h	Rnase1	Tmem184a
4833427G06Rik	Cdca7l	Fads2	Htr1d	Myo7b	Rpl27	Tmem194b
4930427A07Rik	Cdca8	Faim	Hyi	Myod1	Rps15a-ps6	Tmem200a
4930500J02Rik	Cdh1	Fam110c	Igf2	Myog	Runx3	Tmprss2
4930592A05Rik	Cdkn1a	Fam129c	Igf2bp2	Nanos3	S100a11	Tnfrsf22
4933412E12Rik	Cdkn3	Fam189a1	Igsf11	Nap1l2	S100a3	Tnfrsf25
5031414D18Rik	Cenpi	Fam194a	Il12a	Nefl	Sc4mol	Tnnt2
A330009N23Rik	Cenpl	Fam211b	Il12rb1	Nek2	Scml4	Tor1a
AA414768	Cenpw	Fam71b	Il13ra2	Ngfrap1	Scn10a	Tpgs2
Actb	Cep128	Fam71f1	Il18	Nhlrc4	Scrn1	Tpx2
Agmat	Cep164	Fam83d	Il1rn	Nme5	Sdc1	Tram1l1
AI467606	Cfl1	Fancd2	Isx	Nmnat2	Sdsl	Try5
Aif1l	Chaf1b	Fanci	Katnal2	Npas3	Sema4b	Tspan31

Aim1l	Chn1	Fbxl13	Kctd13	Nppb	Serpinb1a	Tspan6
Aim2	Chn2	Fgfbp1	Kctd7	Nptx1	Serpinb9b	Ttll1
Akr1b3	Chrna1	Foxc2	Kif14	Nsdhl	Sesn2	Tuba1a
Apol9b	Chst11	Foxq1	Kif20a	Nudt17	Sfxn3	Tyms
Arhgef39	Chsy1	Frk	Kif23	Nxf3	Sgol1	Ubald2
Arl14epl	Ckap2l	Fstl3	Kif24	Olfml2b	Sh2d7	Ube2c
Asf1b	Cks1b	Fzd2	Kifc5b	Onecut2	Shcbp1	Ube2l6
Atp1a3	Cldn2	Gata4	Klk1b22	Orai2	Sla	Ucp2
Atp6v0e	Cmtm3	Gatm	Knstrn	Ormdl2	Sla2	Uhrf1
AU018091	Cnn3	Gja3	Kremen2	Otx1	Slc23a3	Unc5c
Aurkb	Cnr1	Gjd2	Krt7	P4ha3	Slc25a14	Upp1
Bard1	Cntd1	Gjd4	Lef1	Pabpc1l	Slc44a4	Usp29
Bcl3	Cntrob	Glipr2	Letmd1	Palb2	Slc6a17	Vash2
Bean1	Cpa4	Glis3	Lif	Parpbp	Slc6a19	Zfp239
Bend6	Cpne2	Gm10471	Limk1	Pcdhb21	Slc7a5	Zfp41
Best1	Creb3l1	Gm10494	Lpar1	Pced1b	Slurp1	Zic1
Bgn	Cxcl13	Gm10653	Lpar2	Pcgf2	Smim6	
Birc5	Cxcl5	Gm12191	Lpcat4	Pclo	Soat1	
Bsn	Cxcr4	Gm13139	Lrp11	Pcyox1l	Spag5	
Btbd11	D030025P21Rik	Gm14005	Lrp8	Pcyt1b	Spc25	
C1ql1	Dclk1	Gm14635	Lrrc73	Pdk3	Specc1	
C1qtnf3	Dctd	Gm15706	M1ap	Pdpn	Speer4a	
C920009B18Rik	Defb41	Gm16197	Mab21l3	Peg10	Spg21	
Cacna1b	Depdc1a	Gm17455	Macc1	Peg12	Spink4	
Cage1	Depdc1b	Gm2694	Mad1l1	Pet2	Spn	
Calr	Dhcr7	Gm4371	Mad2l1	Phex	Spock1	