The functional dichotomy of HuR is a key determinant of the fate of skeletal muscle

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For my grandparents, my mom, and my dad, who are the happiest people about the achievement of this thesis.

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Abstract

Skeletal muscle is a tissue composed of oriented and dense myofibers. Upon injury, a repair process, known as myogenesis, is initiated to replace damaged fibers. Impairment of this process may lead to muscle-related disorders and syndromes, such as Duchenne muscular dystrophy and cachexia. The RNA-binding protein Human antigen R (HuR) is a key posttranscriptional master regulator of myogenesis. During this process, HuR regulates the stability of several mRNAs, including *nucleophosmin* (*NPM), myogenin, MyoD,* and *p21,* encoding proteins that mediate the differentiation of muscle fibers. Although the role of HuR in myogenesis is well-established, the mechanisms modulating its function during this process are unknown. Chapter 2 demonstrates that a newly discovered posttranslational modification of HuR, poly(ADP-ribosyl)ation (PARylation), modulates its pro-myogenic function. I showed that poly(ADP-ribose) polymerase 5a (PARP5a), also named tankyrase-1 (TNKS1), is essential for myogenesis, as its depletion and chemical inhibition result in impaired muscle fiber formation. Additionally, I showed that TNKS1 PARylates HuR during muscle fiber formation. My results revealed that TNKS1 PARylates HuR by binding a conserved consensus TNKS1-binding motif located in the hinge region of HuR. Importantly, mutating this motif prevented HuR from rescuing myogenesis following the knockdown of HuR, a condition known to impair myogenesis. I demonstrated that PARylation of HuR promotes its RNA-binding activity, cytoplasmic accumulation, and cleavage, which are crucial events for the pro-myogenic function of HuR. This work reveals a novel regulatory mechanism through which the function of HuR may be targeted in myogenesis, and potentially in other processes. Recent work from my lab shows that HuR also promotes the composition and integrity of skeletal muscle. Interestingly we demonstrated, in that study, that HuR plays a role in promoting cancer-cachexia-induced muscle wasting. In Chapter 3, I identified the key pro-cachectic transcription factor, Signal Transducer and Activator of Transcription 3 (STAT3) as a novel mRNA target of HuR during muscle wasting. I demonstrate that HuR binds a U-rich element in the 3'UTR of *STAT3* mRNA and promotes its translation. My results revealed that HuR does so by inhibiting the ability of miR-330 to repress the translation of the *STAT3* mRNA. Indeed, miR-330 regulates the expression of STAT3 by binding to

a seed element located in the 3'untranslated region (UTR) of the mRNA. These results demonstrate that the competitive interaction of HuR and miR-330 with their respective binding sites in the 3'UTR regulates the translation of STAT3. We, therefore, delineate a new mechanism through which HuR promotes muscle wasting by regulating the expression of STAT3. Overall, in my thesis, I show that HuR plays a dual function in skeletal muscle based on the conditions, and by doing so determines the fate of this tissue.

Résumé

Le muscle squelettique est un tissu composé de myofibres orientés et denses. Lors d'une blessure, un procès de réparation nommé la myogenèse est initié pour remplacer les fibres endommagées. La détérioration de ce procès peut causer des maladies et syndromes reliés aux muscles, tel que la dystrophie musculaire Duchenne et la cachexie. La protéine liant l'ARN Human antigen R (HuR) est une des protéines régulatrices posttraductionelles principales de la myogenèse. Durant ce procès, HuR régule la stabilité de plusieurs ARNs messagers (ARNm), incluant *nucleophosmin* (*NPM), myogenin, MyoD,* et *p21*, qui encodent des protéines qui médiatisent la différentiation des fibres musculaires. Bien que le rôle de HuR dans la myogenèse soit bien établi, les mécanismes qui module sa fonction durant ce procès sont inconnus. Chapitre 2 démontre qu'une nouvelle modification posttraductionelle de HuR, poly(ADP-ribosyl)ation (PARylation), module sa fonction pro-myogénique. J'ai montré que poly(ADP-ribose) polymérase 5a (PARP5a), aussi nommé tankyrase-1 (TNKS1), est essentiel durant la myogenèse, vue que sa déplétion ou son inhibition chimique impaire la formation de fibres musculaires. Mes résultats révèlent que TNKS1 PARylate HuR en liant un motif de liaison de TNKS1 consensus qui est conservé et qui est localisé dans la région charnière (hinge) de HuR. De plus, la mutation de ce motif a empêché HuR de secourir la myogenèse à la suite de la déplétion de HuR, qui est une condition qui détériore le procès. J'ai démontré que la PARylation de HuR promeut sa capacité de lier l'ARN, son accumulation dans le cytoplasme, et son clivage, qui sont tous des évènements essentiels à sa fonction pro-myogénique. Ce travail révèle un nouveau mécanisme régulatoire par lequel la fonction de HuR pourrait être ciblée durant la myogenèse et potentiellement dans d'autres procès. De travaux récents de mon laboratoire montre aussi que HuR promeut la composition et l'intégrité des muscles squelettiques. Notablement, cette étude démontre que HuR joue un rôle dans la promotion du dépérissement des muscles induit par la cachexie du cancer. Dans Chapitre 3, j'ai identifié le facteur transcriptionnel pro-cachectique clé, Signal Transducer and Activator of Transcription 3 (STAT3), comme étant un nouvel ARNm cible de HuR durant l'atrophie des muscles. J'ai démontré que HuR lie un élément riche en U dans le 3'UTR de l'ARNm de *STAT3* et promeut sa traduction. Mes résultats

révèlent que HuR fait ceci en inhibant la capacité de miR-330 de réprimer la traduction de l'ARNm de STAT3. En effet, miR-330 régule l'expression de STAT3 en liant une séquence localisée dans le 3'UTR de l'ARNm de *STAT3.* Ces résultats démontrent que l'interaction compétitive de HuR et miR-330 avec leurs sites de liaison respectifs dans le 3'UTR régule la traduction de STAT3. Ainsi, nous délinéons un nouveau mécanisme par lequel HuR promeut la détérioration des muscles en régulant l'expression de STAT3. En somme, dans ma thèse, je montre que HuR joue une double fonction dans les muscles squelettiques, dépendamment des conditions, et par ceci détermine le sort du tissue.

Acknowledgments

My journey in pursuing graduate studies is no doubt an unforgettable chapter of my life. It was filled with beautiful memories, valuable lessons, and tough times that made me the person I am today. The beauty of my journey was mainly attributed to the people that were involved in this journey and whom I would like to thank from the bottom of my heart because it is with their support that I succeeded. First, I would like to thank my family for their constant support and encouragement. The pride I saw in their eyes kept me going. Next, I would like to thank Imed for accepting me into his lab. I would like to thank him for making me the stronger person I am today, for believing in me, and for challenging me to get out of my comfort zone. I would like to thank him for teaching me the importance of my research and making me aim for the sky and believe that I will reach it. I would like to thank him for his endless support during my hardships. I would also like to thank Sergio for the endless time and support he gave me and for always being there when I needed him. I am so grateful for his presence and for that, he made a huge contribution to my success. I would like to thank Michela for teaching me the techniques that I do best and for the life lessons and wonderful conversations we had. I would also like to thank Xian for his help in my experiments and for teaching me lab techniques and for giving me great professional advice.

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met, it was a pleasure meeting you and working with you. Lastly, To Dr. Thomas and Dr. Teodoro, thank you for your support and advice over the years during my committee meetings. To Dr. Thomas and to Christine, your kindness and support are beyond description, and our small talks always gave me a sense of security.

Contribution to Original Knowledge

Chapter 2: Investigating the role of the Posttranslational Modification PARylation in the pro-myogenic Function of HuR.

- Identified TNKS1-mediated PARylation as an event required for the process of myogenesis.
- Found that HuR is PARylated by TNKS1 *in vitro* and in muscle cells, through the interaction with TNKS1 via a conserved consensus TNKS1-binding motif. This PARylation promotes the RNA-binding activity, the stabilization function, and the cytoplasmic translocation of HuR during myogenesis.
- Demonstrated the importance of the PARylation of HuR by mutating the TNKS1-binding motif in HuR which abolished TNKS1 binding and PARylation of HuR. Mutating this motif fails to rescue myogenesis in HuR-depleted conditions (which is well established to inhibit myogenesis).
- Collectively, this work reveals a new role for TNKS1 in skeletal muscle and identifies the TNKS1-mediated PARylation of HuR as an important regulatory process required for muscle fiber formation.

Chapter 3: Identification of STAT3 as a target of HuR-mediated regulation in Cachexia.

- Identified the key pro-cachectic factor STAT3 as a target mRNA of HuR during muscle wasting. STAT3 mRNA is posttranscriptionally regulated by HuR through a U-rich element in the 3'untranslated region (UTR) to promote its translation.
- Demonstrated that HuR increases the translation of STAT3 during cytokine-induced muscle wasting, by interfering with the miR-330-mediated translation repression.
- Found that mutating the HuR binding site in the 3'UTR of STAT3 prevents binding of HuR while mutating the miR-330 site in the 3'UTR of STAT3 mRNA resulted in increased association of HuR with this message.

- Collectively, this work identifies a new mechanism through which HuR mediates cytokine-induced muscle wasting and provides additional evidence for the role of HuR in muscle wasting, and consequently for its dual and opposite function in skeletal muscle.

Author Contributions

This is a manuscript-based thesis comprising one published article (Chapter 3), and one research manuscript that is in revision for publication (Chapter 2). The contribution of co-authors is outlined below.

Chapter 2 | Investigating the role of the Posttranslational Modification PARylation in the pro-myogenic Function of HuR.

The content of this chapter is under revision as

Mubaid S, Adjibade PM, Hall DT, Lian XJ, Brusque S, Ashour K, Carlile G, Gagné J-P, Di Marco S, Thomas DY, Poirier GG, Gallouzi IE. “Tankyrase-1 regulates RBP-mediated mRNA turnover to promote muscle fiber formation.” 2022.

I was responsible for the experimental design and conducted all experimental investigations, with assistance from other co-authors. I performed the formal analysis and visualization of experimental findings. I prepared the original manuscript. DTH contributed to the conceptualization and conducted the preliminary investigation and validation of experimental findings. PMA contributed to the investigation and validation of the experiments involving the XAV939 inhibitor. XJL and SB assisted with sample collection and processing of experiments. SDM assisted with conceptualization, data analysis, and helped edit and review the manuscript. GC helped in the conceptualization, conduction, and analysis of the in vitro ribosylation assays used in the preliminary investigations. DYT provided technical and experimental expertise with the in vitro PARylation assay. J-PG provided instructions for the material provided or suggested (pADPr antibody and inhibitors, respectively). GGP provided materials. I-EG conceptualized, established, and directed the execution of research goals, interpreted the data, reviewed, and edited the manuscript.

Chapter 3 | Identification of STAT3 as a target of HuR-mediated regulation in Cachexia.

The contents of this chapter were originally published in

Mubaid S, Ma JF, Omer A, Ashour K, Lian XJ, Sanchez BJ, Robinson S, Cammas A, Dormoy-Raclet V, Di Marco S, Chittur SV, Tenenbaum SA, Gallouzi IE. “HuR

counteracts miR-330 to promote STAT3 translation during inflammation-induced muscle wasting." *PNAS* August 2019; 10.1073/pnas.1905172116

I am first co-author in this paper. I was responsible for a significant amount of experimental design and investigations. JFM is the second co-first author and she was responsible for the experimental design, investigation, analysis, and visualization of the preliminary data. She prepared the original manuscript, and I participated in the review and preparation of the final submitted manuscript. AO, KA, XJL, BJS, SR, AC, and VDR assisted with sample processing for both *in vitro* and *in vivo* experiments. SDM assisted with conceptualization, data analysis, and helped edit and review the manuscript. SVC and SAT contributed equipment and performed the microarray experiments. I-EG conceptualized, established, and directed the execution of research goals, interpreted the data, and reviewed and edited the manuscript.

List of Figures and Tables

List of Abbreviations

2-AMG	aminoguanidine
4E-BP1	eIF4E binding protein-1
4E-T	eukaryotic translation initiation factor 4E transporter
AChE	acetylcholinesterase
AChR-β	acetylcholine receptor-β subunit
ADP	adenine diphosphate
ADPr	adenine diphosphate ribose
AGO2	argonaute 2
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis-inducing factor
AKT	protein kinase B
AMPK	AMP-activated protein kinase
AP	homeodomain of antennapedia
AP1 or 2	activator protein
APC	adenomatous polyposis coli
APRIL	A proliferation-inducing ligand
ARC	ankyrin repeat clusters
ARE	AU-rich element
ARH	ADP-hydrolases
ARTD	ADP-ribosyltransferase enzymes
ATG	autophagy-related
AUF1	AU-rich element RNA-binding protein 1
Bax	Bcl-2 associated X
Bcl-2	B-cell lymphoma-2
bHLH	basic helix-loop-helix
BNIP3	Bcl-2 interacting protein 3
C/EBPβ	CCAAT/enhancer binding protein β
C26	colon-26 carcinoma
CAAlnc1	cachexia-related anti-adipogenesis lncRNA 1

CARM1	coactivator-associated arginine methyltransferase 1
CAT-1	cationic amino acid transporter 1
CCNA2	cyclin A2
CCNB1	cyclin B1
CDK	cyclin dependent kinase
CHK2	cell cycle checkpoint kinase
COX2	cyclooxygenase-2
CP	cleavage product
CRM1	chromosome region maintenance 1
D-loop	Donor loop
Db/db	diabetic
DCP	Des-Gamma carboxyprothrombin
DGCR8	DiGeorge syndrome critical region 8
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
DP1	dimerization partner 1
E-box	enhancer box
eIF2α	eukaryotic initiation factor 2α
eIF2AK2	eukaryotic translation Initiation Factor 2 Alpha Kinase 2
eIF3-f	eukaryotic translation initiation factor 3f
eIF4E	eukaryotic translation initiation factor 4E
eIF4G	eukaryotic translation initiation factor 4G
Eif4enif1	eukaryotic translation initiation factor 4E nuclear import factor 1
ELAV1	embryonic lethal abnormal vision 1
ERBB2	erb-b2 receptor tyrosine kinase 2
ERK	extracellular signal-regulated kinase
ETC	electron transport chain
EX527	selisistat
EXOG	exo/endonuclease G
FoxO	Forkhead box O
G3BP	Ras-GTPase-activating protein (SH3 domain)-binding protein

GCN-5	general control non-depressible 5
Glut4	glucose transporter type 4
GM-CSF	granulocyte-macrophage colony-stimulating factor
Gp130	Glycoprotein 130
GSK3β	glycogen synthase kinase 3β
HCT116	human colorectal cancer cells
HD	auto-inhibitory region
HDFs	human diploid fibroblasts
HECT	homologous to the E6-AP carboxyl terminus
HeLa	Henrietta Lacks
HIF-1α	hypoxia-inducible factor 1α
HMGB1	high mobility group box protein 1
HNE	4-hydroxynoneal
HnRNP	Heterogeneous nuclear ribonucleoproteins
HNS	nucleocytoplasmic shuttling sequence
HPS	histidine, proline, and serine
HSC	hematopoietic stem cells
Hsp60	heat shock protein 60
Hsp72	heat shock protein 72
hsrω-RA	heat shock RNA ω
HuA	human antigen A
HuB	human antigen B
HuC	human antigen C
HuD	human antigen D
HuR	human antigen R
IFN-γ	interferon γ
IKK	inhibitor of κB kinase
I/T	IFN-γ/TNFα
Id	inhibitor of differentiation
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IL	interleukin
IRAP	insulin regulated aminopeptidase
IRI	irinotecan
iNOS	inducible nitric oxide synthase
IRES	internal ribosome entry site
JAK	Janus-kinases
KSRP	KH-type splicing regulatory protein
KO	knockout
LA	lupus antigen
LC3B-II	Microtubule-associated protein 1 light chain 3 B isoform II
Let-7	lethal-7
LKB1	liver kinase B1
LLC	Lewis lung carcinoma
lncRNA	long non-coding RNA
LPS	lipopolysaccharide
M7G	7-methylguanosine
MacroD	macrodomain protein
MAFbx	muscle atrophy F-box
MAPK	mitogen-activated protein kinase
MARylation	mono(ADP-ribosyl)ation
MCK	muscle creatine kinase
Mcl-1	myeloid cell leukemia-1
MEF2	myocyte enhancer binding factor 2
MF20	myosin heavy chain
MG132	carbobenzoxy-Leu-Leu-leucinal
MIN	multiple intestinal neoplasia
MiRNA	microRNA
Mix1	homeodomain protein MIX
Mlc1f	myosin light chain 1 fast
MRF	myogenic regulatory factor
mRNA	messenger RNA

mRNP	messenger ribonucleoprotein
MRS	magnetic resonance spectroscopy
mTOR	mammalian target of rapamycin
MuRF1	muscle RING-finger protein-1
muHuR KO	muscle-specific knockout of HuR
Myf5	Myogenic factor 5
MyHC	myosin heavy chain
MyoD	myoblast determination protein 1
NA	nicotinic acid
NAM	nicotinamide
NAD^+	nicotinamide adenine dinucleotide
NCL	nucleolin
NELFE	Negative Elongation Factor Complex Member E
NES	nuclear export signal
NFκB	nuclear factor κ-light-chain-enhancer of activated B cells
NNMT	nicotinamide *N*-methyltransferase
NO	nitric oxide
NPM	nucleophosmin
NR	nicotinamide riboside
Nrf2	nuclear factor erythroid 2-related factor
NSCLC	Non-Small Cell Lung Cancer
NuMa	nuclear mitotic apparatus
OPMD	oculopharyngeal muscular dystrophy
OXPHOS	oxidative phosphorylation
P bodies	processing bodies
p-mTOR	phosphorylated-mTOR
$P16^{INK4}$	cyclin-dependent kinase inhibitor 2A
$P21/P21^{cIP1}/P21^{Waf}$	cyclin-dependent kinase inhibitor 1
P27	cyclin-dependent kinase inhibitor 1B
P53	tumor protein p53
P65	REL-associated protein

Pabpn1	poly(A) binding protein, nuclear 1
pADPr	poly(ADP-ribose)
PAP	poly-A-polymerase
PAR-CLIP	photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation
PARN	Poly(A)-Specific Ribonuclease
PARP	poly(ADP-ribose) polymerase
PARG	poly(ADP-ribose) glycohydrolase
PARylation	poly(ADP-ribosyl)ation
Pax7	paired box gene 7
Pax3	paired box gene 3
PDCD4	programmed cell death 4
PEX14	Peroxisomal Biogenesis Factor 14
PGC1α	peroxisome proliferator-activated receptor gamma coactivator 1-α
PHAP-I	putative HLA-associated protein-I
PHAP-II	putative HLA-associated protein-II
PIAS3	protein inhibitor of activated STAT3
Pitx2	paired-related homeobox 2
PKC	protein kinase C
PKM2	pyruvate kinase M2
PKR	protein kinase R
Pp32	phosphoprotein 32
PPARγ	Peroxisome proliferator-activated receptor γ
Phospho-RB	phosphorylated retinoblastoma protein
Pre-miRNA	precursor microRNA
Pre-mRNA	precursor mRNA
PTP	protein tyrosine phosphatases
RAB10	Ras-related protein Rab-10
RB	retinoblastoma protein
RBM38	RNA-binding motif protein 38
RBP	RNA-binding protein

RhoB	Ras Homolog Family Member B
RING	really interesting new gene
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNF146	Ring Finger Protein 146
RRM	RNA-recognition motif
S6K	S6 kinase
SAM	sterile alpha motif
SC	satellite cell
SH2	Src homology 2
SH3BP2	Src homology 3 Domain Binding Protein 2
SHP	SH2 domain-containing phosphatases
SIRT1	sirtuin 1
SMA	spinal muscular atrophy
SMN1	survival of motor neuron 1
SnRNP	small nuclear ribonucleoproteins
SNAP23	synaptosome associated protein 23
SOCS3	suppressor of cytokine signalling 3
Sort1	sortilin 1
Src	Proto-oncogene tyrosine-protein kinase
STAT3	Signal transducer and activator of transcription 3
TA	tibialis anterior
TARBP2	transactivation-responsive RNA-binding protein 2
TARG	terminal ADP-ribose protein glycohydrolase
TBM	tankyrase-binding motif
TBP	TATA-box binding protein
TCA	tricarboxylic acid
TCPTP	T-Cell Protein Tyrosine Phosphatase
TGF-β	transforming growth factor β
TNFα	tumour necrosis factor α
TNKS	tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase

TOP2A	DNA Topoisomerase II Alpha
TRF-1	Telomeric repeat-binding factor 1
TTP	tristetraprolin
U2AF65	splicing factor U2AF
UCP-1	uncoupling protein 1
ULK1	unc-51 like autophagy activating kinase
UPP	ubiquitin-proteasome pathway
USP25	ubiquitin specific peptidase 25
UTR	untranslated region
Vamp	Vesicle associated membrane protein
Wnt	wingless-related integration site
WT	wildtype
WWE	tryptophan, tryptophan, glutamate
XIAP	X-linked inhibitor of apoptosis
YB-1	y-box binding protein 1

Chapter 1 | General Introduction

1.1 Myogenesis

Skeletal muscle constitutes the largest tissue mass of the human body. The primary functions of skeletal muscle tissue are locomotion, postural behavior, and breathing. As such, compromised muscle function can lead to a severely deteriorated quality of life. The process of muscle fiber formation, also termed myogenesis, is a process in which satellite cells, which are muscle precursor cells, differentiate to commit to becoming mononucleated myoblasts. After that, myoblasts further differentiate and fuse to form multinucleated myotubes. During embryogenesis, skeletal muscles originate from the mesoderm. After birth, the process can be activated by growth, muscle tissue injury, or exercise. The molecular regulation of myogenesis, which includes activation of the precursor cells (named satellite cells, SCs), the proliferation of myoblasts and their differentiation, and the quiescence of non-differentiated cells, is strikingly similar between embryonic and adult muscle stem cells. Muscle regeneration, which is triggered by tissue injury, can be divided into two steps: a degenerative phase, and a regenerative phase [1]. During the degenerative phase, inflammatory and muscle precursor cells residing within the muscle tissue are activated due to the necrosis resulting from the disrupted myofiber sarcolemma. Next, the regeneration phase begins with the expansion of the myoblasts which is followed by their fusion with the damaged fiber or the formation of new fibers that become undistinguishable from undamaged fibers. A subset of myoblasts, that fail to differentiate and remain associated with the surface of the developing myofibers, form the quiescent muscle satellite cell pool.

The commitment of myogenic progenitor cells to myogenesis during development and postnatal muscle regeneration is a multi-stage process controlled by several transcription factors such as the paired domain transcription factor *Pax7* as well as myogenic regulatory transcription factors (*MRFs*) including myogenic factor 5 (*Myf5*), myogenic differentiation antigen (*MyoD*), *myogenin*, and *MRF4* (Figure 1.1) [2-4]. Upon activation of myogenesis during development or in response to injuries, these factors collaborate together to ensure the commitment of SCs to the myogenic program through induction of a muscle-specific transcriptional network [2, 5, 6]. Embryogenic myogenesis

can be divided into 2 phases: early embryonic (or primary) and later fetal (or secondary) phase. In the primary phase, paired box gene Pax3 expressing progenitors form primary myotubes which provide the templates upon which secondary myotubes can form. During the secondary phase, a subset of the $Pax3^{+}$ cells upregulate Pax7 expression and downregulate Pax3 to form secondary myotubes. A subset of the Pax7 expressing progenitor cells will form a pool of adult muscle stem cells, called SCs, which are used during post-natal myogenesis. The majority of SCs in adult muscles are quiescent and express *Pax7* and *Myf5*. The expression of both *MyoD* and *Myf5* is the key step in the commitment of SCs to the myogenic lineage, since the simultaneous disruption of both genes results in the absence of skeletal myoblasts [4, 7-9]. However, mice lacking myogenin have poorly developed skeletal muscle tissue even though myoblasts are present, suggesting that myogenin plays a critical role in the terminal differentiation of myoblasts despite being dispensable for the generation of the myogenic lineage [4, 8-11].

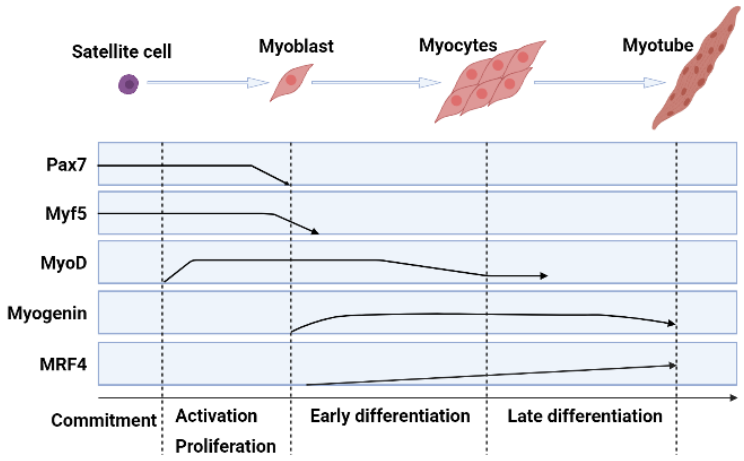


Figure 1.1. The molecular regulation of myogenesis.

When muscle precursor cells, called satellite cells, are activated, they proliferate and differentiate into myoblasts by upregulating the expression of primary myogenic regulatory factors (MRFs), such as Myf5 and MyoD. These myoblasts undergo early differentiation and once enough myoblasts have grown, they become myocytes where they upregulate secondary MRFs, such as myogenin and MRF4 to start late differentiation. At that stage, they irreversibly withdraw from the cell cycle and fuse to form myotubes. These MRFs are transcription factors that lead to the sequential expression of each other. Image adapted from [12].

1.1.1 The molecular regulation of Myogenesis

The myogenic process requires tight regulation, which is achieved by the sequential expression of the MRFs described above. MRFs are basic helix-loop-helix

(bHLH) transcriptional activators which are upregulated to allow commitment to the myogenic lineage [13, 14]. The bHLH domain recognizes the E-box DNA sequence (CANNTG) in the regulatory regions of target genes. Proliferating myoblasts that are committed to the myogenic lineage express the primary MRFs MyoD and Myf5 [7, 15]. Once these proliferating myoblasts have reached a sufficient number of cells, they withdraw from cell cycle and become terminally differentiated myocytes that express secondary MRFs which include myogenin and MRF4. Subsequently, the fused myocytes (now myotubes) will express myosin heavy chain (MyHC) and muscle creatine kinase (MCK). Expression of different isoforms of MyHC proteins determines the specification/composition of muscle fibers, which are named after the isoforms that they express [16]. Muscle fibers, therefore, can be classified, based on the expression of these myosin heavy chain (MyHC) isoforms as being either Type I oxidative, slow-twitch or Type II glycolytic, fast-twitch [17, 18].

The commitment of cells to the myogenic lineage and their differentiation into muscle fibers is mediated by the sequential activation of the MRFs described above. During embryonic or regenerative myogenesis, satellite cells are activated by signals from their surrounding environment [19]. These cells proliferate in a Myf5-dependent manner due to the transcription of genes involved in cell cycle progression. Subsequent to the rapid upregulation of Myf5 expression during this process, MyoD expression is initiated, and satellite cells undergo cell division.

MyoD is a key MRF that mediates the expression of muscle-specific genes, such as myogenin, as well as genes involved in the inhibition of cell proliferation. Importantly, ectopic expression of MyoD in non-muscle cell types reprogrammes them to have features of muscle lineage [20, 21]. During differentiation, MyoD contributes to cell cycle arrest by complexing with various proteins, including the transcription factors E12/E47, to promote the transcription of myogenin and cell cycle arrest genes. During proliferation, the complex is disrupted by Id (inhibitor of differentiation) proteins which compete with MyoD for the binding of E12/E47 or bind myogenic factors to produce transcriptional inactive complexes. While the expression of Id is activated in response to growth factors, it decreases upon differentiation. Another factor that MyoD interacts with

to stop cell cycle is retinoblastoma protein (Rb). MyoD prevents phosphorylation of Rb, allowing it to interact with E2F and prevent cell cycle irreversibly, by preventing E2F from complexing with DP1 (dimerization partner 1) to promote the transcription of cell cycle genes. During proliferation, the anti-proliferative activity of MyoD is prevented by cyclin D1, whose expression leads to increased cyclin-dependent kinase (CDK) 4 (CDK4) nuclear localization, where it complexes with MyoD and prevents it from performing its transcriptional activity by disrupting its association to DNA [22]. During differentiation, p21 and p16 inhibit the formation of the MyoD/CDK4 complex thus stimulating the transcriptional activity of MyoD. MyoD induces the expression of p21, providing a positive feedback loop to enhance its own activity. Additionally, during differentiation, the transcriptional activity of MyoD upregulates myogenin transcription [23]. Therefore, MyoD induces early differentiation of myoblasts by preventing the transcription of proliferation genes and, furthermore, promoting the transcription of genes that induce the differentiation of muscle cell progenitors [24, 25].

The myogenic differentiation process is tightly linked to the cell cycle, as one of the early steps of the process is the arrest of the cell cycle, which is an irreversible step during differentiation. As previously mentioned, cell cycle inhibition is initiated by the activity of MyoD, and it is thought to mainly occur due to the increased transcription/expression of p21. p21 is a G_1-specific, cyclin-dependent kinase inhibitor which has emerged as a key regulatory component involved in regulating cellular proliferation and differentiation in various systems. During myogenesis, p21 transcription is upregulated during muscle cell differentiation by both MyoD and myogenin. Mammalian cell cycle progression is regulated by CDKs and their regulatory subunits cyclins [26]. Fluctuations in cyclins lead to activation of CDKs. CDK-cyclin complex partially phosphorylate Rb. p21 can bind both CDKs and cyclins and thereby can irreversibly inhibit cell cycle by inhibiting CDK4/cyclin D and CDK2/cyclin E, and by disrupting the CDK-cyclin-Rb interaction [27, 28]. The importance of p21 in the myogenic process is underscored by the delay in muscular regeneration observed in p21 KO mice [29].

Once myogenin is expressed, myoblasts rapidly exit cell cycle [30]. MyoD and myogenin synergize to restructure/open the chromatin at the loci of *p21*, *p16, p57* and *RB* genes to enhance their expression [31]. Myogenin is required for the assembly of the transcription machinery through the recruitment of RNA polymerase II and TATA-box binding protein (TBP) [32]. Myogenin also upregulates the transcription of a cluster of small non-coding RNA (microRNAs, miRNAs) named miR17-92 [33-35], as well as the Lats2 kinase [30]. In doing so, myogenin, through the regulation of both the miRNA17-92 cluster and Lats2 kinase, affects the expression of the transcription factors E2F1, E2F2, and E2F3 by inhibiting their translation and their transactivation, respectively [13].

After fusion of myoblasts, MRF4 becomes highly expressed and triggers the downregulation of myogenin expression [36]. In fact, MRF4 is the most highly expressed MRF in healthy mature muscle fibers [37]. Not much is known about the role of MRF4 in myogenesis, however it was recently shown that MRF4 is involved in muscle growth regulation by interacting with the myocyte enhancer binding factor 2 (MEF2) family of proteins [38]. Although their function in skeletal muscle remains poorly understood, MEF2 proteins are upregulated during regeneration and their absence inhibits the myogenic process. MRF4 knockdown results in an increase in muscle fiber size, with an upregulation of genes involved in the sarcomere, membrane cytoskeleton, contraction, and energy metabolism. MRF4 depletion was also accompanied by an increase in MEF2 expression, and these effects were abolished by the expression of a dominant negative MEF2, suggesting that MRF4 regulates the growth of muscle fibers by repressing MEF2 activity.

1.2 Cachexia-induced muscle wasting

Similar to any tissue in the body, pathologies associate to skeletal muscle may have a detrimental affect on the well-being of individuals. Patients affected by chronic diseases such as cancer, AIDS, chronic obstructive pulmonary disorders, as well as severe burns can develop a syndrome, named cachexia, that is characterized by a dramatic weight loss which occurs due to the wasting of skeletal muscle [39-43]. The main trigger of cachexia is the induction of a chronic, proinflammatory response which is

a common hallmark of these diseases [44-46]. This loss of muscle reduces the quality of life by impairing function and activities of daily living and increases the toxicity and complications of anti-cancer treatments [47, 48]. If persistent, cachexia leads to reduced mobility, impaired respiration, and eventually death. As mentioned above, cachexia is characterized by a progressive loss of skeletal muscle mass, which may or may not include adipose tissue loss. It may be accompanied by anorexia and the wasting of other organs, including the brain, gut, and liver. It is a complication of cancer which occurs in 30 to 80% of patients, with the highest frequencies in pancreatic and gastric cancer [45]. Importantly, the weight loss is an important prognostic factor in cancer, which negatively correlates with survival time [40, 49, 50].

The chronic inflammation initiating cachexia is caused by tumour- and host-derived factors that will cause a systemic inflammation, which leads to abnormal metabolism of carbohydrate, lipid, proteins, and an overall hyper-catabolic state [45, 51-53]. Despite the clear impact of cachexia, due to the numerous factors that render it a complex syndrome, no approved therapies exist yet. Therefore, understanding mechanisms that lead to cachexia is important for exploring new strategies to treat this condition.

1.2.1 Mechanisms of Muscle Atrophy

Muscle atrophy occurs due to the inflammation-induced activation of several pathways in skeletal muscle. Chronic inflammation affects the body homeostasis and creates a catabolic environment that leads to muscle atrophy. As such, cachexia results in decreased anabolism and increased catabolism which accounts for the loss of muscle mass. Indeed, proinflammatory factors named cytokines mediate the inhibition of general protein synthesis (resulting in decreased anabolism) while simultaneously activating the ubiquitin-proteasome (UPP) and autophagy pathway which mediated the degradation of proteins (resulting in increased catabolism). In doing so, these cytokines mediate muscle wasting by inducing an overall negative disbalance in protein levels. Additionally, the loss of muscle mass is also mediated by an impairment in the regeneration potential of satellite and muscle precursor cells that, as described above, is needed for the repair of muscle.

1.2.1.1 Decreased Protein Synthesis

The process of protein synthesis through mRNA translation is highly regulated in skeletal muscle by the serine/threonine kinase AKT. AKT controls translation through the mTOR kinase, which phosphorylates eIF4E binding protein-1 (4E-BP1) and p70 S6 kinase (S6K) to promote the initiation of general translation [17]. 4E-BP1 regulates the initiation of translation by associating/inhibiting the function of the initiation factor eIF4E. The phosphorylation of 4E-BP1, however, releases it from eIF4E, allowing the recruitment of the initiation factor eIF4G to increase translation initiation rates [45, 54-56]. In parallel, phosphorylated S6K phosphorylates and activates the ribosomal protein S6 to promote protein synthesis. Muscle tissues of cachectic patients exhibit decreased levels of total AKT, total mTOR, and p-mTOR levels, indicative of decreased protein synthesis [57]. Additionally, muscle and adipose tissues of colon-26 (C26)-tumour bearing mice, an animal model of cachexia-induced muscle wasting, are characterized by decreased levels of phospho-AKT [58]. AKT also regulates translation through glycogen synthase kinase 3β (GSK3β), which phosphorylates the initiation factor eIF2α to inhibit its activity, since it is an important step in the process of protein synthesis and represents a checkpoint through mediation of translation elongation initiation [17, 45, 55]. Other kinases can regulate this step, including PKR, which was shown to phosphorylate eIF2α in response to pro-inflammatory cytokines tumour necrosis factor-α (TNFα) and interferon γ (IFNγ), and thereby results in decreased global translation [59, 60]. Interestingly, in cachectic patients, there is an increase in phosphorylation of PKR and phosphorylation of eIF2α.

1.2.1.2 Increased Protein Degradation

The pathways that trigger protein degradation in cachexia are stimulated by Forkhead box O3 (FoxO3), which activates lysosomal and proteasomal pathways. The UPP plays a predominant role in the degradation of myofibrillar proteins. In this pathway, proteins are targeted for protein turnover due to the posttranslational modification/addition of ubiquitin. This modification is mediated by a cascade of reactions carried by three groups of enzymes: E1, E2, and E3 [61]. E1 activates ubiquitin, which is then transferred to the ubiquitin-conjugating enzyme E2. E2 interacts with the ubiquitin-ligase enzyme E3, which catalyzes the transfer of ubiquitin to the

amino acid acceptor, typically lysine. There exist hundreds of E3 enzymes, which can interact with substrates and recognize them through the homologous to the E6-AP carboxyl terminus (HECT) domain and the really interesting new gene (RING) domain. Ubiquitin chains can be subsequently formed and recognized by the 26S proteasome complex, which degrades targeted proteins [61]. Two E3 muscle-specific ubiquitin ligases, atrogin1/muscle atrophy F-box (MAFbx) and muscle RING-finger protein-1 (MuRF1) are highly expressed during muscle atrophy [45]. MuRF1 ubiquitylates myosin heavy chain proteins and other thick filament proteins, as well as actin and other thin filament proteins, suggesting that it promotes protein degradation [62-64]. While Atrogin-1/MAFbx ubiquitylates the eukaryotic initiation factor 3, subunit 5 (eIF3-f), suggesting that it controls protein synthesis [65]. Consequently, both MuRF1 and Atrogin-1/MAFbx target proteins for degradation by the 26S proteasome, leading to a net protein degradation balance shift [66].

Macroautophagy (autophagy) is a mechanism used by cells, including skeletal muscle, to transport organelles, and proteins to lysosomes for degradation [67]. Autophagy is induced through the activation of the energy sensor, AMPK, which mediates autophagy through two pathways. The first pathway is the activation of FoxO3, which leads to the transcription of autophagy-related (ATG) proteins and E3 ubiquitin ligases such as muscle RING-finger protein-1 (MuRF1) [68]. The second pathway is alleviating the mTOR-mediated inhibition of autophagy [69]. mTOR inhibits autophagy through the phosphorylation of unc-51 like autophagy activating kinase (ULK1), which is the mammalian orthologue of ATG1, sequestering it in an inactive state. Therefore, inhibition of mTOR activates autophagy initiation complex by activating ULK1. Evidence shows that both the hyper and hypoactivation of autophagy result in muscle atrophy [70]. Indeed, under normal conditions, autophagy serves as a tumour suppressor by negating the effects of harmful stimuli, including oxidative stress, inflammation, and genome instability to preserve cellular function and limit cell growth [71]. In contrast, in cancer-induced conditions, where nutrient availability is reduced in the environment of the tumour, autophagy is activated to serves as a survival factor, which in addition to mobilizing nutrients, protects tumour cells from radiation, mutations, and chemotherapy. In fact, tumour cells accelerate autophagy in myotubes during

cachexia by secreting the interleukin-6 (IL-6) (a proinflammatory cytokine which is known to induce wasting) [72]. The neutralization of IL-6, indeed, was shown to prevent activation of autophagy. The pro-cachectic role of autophagy is further supported by the increase in autophagy-related factors, such as beclin 1 and LC3B-II, in cachectic cancer patients and animals [70, 73]. The role of autophagy in promoting cachexia, nonetheless, remains disputed since exercise and treatments that trigger autophagic flux (AICAR or rapamycin) have also been shown to reduce cachexia-induced wasting, suggesting that this pathway is rather beneficial for countering muscle atrophy [74-76]. More importantly, the phenotype of mice containing muscle-specific inactivation of autophagy-related genes, such as *Atg7* and *Atg5*, result in muscle atrophy and dysfunction [67]. Thus, further research is needed to understand the impact of autophagy in cachexia-induced muscle wasting.

1.2.1.3 Regeneration Deficit

In addition to promoting muscle atrophy via the aforementioned mechanisms, evidence show that cachectic muscle fibers display impaired regenerative capacity. As previously mentioned, during muscle regeneration, satellite cells, which express Pax7, are activated to differentiate and sequentially express MRFs to form muscle fibers. In genetic myopathies such as Duchenne muscular dystrophy, the function of satellite cells is impaired and contributes to muscle wasting [77]. Studies have shown that similarly, during cachexia, there seems to be a regenerative deficit. The damage induced by cachexia causes activation of satellite and muscle precursor cells. Although activated, these cells cannot completely differentiate, due to the persistent activation of Pax7 (self-renewing factor) [78]. In fact, overexpression of Pax7 in normal muscle results in muscle atrophy. Activation of the NFκB pathway in cachectic mice and patients results in the secretion of serum factors that induce Pax7 expression. Although this study does not identify these factors, incubation of C2C12 cells with serum from cachectic animals and patients induced Pax7 expression, and consequently led to a reduction in the expression of the MRFs MyoD and myogenin, resulting in impaired regeneration of muscle tissue. Another study subsequently showed that similar to NFκB, the transcription factor CCAAT/enhancer binding protein β (C/EBPβ) also increases Pax7 expression and leads to a regeneration deficit during cancer cachexia [79]. While it has

been previously shown that C/EBPβ directly regulates the transcription of Pax7, it is unknown whether NFκB directly or indirectly regulates the transcription of Pax7 [80]. In a later study, it was shown that the myogenic capacity of satellite cells in tumour-bearing C26 mice was reduced, due to the severe impairment of their proliferation and differentiation [81]. The regenerative capacity of muscle during cachexia is another mechanism affecting the integrity/maintenance of skeletal muscle.

1.2.2 Mechanisms of Cachexia-induced Muscle Wasting

The chronic elevation of pro-inflammatory cytokines is one of the main triggers of cachexia-induced muscle atrophy. In fact, pro-inflammatory cytokines that include IL-6, IL-1, IL-8, TNFα, and interferon-γ (IFNγ) have been shown to be elevated in patients that have cachexia [82-84]. Additionally, the treatment of muscle cells with these cytokines *in vitro* can mimic the wasting phenotype [44]. Prolonged exposure to these cytokines results in the constant activation of inflammatory signalling in muscle cells, which induce the expression of pro-cachectic genes.

1.2.2.1 Proinflammatory mediators of Cachexia induced muscle wasting

1.2.2.1.1 The role of IL-6 in Cachexia-induced Muscle Atrophy

IL-6 is one of the most established pro-inflammatory cytokines in cachexia-induced muscle atrophy. Indeed, numerous studies have shown that IL-6 levels become elevated in cancer patients that develop cachexia [85]. Indeed, IL-6 level augmentation has been shown to correlate with weight loss and reduced survival in pancreatic cancer and Non-Small Cell Lung Cancer (NSCLC) patients [52, 86]. Murine models of cachexia show similar implication of IL-6 in muscle wasting to what was observed in cancer patients. For example, a study using the $APC^{MIN/+}$ (adenomatous polyposis coli (APC) carrying the multiple intestinal neoplasia (MIN) point mutation) mouse model of muscle wasting showed that there is a 10-fold increase in IL-6 levels compared to wildtype mice [87]. The genetic ablation of IL-6 in these mice, interestingly, reduced tumour burden and muscle wasting. Although rescue of IL-6 expression in these KO mice reversed this effect, it did not induce wasting in non-tumour bearing mice suggesting that IL-6 indirectly may cause muscle wasting by increasing the tumour burden. Furthermore, the usage of anti-murine IL-6 receptor antibody in the C26 murine model of cancer-induced

cachexia attenuated the muscle atrophy further supporting the role of IL-6 in promoting cachexia-induced wasting [88]. The pro-cachectic activity of IL-6 has been attributed to its ability to increase the expression of ubiquitin and the activation of the proteasomal proteolytic cleavage pathway [89]. Additionally, IL-6 signalling seems to be involved in the activation of the STAT3 pathway (described below), which is implicated in muscle wasting [90, 91].

1.2.2.1.2 The role of TNFα in Cachexia-induced Muscle Atrophy

TNFα is secreted by the tumour and is a well-established cytokine that is involved in cachexia. In fact, it was one of the first humoral factors that was discovered to induce cachexia and it was therefore identified as cachectin [92]. Numerous reports have shown that the induction of an inflammatory state by TNFα promotes cachexia and leads to reduced food intake, loss of body weight, and skeletal muscle loss [93, 94]. Furthermore, mice with TNFα-secreting tumours develop cachexia and display severe weight loss [95]. In support with these findings, mice bearing the Lewis Lung carcinoma (LLC) cells which develop cancer-induced cachexia, display reduced muscle wasting when they are transgenically induced to express a soluble TNFα receptor protein [96]. This demonstrates that in this model, TNFα is necessary for the development of cachexia. More importantly, human patients with cachexia display higher levels of TNFα mRNA and protein in their muscles compared to healthy individuals (controls) [97]. They also have higher levels of the active form of the TNFα receptor. TNFα is known to mediate its pro-cachectic effects by activating NFκB, which promotes the transcription of pro-cachectic genes [98, 99]. Indeed, cancer cachexia patients that have increased levels of TNFα also exhibited increased activation of NFκB and upregulation of iNOS (a known target of NFκB) [97]. Interestingly, it seems that TNFα has a bivalent role in skeletal muscle depending on the exposure, since TNFα null mice and TNFα receptor mutant mice display impaired regeneration capacity, accompanied by lower levels of MyoD and MEF2 expression compared to control mice [100]. Although numerous studies have clearly demonstrated the implication of TNFα in cancer-induced cachexia, TNFα is insufficient to mediate a major cachectic phenotype on its own. Indeed, the administration of therapies that target TNFα does not ameliorate appetite or body weight loss induced by cachexia [101-103]. TNFα, therefore, is suggested to mediate wasting

by synergizing with other pro-inflammatory factors, such as IFNγ [59, 104-108]. Thus, it is likely that TNFα promotes cachexia-induced muscle wasting in conjunction with other cachectic factors.

1.2.2.1.3 The role of IFNγ in Cachexia-induced Muscle Atrophy

IFNγ is another pro-inflammatory cytokine that has been shown to be involved in cachexia. IFNγ can be produced by tumour cells and can contribute to systemic inflammation which results in wasting. Indeed, implantation of tumour cells that overexpress IFNγ in mice resulted in severe cachexia, while inhibition of IFNγ, using neutralizing anti-IFNγ antibodies, before the implantation of the cells prevented the body weight loss [109]. This confirmed that in the presence of the tumour, the weight loss observed in these mice is due to IFNγ. Additionally, in non-tumour-bearing rats, IFNγ was shown to induce cachexia-like symptoms, such as decreased food intake and body weight loss [110]. Whereas the immunization against the recombinant (murine) IFNγ administered to tumour-bearing rats recovered their appetite and their body weight loss and increased their lifespan and the size of the tumour they can tolerate. Therefore. similar to TNFα, IFNγ promotes cachexia in the presence of a tumour and other pro-inflammatory factors.

1.2.2.1.3.1 Nitric Oxide/iNOS

The inducible Nitric Oxide Synthase (iNOS) enzyme is one of three NOS genes, along with endothelial NOS (eNOS) and neuronal NOS (nNOS), which produce nitric oxide (NO) from the conversion of the amino acid arginine to citrulline [111]. While eNOS and nNOS are, in general, associated with basal activities and low levels of NO production, iNOS is rapidly induced under certain stress conditions and leads to the production of higher levels of NO [111]. These higher NO levels are an important part of the cytotoxic immune response but have also been associated with numerous pathological conditions [112]. Furthermore, co-committed production of nitric oxide with oxygen radicals leads to the production of peroxynitrite ($ONOO^-$). $ONOO^-$ is a highly reactive molecule that can cause oxidative damage and has been associated with many of the pathological effects of NO production [113, 114]. Indeed, cachexia has been associated with nitrosylative stress [115]. In addition, our lab has previously shown that

the production of $ONOO^-$ in response to cytokine treatment causes the loss of MyoD mRNA expression in C2C12 myotubes [104]. In a murine model of cachexia, Buck *et al.* demonstrated that chemical inhibition of iNOS could reduce skeletal muscle wasting [116]. We have also demonstrated that compounds that are able to inhibit iNOS expression are also able to prevent cytokine-driven muscle wasting both *in vitro* and *in vivo* [59]. Recently, iNOS was shown to promote cachexia-induced muscle wasting by altering metabolic pathways as well as causing mitochondrial dysfunction and energy crisis [117]. Indeed, during muscle-wasting, iNOS was shown to interfere with cellular energy production and mitochondrial function by impairing glycolysis and preventing entry of pyruvate and fatty acids (through inhibition of carnitine palmitoyltransferase1 and 2) into the TCA cycle [117]. iNOS inhibition prevented the cytokine-induced glycolytic metabolism shift and reduced the compromise of the integrity of complex 2 and complex 4 of the ETC observed in cultured myotubes treated with cytokines. Moreover, LPS-induced muscle wasting display loss of mitochondrial cristae in WT mice, which are the site of OXPHOS. This loss was not observed in iNOS KO mice injected with LPS or mice injected with C26 tumours (which cause cachexia) treated with iNOS inhibitor. As such, genetic ablation, or pharmacological inhibition of iNOS in mice prevent these effects and prevent muscle loss [117].

1.2.2.2. Inflammatory signal transduction pathways activated in skeletal muscle during cachexia

Skeletal muscle is the tissue mostly affected by cachexia and is the main reason for the total body mass loss observed in patients affected by this condition. As aforementioned, the chronic inflammation caused by the tumour is one of the central reasons behind this loss. In response to inflammatory cytokines, signalling pathways are activated in muscle cells and lead to the expression of cachectic genes that trigger the atrophy and dysfunction of skeletal muscle. Several pathways have been deciphered in the process of cachexia-induced muscle wasting, including the NFκB, Foxo-3/Atrogin-1, and STAT3 pathways.

1.2.2.2.1. FoxO3 pathway in cachexia

Forkhead box O (FoxO) proteins, which include FoxO1 and FoxO3, are a family of transcription factors that have established roles in promoting the expression of genes that mediate protein degradation [118-120]. Specifically, FoxO proteins promote the transcription of factors involved in ubiquitin-mediated proteasomal degradation, including MuRF1 and atrogin1, as well as factors involved in autophagy. In muscle fibers, under normal conditions, AKT phosphorylates FoxO3 leading to its localization to the cytoplasm in an inactive state. During cachexia, however, the decreased activation of AKT results in the decreased phosphorylation of FoxO3 that, in turn, leads to its localization to the nucleus where it transcribes pro-cachectic genes such as *MuRF1* [121]. Interestingly, the activity of FoxO proteins is regulated by several signalling pathways triggered by factors which are involved in cachexia, such as TNFα and myostatin. FoxO3, therefore, constitutes a major pathway through which protein degradation is mediated [17].

1.2.2.2.2 NFκB pathway in cachexia

Nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) is a transcription factor that is activated in response to numerous inflammatory cytokines, such as TNFα, IL-1, and IL-6. It is a master regulator of inflammatory genes expression in pro-inflammatory conditions and oxidative stress. Under normal conditions, NFκB is bound to IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) and is sequestered in the cytoplasm. During inflammatory conditions, however, the inhibitor of κB kinase (IKK) is activated and subsequently phosphorylates IκBα leading to its UPP-mediated degradation. This leads to the release of NFκB which translocates to the nucleus to initiate its transcriptional activity. The importance of NFκB in mediating cachexia is underscored by the fact that muscle-specific NFκB knockout mice and mice that are treated with pharmacological inhibitors of NFκB are protected from inflammation-induced muscle wasting [122, 123].

The pro-cachectic activity of NFκB is mediated, in part, due to the transcription and expression of MuRF1 and atrogin1 [46]. In addition, NFκB activity in cachexia has been linked to reduced regenerative potential and reduced expression of promyogenic

genes, including MyoD and myogenin [78, 104, 108]. Furthermore, NFκB promotes the expression of inducible nitric oxide synthase (iNOS) (as described above) in cachectic muscle, which leads to the production of reactive nitrogen species that induces oxidative stress [59, 106, 124]. In C2C12 murine myotubes, iNOS results in the production of NO, which S-nitrosylates Sirt1 and inhibits its activity, preventing it from deacetylating (and thereby inactivating) NFκB [125]. As such, NFκB positively regulates it own activity through this feedback loop which allows it to be activated. Moreover, NFκB seems to result in metabolic dysfunction during cachexia, since treatment of C26-bearing mice that develop cachexia with a specific inhibitor of NFκB prevents this effect [126]. As such, the activation of the NFκB pathway due to the increased levels of inflammatory cytokines such as TNFα and IL-6, leads to muscle atrophy.

1.2.2.2.3 STAT3 pathway in cachexia

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT family of mammalian transcription factors that are involved in development, proliferation, and immune response [127]. Interestingly, the genetic ablation of STAT3, unlike the other STAT family members, is embryonic lethal [128]. Under normal physiological conditions, STAT3 is located in the cytoplasm in an inactive state. In response to inflammatory stimuli, such as IL-6, STAT3 shuttles between the nucleus and the cytoplasm, with a predominant localization in the cytoplasm. Under these inflammatory conditions, the localization of STAT3 is regulated by phosphorylation on the SH2 domain (on the Y705 residue) by Janus-kinases (JAK) as well as Src family kinases. The phosphorylation of STAT3 on the Y705 residue, consequently, allows it form homodimers that accumulate in the nucleus to mediate transcription of inflammatory response genes (Figure 1.2) [129]. The dimerization of STAT3, furthermore, alters its conformation to facilitate DNA binding. Although the activation of the STAT3 pathway is mainly attributed to IL-6, it can also be activated by other cytokines, such as TNFα and IFNγ [91, 106, 130]. Indeed, we have shown that treatment of muscle fibers with TNFα and IFNγ induces muscle wasting by mediating the phosphorylation of STAT3 in an IL-6 independent manner. Under these conditions, the phosphorylated STAT3 forms a heterodimer with NF-kB that translocates to the

nucleus to induce the transcription of pro-cachectic genes such as *iNOS* (Figure 1.2) [106].

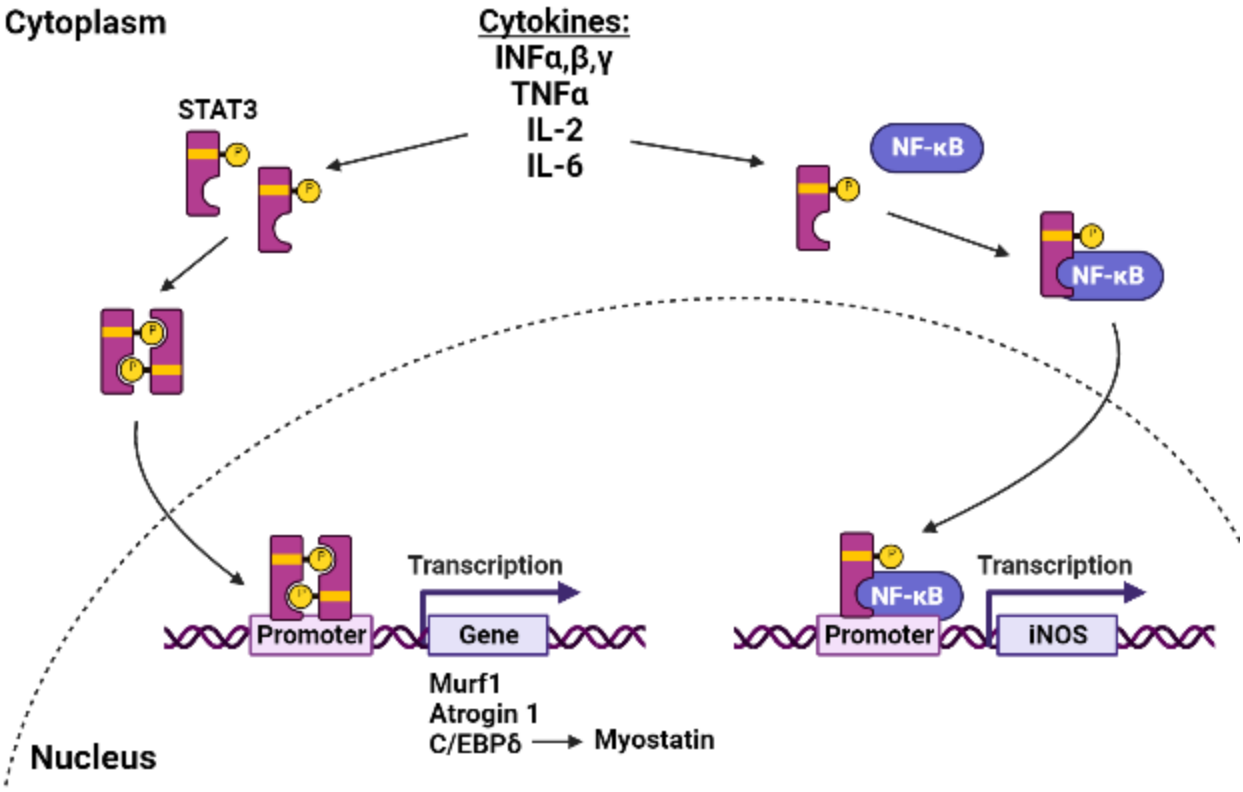


Figure 1.2. Mechanisms of STAT3 in muscle wasting.

In response to the elevation of pro-inflammatory cytokines, such as TNFα, IFNγ and IL-6, STAT3 translocates to the nucleus to mediate the transcription of genes involved in muscle wasting in a phosphorylation-dependent manner. Phosphorylated STAT3 dimerizes and translocates to the nucleus to promote the transcription of MuRF1, atrogin1, and C/EBPδ [129-132]. Phosphorylated STAT3 can also dimerize with NFκB to translocate to the nucleus and promote the transcription of iNOS [106, 133].

The importance of the role of STAT3 in cachexia was evidenced by the fact that chemical and genetic inhibition of STAT3 prevented cachexia in various models, while the muscle-specific overexpression of STAT3 spontaneously induces muscle atrophy [91]. STAT3 mediates muscle wasting through various mechanisms. In addition to iNOS, STAT3 also induces the transcription of atrogin1 and MuRF1 to promote protein degradation. Additionally, STAT3 induces the transcription of caspase-3, which

promotes proteasomal activity [131]. Moreover, STAT3 increases the expression of C/EBPδ, which is another transcription factor that promotes the transcription of myostatin, atrogin1, and MuRF1 [132].

STAT3 can also promote muscle wasting through the regulation of autophagy [67]. During starvation-induced muscle wasting, STAT3 is phosphorylated by Fyn, which is a member of the Src kinase family [134]. This phosphorylation results in the decreased expression of Vps34, which is a key component of autophagic flux (rate of autophagic degradation). STAT3 also promotes autophagy through the regulation of IL-6, and inhibits autophagy through the interaction with protein ligands including PKR/eIF2AK2 and FoxO1/3 and sequestering them, or through its localization to the mitochondria to repress ROS production, which itself induces autophagy [135, 136]. In oncogenesis, STAT3 was shown to be the main transcriptional enhancer of several autophagy-related genes such as *BCL2* (anti-apoptotic) or *BNIP3*, through which it fine-tunes autophagy by inhibiting or promoting it, respectively. However, the importance of this role of STAT3 in autophagy has not been assessed in the context of muscle wasting. Recently, STAT3 was also shown to regulate the biogenesis of tumour-derived exosomes during cancer-cachexia-induced muscle wasting [137]. Recently, exosomes have emerged as mediators of cancer cachexia, through the transport of cargo from tumors to tissues like skeletal muscle [137-139]. The levels of exosomes are correlated with the activation of PKM2 and SNAP23 (assessed by their phosphorylation), which are downstream of STAT3. Under these conditions, the secretion of the exosomes was increased by STAT3 overexpression and, furthermore, reduced by the inhibition or genetic ablation of STAT3. As such, STAT3 is a key mediator of cachexia-induced muscle wasting.

1.3 Posttranscriptional regulation of gene expression during myogenesis and muscle wasting

It is well-established that the ***tight*** and ***coordinated*** regulation of the expression levels of the promyogenic and pro-cachectic factors described above is required for myogenesis and cachexia induced muscle wasting respectively [1, 3, 6, 8, 9, 12, 107, 140-145]. Numerous observations have indicated that this control occurs at the

transcriptional level in response to various signals [1, 3, 6, 8, 9, 140-143]. Recently, the importance of posttranscriptional events, such as mRNA turnover (stability and decay), localization, and translation, was also shown to play major roles in controlling the expression of *MRFs* and pro-cachectic factors [6, 59, 104, 107, 144-152].

1.3.1 The role of cis-acting sequences in the posttranscriptional regulation of promyogenic and pro-cachectic mRNAs

Transcription modulation has been suggested for a long time as the main regulator of the expression of the messenger RNA encoding for promyogenic and pro-cachectic proteins [153-156]. However, transcription alone does not always explain the observed high level of these proteins during myogenesis and in muscle fibers undergoing wasting. The posttranscriptional regulation of many short-lived mRNAs encoding many cytokines, protooncogenes and cell cycle regulatory proteins involves cis-elements including AU-rich (AREs) or GU-rich elements (GRE) found in the 3'untranslated region (3'UTR) of these messages [157]. These AREs have been shown to mediate the stability, cellular localization, and translation of their host messages [158-164]. Many of the ARE-containing mRNAs are labile, such that they can be maintained within critical levels to ensure proper cell growth, differentiation, and response to external stimuli [157, 165-167]. Many promyogenic factors including *p21*, *MyoD* and *myogenin* as well as pro-cachectic messages such as *IL-6, TNFα* and *iNOS* mRNAs are known to contain AREs/GREs that regulate their expression posttranscriptionally [159, 160, 168]. This regulation involves the association of these mRNAs with RNA-binding proteins (RBPs) and/or non-coding RNAs such as miRNAs. Among these trans-acting factors, our laboratory has shown that the RBP HuR, a positive master regulator of mRNA stability and/or translation, plays a prominent role in the posttranscriptional regulation of factors that promote either myogenesis or cachexia-induced muscle wasting.

1.3.2 The role of HuR in regulating the stability and/or translation of mRNA

The following section provides a comprehensive overview of the function of HuR. It describes the known mechanisms through which HuR drives the myogenic or cachectic process. Additionally, I describe the trans-acting factors, including RBPs and

miRNAs as well as the posttranslational modifications that are known to modulate the function of HuR [152, 169-177]. Recent studies show that similar to the bivalent function of HuR in cell survival and cell death, HuR is also a regulator of skeletal muscle formation and atrophy [6, 107, 146, 149, 158, 178-183]. The identification of regulatory mechanisms of the function of HuR, therefore, may be beneficial for targeting it in tissue-specific or condition-specific scenarios. This is especially important due to the toxicity of HuR inhibitors and the lack of treatments for muscle-related diseases and syndromes [184, 185].

1.4 Human Antigen R (HuR)

HuR is a well-characterized RBP that was first cloned and characterized in 1996 [186]. It belongs to the embryonic lethal abnormal vision phenotype (ELAV) family and is therefore also known as ELAV1 [186-189]. This family of RBPs comprises four members, HuR (also known as HuA), HuB, HuC, and HuD. While HuR is constitutively expressed, the other members of the family are primarily neuronal proteins. HuR is known to posttranscriptionally mediate gene expression through the regulation of mRNA splicing, stability, translation, and localization [163, 190-193]. HuR exerts these functions by associating, for the most part, to AREs and GREs (as described above) in the 3' untranslated region (UTR) of its target messages [170, 194-198]. HuR, nonetheless, has also been shown to bind regions in the 5'UTR and coding sequence to respectively control the translation and splicing of its mRNA targets [164, 180, 199-201]. Unlike most other RBPs that bind to AREs in mRNAs to negatively impact their expression [172, 187, 193, 202, 203], HuR has been shown to regulate a target mRNA positively or negatively. The differential ability of HuR to affect the expression of its target mRNAs is dependent on its association with other trans-acting factors, including miRNAs and protein ligands [152, 170-173, 176, 204, 205].

The ability of HuR to bind mRNAs is mediated by RNA-recognition motifs (RRMs). In fact, HuR contains three highly conserved RRMs named RRM 1-3. RRM1 and RRM2 preferentially bind AREs while RRM3 preferentially binds U-rich sequences [170, 194-196]. In addition to binding RNA, RRM3 was also shown to mediate the formation of oligomeric complexes of HuR proteins on target messages, which have

been suggested to interfere with the binding of trans-acting factors (RBPs and miRNAs) [195, 206]. Moreover, RRM3 can also mediate protein-protein interactions (HuR and RBM38 (RNA-binding motif protein 38) interaction to stabilize p21 mRNA), poly(A) binding, and stabilization of the HuR-RNA complex [171, 174, 195, 207, 208]. HuR also contains a 52-amino acid flexible hinge region (between RRM2 and RRM3) that includes the nucleocytoplasmic shuttling (HNS) domain (described below) [209]. The HNS is known to mediate both the import and export of HuR, in and out of the nucleus respectively [162, 193]. The export of HuR out of the nucleus occurs through the association of the HNS domain with adaptor proteins for nuclear export, such as pp32/PHAP-I, APRIL/PHAP-II, and CRM1. It has also been shown by our group that the import of HuR into the nucleus is mediated by its interaction with transportin-2 via the HNS [171, 181, 191, 210]. While HuR is predominantly nuclear, its shuttling allows it to bind to its target mRNAs in the nucleus and export them to the cytoplasm where they are actively translated. While bound to mRNAs in the cytoplasm HuR is able to influence the translation and/or stability of its target messages [162, 181, 191, 211].

1.4.1 HuR regulates pre-mRNA splicing in the nucleus

Studies have shown that the vast majority of pre-mRNAs contain exons that can be alternatively included or removed from the mature mRNA, and thus are alternatively spliced. The spliceosome complex, which is composed of 170 protein and five small nuclear ribonucleoprotein (snRNP) complexes, recognizes the splice sites in introns and assembles in a stepwise manner to mediate the cleavage and ligation reactions that are necessary for the removal of introns [212, 213]. Although the cytoplasmic translocation of HuR has been shown to be essential for its function, HuR has been shown to regulate precursor mRNA (pre-mRNA) splicing in the nucleus. Indeed, numerous studies have shown that HuR is involved in regulating mRNA splicing [206, 214, 215]. For example, HuR was shown to mediate the alternative splicing of the Fas pre-mRNA [206]. The alternative splicing of the Fas pre-mRNA, which results in a transcript that lacks exon 6, encodes a soluble form of the receptor which inhibits Fas signalling-induced apoptosis [216]. HuR was shown to prevent Fas splicing by interfering with the association of the splicing factor U2AF65 binding to the 3’ splice site region [206, 217]. In another study, HuR was shown to mediate the alternative splicing of eukaryotic

translation initiation factor 4E nuclear import factor 1 (Eif4enif1) mRNA, which encodes the eukaryotic translation initiation factor 4E transporter (4E-T) protein [218, 219]. The depletion of HuR was shown to induce the splicing of exon 11 in the *Eif4enif1* mRNA, leading to the expression of the short stable 4E-T isoform, which represses the translation of ARE-bearing mRNAs by targeting them to processing bodies for degradation [214]. In another example, HuR was shown to regulate the splicing of nuclear factor erythroid 2-related factor (*Nrf2*), a transcription factor that transcribes stress-related defense genes in response to oxidative stress [215, 220]. The knockdown of HuR, under these conditions, resulted in the decreased expression of Nrf2 due to the accumulation of unspliced, unstable, premature *Nrf2* mRNA in the nucleus.

1.4.2 HuR mediates the nuclear export of target messages

The nucleocytoplasmic shuttling capability of HuR allows it to transport target mRNAs from the nucleus to the cytoplasm. The transport of mature mRNAs out of the nucleus and their localization to the cytoplasm enables the translation of mRNAs in a spatial-temporal-specific manner. It also, in addition, facilitates the sorting of proteins to organelles, since mRNAs coding for mitochondrial, endoplasmic reticulum, and peroxisomal proteins are enriched at these organelles through harbored cis-elements [221]. HuR was previously shown to act as an adapter that binds target mRNAs and interacts with export proteins to enable the transport of mRNAs to the cytoplasm [163, 171, 222]. The shuttling of HuR and associated mRNAs seem to occur via two distinct pathways that are dependent or not on the nuclear pore receptor CRM1 (chromosome region maintenance 1). The first pathway involves the interaction of HuR with the nuclear phosphoprotein ligands, pp32 and APRIL, which contain Nuclear Export Signals (NES) [163, 171]. The binding of this complex to CRM1 mediates the export of HuR out of the nucleus [163, 171, 223]. Although CRM1 inhibition does not impair the nuclear export of HuR, it increases its binding to pp32 and APRIL, where approximately 50% of HuR is bound by them, as well as its binding to poly(A) containing mRNAs in both the nucleus and cytoplasm, whereas in normal conditions the binding of HuR to these mRNAs occurs only in the cytoplasm. This suggests that the interaction of HuR with these protein ligands modulates its interaction with target mRNAs. Interestingly however, leptomycin B treatment caused the selective retention of the ARE-containing

c-fos mRNA, an early response gene whose mRNA is a target of HuR, in the nucleus. This led the discovery of the second HuR shuttling pathway that is CRM1-independent [163].

The other pathway, which occurs in a CRM1 independent manner, is thought to be dependent on the binding of HuR to Transportins 1 (TRN1) and 2 (TRN2) [224]. The nuclear import of HuR by transportins was shown to be mediated by the HNS, which is a sequence that bears similarity to the M9 shuttling sequence of heterogeneous nuclear ribonucleoproteins A1 (hnRNP A1) [224, 225]. Several studies have shed light on the regulation of the localization of HuR, and through one of these studies, HuR was found to interact with the nuclear import adaptor protein importin α1 [226]. This interaction was found to be regulated by the AMPK-mediated phosphorylation of importin α1, and the AMPK-dependent-p300-mediated acetylation of importin α1 [226, 227]. Indeed, mutating the phosphorylation (S105) or acetylation (K22) sites of importin α1 resulted in the cytoplasmic localization of HuR [226]. The localization of HuR was extensively shown to be regulated by phosphorylation, as will be explained later in Section 1.5 [198, 209, 228-232]. The nuclear import of HuR is associated with reduced stability of target messages, supporting the role of HuR as an adaptor protein which escorts target ARE-containing mRNAs from the nucleus to the cytoplasm [158, 183, 233].

The nuclear export of HuR and the HuR-mediated cytoplasmic trafficking of mRNA cargo were recently shown to be dependent of actin and microtubules cytoskeleton [234-236]. Indeed, disruption of cytoskeleton transport systems was shown to prevent the cytoplasmic accumulation of HuR [234-236]. Additionally, it has been shown that RNA transport by HuR can be achieved by the association of HuR and a subset of target mRNAs with a microtubule-associated protein, and this complex in turn can associate to polysomes [237]. The association of HuR with the cytoskeleton (which mediates the trafficking of mRNA targets to polysomes) was shown to be dependent on its phosphorylation on the S318 residue. The phosphorylation of HuR at this residue mediates its interaction with the cytoskeletal protein myosin IIa (via RRM3) in an RNA-dependant manner [234]. The treatment of cells with microfilament inhibitors, additionally, was shown to decrease the expression/stability of HuR mRNA targets due

to the decreased recruitment of these messages to free cytoplasmic polysomes and polysomes associated with the endoplasmic reticulum.

1.4.3 HuR mediates the mRNA turnover of target messages

The stabilization/decay and translation of mRNAs are closely related to their localization to the cytoplasm. HuR is a well-established posttranscriptional regulator of mRNA turnover. The rate of decay of an mRNA, which is also called “mRNA turnover”, is an important process that determines the abundance of mRNA in a cell at any given time. In order to establish rapid responses to certain stimuli, there is differential turnover of mRNAs whereby those with shorter half-lives (a measure of the longevity of a mRNA) respond more rapidly to changes in transcription. mRNA decay is achieved by the orchestrated interactions between the mRNA structural components with trans-acting factors [238]. Such components include the 3’ and 5’-UTRs, the 5’cap, the poly(A) tail, as well as the coding region. Various studies have shown that deadenylation (removal of the poly(A) tail) is the first step in many mammalian mRNA decay pathways. During this process, enzymes such as poly(A) ribonuclease (PARN) shorten the poly(A) tail [239, 240]. The decapping of the 5’end constitutes another major decay pathway and is mediated by enzymes such as DCP1-DCP2 [238]. Importantly, the 3’UTR is also well known for its role in regulating the stability and decay of mRNAs due to the presence of cis-elements including AREs. Approximately 5 to 8% of human genes code for mRNAs that contain AREs and most ARE-binding proteins identified promote the decay of these mRNA [241, 242]. As mentioned above, HuR is a master regulator of the stability of mRNA containing AREs. The importance of the role of HuR in mRNA stability is underscored by the fact that the overexpression of HuR was shown to increase the half-life of many short-lived mRNAs [187].

One mechanism through which HuR stabilizes mRNAs is by protecting the message from degradation by preventing the binding of RBPs that trigger the decay of the message. In fact, HuR has been shown to compete with RBPs that promote decay of mRNA, including KSRP, TTP, and AUF1 which associate with the exosome to promote the degradation of ARE-containing mRNAs [243]. For example, in response to cytokine treatment, HuR was shown to compete with KSRP for the binding of the *iNOS*

message, thereby promoting its stabilization by preventing its KSRP-mediated decay in DLD-1 cells (colorectal adenocarcinoma cells) [244]. In fact, increasing the amount of HuR resulted in decreased binding of KSRP to the transcript, and vice-versa [244]. One mechanism through which HuR is known to compete with these RBPs is by binding its target messages and forming oligomers via its RRM3 and hinge regions, thereby physically hindering other RBPs from binding [245]. One such example is the binding and cooperative assembly of HuR oligomers on the ARE of the *TNFα* mRNA [195]. HuR was found to form oligomers on an ARE sequence that is bound by AUF1, which causes its destabilization, suggesting that HuR protects TNFα mRNA from decay by oligomerizing and competing with AUF1 for the binding of the message [232, 246]. This study also showed that the HNS contributes significantly to the binding energy in the formation of HuR:ARE complexes in a length dependent manner, requiring an ARE of at least 18 nucleotides. In addition, HuR was shown to compete with TTP to override the destabilization of the *TNFα* mRNA by binding an ARE in the 3'UTR of *TNFα* mRNA in macrophages [247].

Although HuR is widely regarded as a positive regulator of mRNA stability, several studies have shown that it may also cooperate with decay promoting RBPs to promote the decay of target mRNAs. HuR was shown to cooperate with AUF1 to promote the decay of *p16^{INK4}* mRNA [173]. In this case, the destabilization seemed to depend on stem loop structures found in the 3'UTR of the *p16^{INK4}* mRNA that bound both AUF1 and HuR. The HuR/AUF1 complex, consequently, recruited the RNA-induced silencing complex (RISC) to mediate the decay of the message. Therefore, it appears that the interaction of HuR with other RBPs and the impact of that interaction on its function is complex and is probably regulated by other factors, such as posttranslational modifications or trans-acting factors.

1.4.4 HuR mediates the translation of target messages

Numerous studies reported the function of HuR in promoting translation of target mRNAs. In fact, it has been shown that a substantial fraction of cytoplasmic HuR is associated with polysomes, further supporting its role in translation [222]. Translation is the step in which mRNAs are translated into proteins. Eukaryotic mRNAs contain four

structural elements that are important for their translation [248]. First, the 5'end of cellular mRNAs is capped with 7-methylguanosine (5' m7G), which interacts with eIF4E to enhance ribosome binding to the mRNA [249-251]. The second structure is the sequence flanking the AUG start codon, such as the GCCRCC motif which precedes the AUG codon, and which optimizes the positioning of the ribosome on the AUG codon and augments translation initiation [252]. The third structure is the position of the AUG codon relative to the 5'end of the mRNA, where translation favorably initiates at the AUG codon that is closest to the 5'end [253]. The fourth structure are the secondary structure within the mRNA leader sequence, such as the secondary structures that form by the GC-rich sequence of the 5'UTR and block ribosome entry [254-256].

Numerous studies have demonstrated the ability of HuR to regulate the translation of target mRNAs, although much remains unclear about the mechanisms driving this function of HuR. It was previously shown that HuR synergizes with TIA-1 to inhibit the translation of *TNFα* mRNA through the binding of an ARE in the 3'UTR of the message in bone marrow-derived macrophages [247]. Conversely, HuR was also shown to compete with TTP to initiate the translation of *TNFα* mRNA, where phosphorylation of TTP decreases its affinity to the mRNA and allows HuR to compete for the binding during immune responses in macrophages [232]. In addition to the binding of the 3'UTR of target messages, HuR has been shown to regulate translation through the binding of the 5' UTR of mRNAs. As previously mentioned, one of the mechanisms through which HuR positively regulates the translation of target mRNAs is by recruiting them to polysomes. In fact, HuR was shown to bind the 3'UTR of *p53* and *cytochrome c* mRNAs, thereby increasing their recruitment to polysomes and their translation [176, 257]. Similarly, HuR was shown to bind the 5'UTR of hypoxia-inducible factor 1α (HIF-1α) mRNA, leading to its enhanced association with polysomes [201]. Conversely, HuR was also shown to suppress translation of target mRNAs. So far, the best characterized examples for the translation repression function of HuR involves its binding to the 5'UTR of target mRNAs. For example, the binding of HuR to the 5'UTR of the caspase-2 transcript represses its translation in adenocarcinoma cells, which leads to a resistance to apoptosis [258]. HuR was also shown to bind an U-rich element in the 5'UTR of the p27 mRNA, leading to its repressed translation [199]. Interestingly,

HuR can also inhibit the cap-independent translation of p27, which is mediated by the internal ribosome entry site (IRES) in the 5'UTR of the message [164]. HuR is also able to repress both IRES- and cap-dependent translation of the insulin-like growth factor I receptor (IGF-IR) [200]. However, it seems that IRES-mediated translation is permanently blocked by HuR, while the cap-dependent inhibition is transient. Intriguingly, HuR was found to stimulate the IRES-mediated translation of the intrinsic cellular caspase inhibitor XIAP by recruiting it to polysomes, as part of its role in cell survival regulation [180].

Therefore, the role of HuR in regulating translation is complex and mechanisms governing this process require further investigation.

1.4.5 MicroRNAs modulate the function of HuR

Of note, various studies have shown that the interplay between HuR and microRNAs (miRNAs) regulate the stability and translation of target mRNAs [259]. MicroRNAs (miRNAs) are conserved small non-coding RNA regions that are approximately 22 nucleotides long, and originate from intergenic or intragenic regions [260]. RNA polymerase II transcribes them as primary miRNAs which contain a 5' cap and a 3' poly(A) tail (Figure 1.3). They base pair with themselves and form stem-loop structures that are then processed by a microprocessor complex DGCR8/Drosha to form an intermediate precursor (pre-miRNA). This intermediate is transported to the cytoplasm by exportin 5, where it is further processed by the RNase III endonuclease Dicer1 and the double strand RBP TARBP2 to produce the double stranded miRNA. The miRNA is then unwound by helicases, where one strand is degraded, and the other strand is transferred to the RISC. In this complex, argonaute 2 (AGO2) serves as the catalytic engine for RNA interference [261].

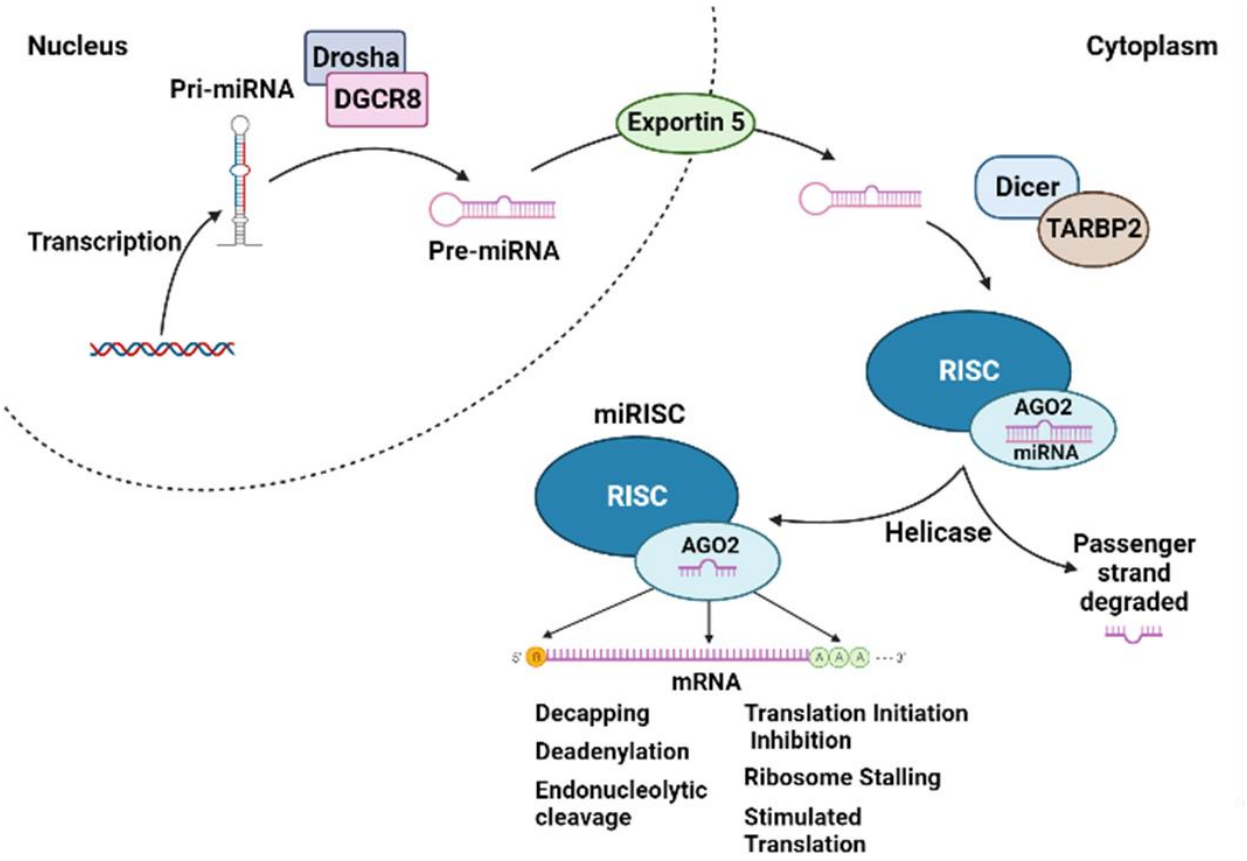


Figure 1.3. miRNA biogenesis.

miRNAs are transcribed by RNA polymerase II from intergenic or intragenic regions as primary miRNAs which contain a 5'cap and a 3' poly(A) tail [260]. They base pair to form stem-loop structures that are processed by the microprocessor complex DGCR8/Drosha, forming an intermediate precursor (pre-miRNA). The pre-miRNA is transported to the cytoplasm by exportin 5. RNase III endonuclease Dicer1 and TARBP2 process the pre-miRNA into the double-stranded miRNA. Helicases unwind the miRNA. One strand is degraded (passenger strand), while the other strand is transferred to the RNA-induced silencing complex (RISC), forming the miRISC. The miRISC regulates mRNA turnover by decapping, deadenylation, or endonucleolytic cleavage, and regulates translation by inhibiting translation initiation, ribosome stalling, and stimulating translation.

MiRNAs have mostly been shown to function as negative posttranscriptional regulators of gene expression through the interaction with mRNAs, resulting in mRNA degradation or inhibition of mRNA translation [262]. However, miRNAs have also been shown in some instances to promote translation by increasing ribosome loading to

target mRNAs [263, 264]. miRNAs can target various mRNAs and conversely, a single mRNA can be targeted by several miRNAs. miRNAs bind complementary sequences, named seed elements, that are usually present in the 3'UTR of RNA, but can also be present in the 5'UTR or the coding sequence of target mRNAs. As such, miRNAs can regulate mRNA turnover and translation in various ways, such as by promoting the deadenylation or decapping of mRNAs, by inhibiting the assembly of ribosome subunits or by causing ribosome stalling.

Studies have shown that AGO2, a component of the RISC that mediates endonucleolytic cleavage of target mRNAs, shares numerous mRNA targets with HuR, suggesting that HuR and miRNAs compete/collaborate to regulate gene expression [265, 266]. The first report evidencing this interplay showed that HuR competes with miR-122 for the binding to the 3'UTR of the cationic amino acid transporter 1 (CAT-1) mRNA [267]. Under normal conditions, miR-122 base-pairs with and represses the translation of the CAT-1 message, which is sequestered in processing bodies (P bodies). During starvation-induced stress, HuR binds an ARE present in the 3'UTR of the message which leads to the release of the mRNA from P-bodies and its translation. Other studies showed that HuR competes with miRNAs to promote the expression of *COX2, GM-CSF, TOP2A, NCL*, and *ERBB2* mRNAs [268-272]. These studies show that HuR and the miRNAs compete with each other since the HuR binding site and the miRNA seed element overlap. miRNA seed elements, however, do not necessarily overlap with the binding site of HuR for the competition to occur. Furthermore, it was suggested that while overlapping miRNA and HuR binding sites result in direct competition for the regulation of the target message, non-overlapping binding of HuR and the miRNAs can drive competition through steric hinderance or hinderance involving changes in the secondary structure of the mRNA [259, 273]. In addition, the oligomerization of HuR (through the HNS-RRM3 region) was shown to be important for its function in interfering with the miRNA-mediated translation inhibition of mRNAs [274]. Oligomerization-competent HuR can displace already bound RISC from the RNA target. As such, although HuR does not interact with the RISC, its ability to oligomerize allows it to get near the complex and interfere with it. The oligomerization of HuR was also

shown to be important for the HuR-mediated alleviation of the deadenylation of RNA by miRISC.

Conversely, HuR was shown in several instances to collaborate with miRNAs for the repression of mRNA stability and translation [259]. For example, HuR was shown to bind the 3'UTR of the *c-Myc* mRNA and promote the recruitment of let-7 miRNA (whose seed sequence is nearby the HuR-binding site) to repress the translation of the message [177]. In a similar instance, although the binding of HuR to the *RhoB* mRNA is distal to the binding of miR-19 to its seed element, the association of HuR promotes the recruitment of the miRNA to the message resulting in translational repression [275]. Importantly, in both examples, HuR facilitated the recruitment of the miRNAs leading to the translational repression of its mRNA targets [177, 275].

1.4.6 HuR is involved in various cellular processes

The aforementioned functions of HuR lead to the regulation of various cellular processes such as cell proliferation, apoptosis, differentiation, as well as adipogenesis and response to stress and immune agents [158, 165, 178, 181-183, 222, 276-278]. HuR has been implicated in several disease processes including cancer and chronic inflammation as well as cardiovascular, neurological, and muscular associated pathologies [279-281]. Furthermore, the importance of HuR is underscored by the fact that HuR knockout mice are embryonic lethal [282]. Experiments involving HuR knockout mice have shown the importance of HuR in the early repair of damaged mucosa [283], spermatogenesis [284], angiogenesis [205], motor neuron cells survival and proliferation [285], and muscle function [149]. Although HuR is ubiquitously expressed, its expression is higher in brain, muscle and thymus tissues [186].

1.4.6.1 HuR regulates both cell survival and cell death genes

HuR is overexpressed in various cancers, including breast, ovarian, liver, gastric, esophageal, pancreatic and colorectal cancer, which is why its role in cell survival and death is actively studied [158, 185, 286-291]. HuR is known to regulate the expression of genes that promote cell survival in certain conditions, while it regulates the expression of pro-apoptotic genes in other conditions. This paradoxical role has been a popular area of research, due to its relevance in cancer. Numerous studies have

revealed that HuR has a pro-apoptotic role via the regulation of genes that include *c-myc, caspase-9, Bax, p53* and *p27* [182, 186, 257, 292]. More extensive studies on HuR later demonstrated that it is also involved in promoting cell survival through the regulated expression of anti-apoptotic genes as well, such as *Bcl-2, Mcl-1, cyclin A, cyclin B1, cyclin D1, p21*, and *prothymosin α* [178, 179, 182, 186]. This paradox in the function of HuR can be explained by the fact that although HuR has a pro-survival function during mild stress conditions, it promotes cell death if the stess reaches a lethal stage. Our group noticed that in response to lethal stress, HuR undergoes caspase-3 and -7 mediated cleavage, resulting in the generation of two cleavages products (CPs), named HuR-CP1 and -CP2 which promote cell death (Figure 1.4) [182]. The cleavage of HuR, therefore is responsible for the functional switch whereby HuR differentially affects cell fate under mild or lethal conditions. In fact, the cleavage products of HuR (HuR-CP1,2) have been shown to favour one fate (cell death) over the other (cell survival), by preferentially binding pro-apoptotic target mRNAs, such as *caspase-9*, rather than binding anti-apoptotic target mRNAs, such as the anti-apoptotic effector *prothymosin-α* [179, 280]. The mechanism of HuR cleavage and the resulting HCPs were found to have an important effect over the activation of the apoptosome, resulting in an amplification of its activation [181]. Indeed, the addition of exogenous HuR-CPs to cells induce apoptosis under non-lethal stress conditions [182]. Subsequent studies have shown that in cancers where HuR is overexpressed, HuR fails to be cleaved further confirming the importance of the cleavage of HuR in its pro-apoptotic function [293, 294].

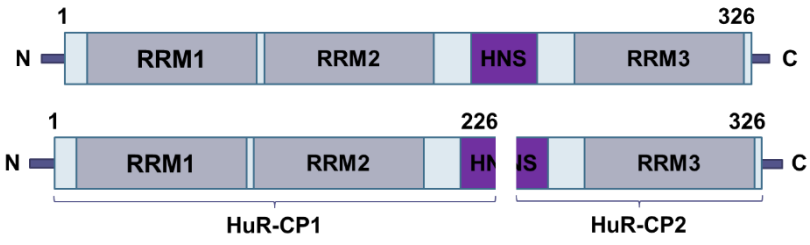


Figure 1.4. Schematic of the structure of HuR.

(*Top panel*) HuR contains three RNA recognition motifs (RRMs) and a hinge region (HNS) containing the HuR nucleocytoplasmic shuttling sequence (located between RRM2 and RRM3) [295-297]. Its coding region contains 326 amino acids [295]. (*Bottom panel*) HuR can be cleaved at residue aspartate 226 located in the hinge region, generating two cleavage products: HuR cleavage product 1 (HuR-CP1) and HuR-CP2 [298].

1.4.6.2 HuR regulates inflammatory responses

The activity of HuR in promoting inflammation is another actively studied area of research [281]. One of the rare instances where an increase in the expression of HuR was noted is in activated T cells [299, 300]. Indeed, the expression of HuR was shown to increase in activated and TH17 polarized $CD4^+$ T cells [299, 300]. HuR has been shown to associate to mRNAs encoding pro-inflammatory cytokines and chemokines, such as TNFα, IL-6, IL-8, TGF-β, and IFNγ, and upregulate their expression in various cell types [161, 281, 301-306]. Additionally, HuR binds and stabilizes *COX-2* and *iNOS* mRNAs, which are major pro-inflammatory mediators. HuR was shown to stabilize *COX-2* mRNA in macrophages, renal mesangial cells, and various cancer cells [161, 307-309]. Similarly, HuR was shown to stabilize the *iNOS* mRNA, leading to the production NO in muscle cells (described below) and hepatocytes as well as lung and colon cancer cells [104, 244, 310]. The importance of HuR in promoting inflammation is further evidenced by the fact that anti-inflammatory cytokines, such as IL-10 and IL-19, were shown to repress the function of HuR [311-313]. In fact, while HuR expression increased in the myocardium of mice with myocadial infarction (MI), and LPS-treated U937 monocytes, IL-10 treatment dramatically reduced HuR protein levels, leading to the reduction of HuR-target pro-inflammatory cytokines expression, such as TNFα [311, 312]. This resulted in attenuated myocardial inflammation, leading to improved left ventricular function and remodeling by inhibiting fibrosis [311]. On the other hand, in injured vascular smooth muscle cells, IL-19 suppresses the function of HuR by reducing its abundance and its PKCα-mediated serine phosphorylation, preventing its localization to the cytoplasm. These effects resulted in the reduced pro-inflammatory response that is deleterious in vascular pathophysiological processes [313]. The importance of HuR in immunological processes is also demonstrated by its implication in various diseases [281]. For instance, HuR is involved in arthritis through the upregulation of TNFα and COX-2 expression [314-316]. HuR-mediated COX-2 upregulation has also been linked to inflammatory bowel disease [317]. HuR was also associated to asthma through its ability to upregulate the expression of TNFα and GM-CSF [318].

In addition to inflammatory responses, HuR is well-established as key mediator of skeletal muscle homeostasis under both normal and inflammation-induced

conditions. HuR has been shown to regulate the expression of many of the aforementioned key factors to drive the myogenic process or promote cancer-induced muscle wasting. Intriguingly, HuR regulates genes involved in stopping cell proliferation, and those involved in promoting differentiation. In the section below, I provide a literature review of what is known about the function of HuR in myogenesis.

1.4.7 The role of HuR in myogenesis

The role of HuR in skeletal muscle fiber formation has been extensively studied *in vitro* and *in vivo*. In fact, HuR has been proven to be crucial for the myogenic process to occur and its depletion inhibits muscle fiber formation [146, 152, 319-321]. Moreover, myogenesis is one of the few processes where the expression of HuR is increased [183, 322]. In fact, the protein expression of HuR was found to increase during *in vivo* regeneration, and this increase was later found to be affected by methylation [183, 323]. HuR is involved in both cell cycle withdrawal and differentiation of myoblasts. Through its various functions, it promotes the expression of factors required for these two processes, and the downregulation of factors involved in cell cycle promotion.

1.4.7.1 The role of cytoplasmic translocation of HuR in myogenesis

The cytoplasmic accumulation of HuR during myogenesis is essential for its pro-myogenic function. The localization of HuR to the cytoplasm is correlated with an augmented stabilization of MRFs and improved myogenic phenotype [6, 319, 324]. Our group showed that, during myogenesis, HuR is cleaved by caspase-3 on an aspartic acid residue (D226) located in the hinge region (Figure 1.5) [181]. This cleavage results in the generation of HuR-CP1 and -CP2, and is responsible for the cytoplasmic accumulation of HuR during muscle cell differentiation [324]. The accumulation of HuR in the cytoplasm occurs by the competition of HuR-CP1 with the full length HuR for the binding to TRN2. Indeed we demonstrated that HuR-CP1 has higher affinity for TRN2, since mutating the D226 residue (HuR_{D226A}) into an alanine, such that it cannot be cleaved by caspase-3 did not disrupt the interaction of HuR-CP1 and transportin-2 [6, 324]. Importantly, the HuR_{D226A} mutant failed to rescue myogenesis after the depletion of HuR, further underscoring the importance of the cleavage of HuR in its pro-myogenic function [6]. In doing so, HuR-CP1 interferes with the ability of TRN2 to import HuR

back to the nucleus. This therefore allows HuR to retain its association to its target mRNAs, thus stabilizing them, in the cytoplasm (Figure 1.5) [324].

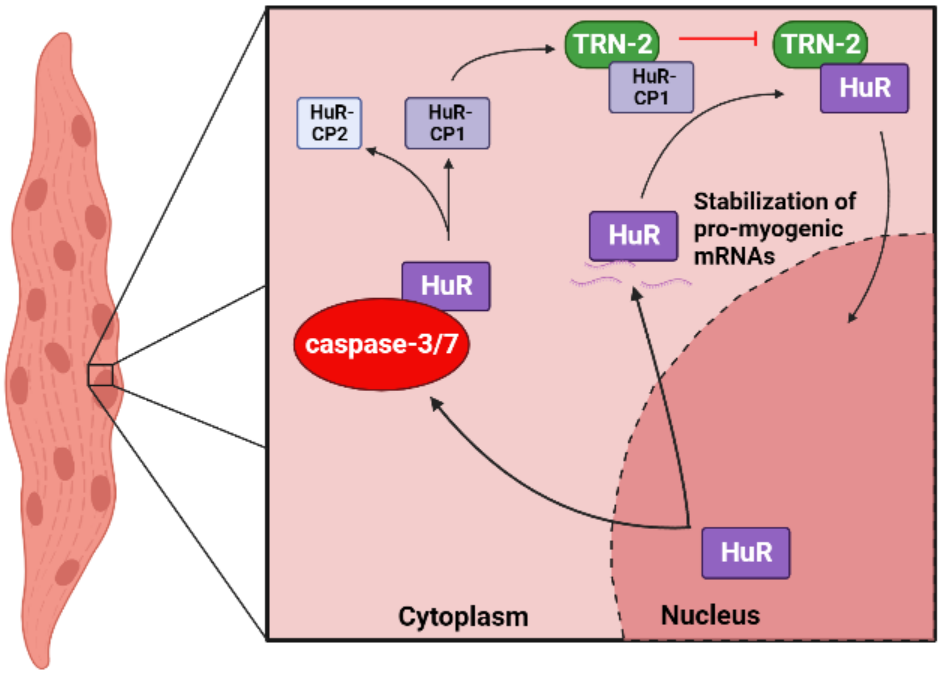


Figure 1.5. The cleavage of HuR is important for its function in myogenesis. HuR is a shuttling RBP that mediates RNA stability in the cytoplasm and gets imported back to the nucleus by transportin-2 (TRN2) [324]. During myogenesis, HuR gets cleaved by caspase-3 and caspase-7, generating the two cleavage products, HuR-CP1 and HuR-CP2. HuR-CP1 competes with HuR for the binding of TRN2, which results in the accumulation of HuR in the cytoplasm where it stabilizes pro-myogenic messages. This event is essential for the pro-myogenic function of HuR.

1.4.7.2 The role of mRNA stabilization and decay by HuR in myogenesis

The best-known function of HuR in myogenesis is the stabilization of pro-myogenic messages. In fact, HuR has been shown to regulate the stability of *p21*, *MyoD*, and *myogenin* mRNAs (Figure 1.6) [146, 183, 325]. During muscle fiber formation, HuR binds AREs in the 3'UTRs of these target MRFs mRNAs to regulate their stability. Indeed, overexpression of HuR was shown to increase the stability of *p21*, *MyoD*, and *myogenin* mRNAs upon induction of muscle cell differentiation [147, 183]. Since these messages are targeted for decay by the RBP KSRP, it is believed that HuR protects them by negating the action of KSRP [326].

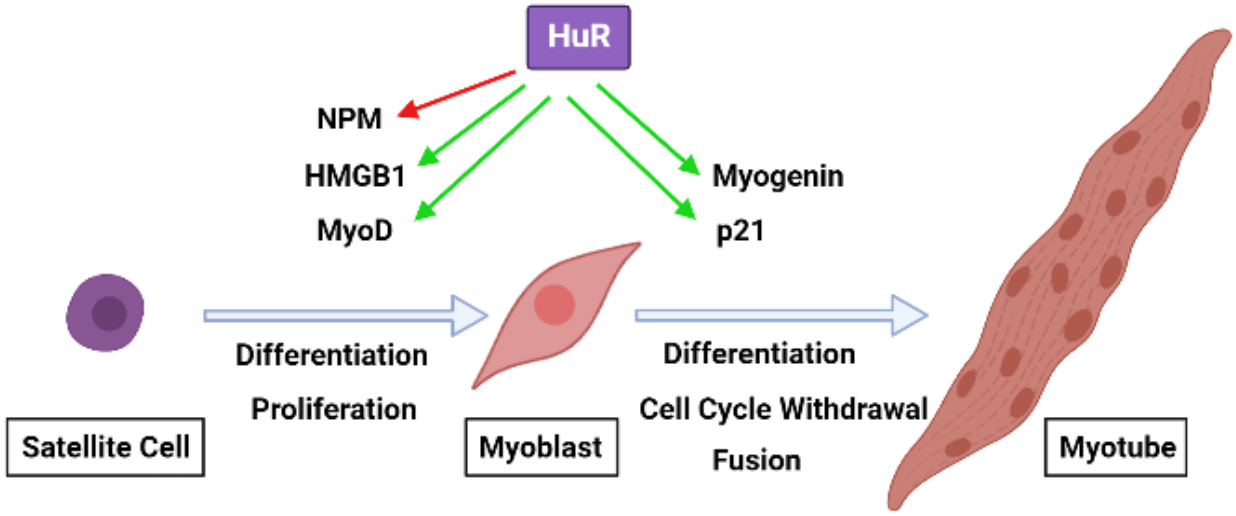


Figure 1.6. The role of HuR in regulating myogenesis.

HuR promotes myogenesis by increasing the translation of the HMGB1 mRNA inducing the decay of the NPM mRNA during the early stages of myogenesis as well as increasing the stability of p21, MyoD, and myogenin upon induction of muscle differentiation. [152, 183, 325].

Recently, HuR was found to regulate, in part, the stability of MyoD and myogenin mRNAs by interacting with the long non-coding RNA (lncRNA) *lncMGPF*, which is enriched in skeletal muscles [327]. *LncMGPF* was the highest of 19 enriched lncRNA found in a microarray screen of lncRNA correlating with the expression of MyoD in differentiating C2C12. Its knockdown decreased the mRNA stability of *MyoD*, *myogenin*, *MyHC*, and *β-1 integrin*, leading to the decrease of their mRNA and protein levels, resulting in reduced fusion of myoblasts. Overexpression of this lncRNA increased the expression of these mRNAs. Importantly, genetic ablation of *lncMGFP* in mice decreased skeletal muscle growth, function (contractility and endurance), and regeneration (following cytotoxin-induced injury in the TA muscle). HuR was identified as a *lncMGPF* binding protein. Knockdown of HuR negated the effect of *lncMGPF* overexpression on *MyoD* and *myogenin* mRNA stability. Inversely, the knockdown of *lncMGPF* significantly decreased the ability of HuR to bind MyoD and myogenin mRNAs. Further investigation showed that the knockdown of *lncMGPF* reduced the cytoplasmic accumulation and the cleavage of HuR, which might explain how it affected the expression of these mRNAs and, furthermore, the differentiation of muscle cells.

HuR is also implicated in the cell cycle arrest of differentiating myoblasts by stabilizing the *p21* mRNA resulting in its increased expression [183, 325]. Similar to MRFs, HuR binds the 3'UTR of *p21* mRNA, increases its nuclear export and stabilizes its mRNA.

HuR was also shown to regulate proliferation of myoblasts by interacting with the transcription factor paired-related homeobox 2 (Pitx2), which promotes cell proliferation [328, 329]. While Pitx2 promotes the expression of cell cycle genes through transcriptional regulation, its has been shown to be involved in regulating the mRNA stabilization of its own and other genes, including *cyclin D1* mRNA, by interacting with HuR [330]. Similarly, in myoblasts, HuR and Pitx2 were found to interact and stabilize the mRNA encoding *cyclin D1*, which is a growth regulating factor, thereby promoting cell proliferation [328]. However, during differentiation, Pitx2 is phosphorylated by AKT, leading to the dissociation of the complex from the *cyclin D1* mRNA and decay of the message. Our group has recently shown that during early differentiation, HuR mediates myogenesis by interacting with the decay factor KSRP to regulate the stability of the *nucleophosmin (NPM)* mRNA (Figure 1.6) [172]. The complex regulates the expression of NPM, a known regulator of the cell cycle, by recruiting the deadenylase poly(A)-Specific Ribonuclease (PARN) and the exosome to destabilize *NPM* mRNA. Disruption of this complex by the knockdown of either HuR or KSRP resulted in the diminished binding of the other to *NPM* mRNA.

In addition to muscle-related genes, HuR was found to regulate the expression of acetylcholinesterase (AChE), which is an enzyme found at postsynaptic neuromuscular junctions in muscles and nerves to terminate neurotransmission at cholinergic synapses of the central and peripheral nervous systems [331]. The insufficient expression of AChE impaired skeletal muscle activity in myasthenic syndrome patients [332, 333]. Additionally, AChE expression is upregulated during muscle differentiation prior the occurrence of nerve-muscle interactions [334-336]. The increased expression of AChE was found to occur posttranscriptionally at the stability level due to augmented binding of HuR to AREs in the 3'UTR of its mRNA [331].

1.4.7.3 Regulation of mRNA translation by HuR in myogenesis

HuR was also shown to enhance the translation of the high mobility group box protein 1 (Hmgb1) during the early stages of myogenesis (Figure 1.6) [152]. Hmgb1 is a cytokine secreted by injured tissues to initiate a cascade of events that upregulate MyoD and myogenin expression. Our group showed that HuR regulates the translation of the Hmgb1 mRNA by negating the action of miR-1192. HuR binds to the *Hmgb1* mRNA in proximity to the miR-1192 seed sequence in the 3'UTR of the transcript. Although both HuR and miR-1192 can bind the mRNA simultaneously, the binding of HuR prevents the recruitment of AGO2 by miR-1192, which favors the translation of the *Hmgb1* mRNA.

1.4.7.4 The *in vivo* role of HuR in myogenesis

Recent studies on the *in vivo* role of HuR in skeletal muscle homeostasis/function expanded our knowledge about the multifunctionality of HuR in this cellular process. For instance, HuR was shown, in skeletal muscle, to regulate lipid metabolism [337, 338]. Indeed knocking out HuR specifically in skeletal muscle, using the Cre recombinase system under the control of the myosin light chain 1 fast (*Mlc1f*) promoter (which is expressed in the late stages of myogenesis) increased fat mass as well as blood glucose and basal insulin levels, indicative of insulin resistance [338]. Further investigation indicated, in these mice, a decrease in the levels of genes involved in skeletal muscle fatty acid metabolism and oxidative phosphorylation. Interestingly, the aforementioned effects were shown to be sex-specific as these were observed in male but not female mice. A later study by the same group showed that female knockout mice, unlike their male counterparts, have enhanced cellular clearance of glucose [339]. Their observations suggest that the increase in fat mass noted in male HuR knockout mice is a driver of decreased glucose clearance, and therefore glucose and insulin resistance. This effect is not observed in female animals due to their metabolic flexibility, where they are able to switch from lipid oxidation to carbohydrate metabolism more readily. This difference was attributed to the fact that the levels of expression of the metabolic regulator peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1α) was affected in males but not female mice. It is important to note that both studies use mice where HuR is knocked out under the control of *Mlc1f*, which means

that these mice lack HuR in already formed skeletal muscle fibers, and express HuR in differentiating SCs and myoblasts/myocytes. Therefore, these studies provide an understanding of the role of HuR in skeletal muscle tissue, beyond its well-established role in skeletal muscle cell differentiation.

Recently, our group generated an *in vivo* muscle specific HuR knockout mouse model to study the function of HuR during the early steps of skeletal muscle formation [149]. These muscle-specific knockout of HuR (muHuR KO) were generated using the Cre-LoxP system by crossing mice containing floxed ELAVl1 alleles (Elavl1$^{fl/fl}$ allele) with mice expressing Cre recombinase under the control of the *MyoD* promoter. We demonstrated in generating these mice that genetic ablation of HuR, under these conditions, increased endurance and oxidative metabolic capacity. Further investigation showed that HuR is involved in the promoting the decay of the *PGC1-α* mRNA in a KSRP-dependent manner. These results were similar to those described above in regard to the function of the HuR/KSRP complex in the regulation of NPM expression [172]. The increased expression of PGC1α in these muHuR-KO mice favored the formation of type 1, oxidative fibers over type 2, glycolytic fibers (explaining the oxidative phenotype of these mice).

Interestingly, these muHuR-KO mice showed resistance to cancer cachexia-induced muscle wasting. This might be partially due to their muscle fiber type specification which was characterized by the increased formation of oxidative fibers which, unlike glycolytic fibers, are resistant to muscle wasting. As such, in addition to promoting skeletal muscle function and formation, HuR is also implicated in muscle atrophy, similar to its paradoxical role in cell death and survival.

1.4.8 The role of HuR in muscle-related diseases

The first evidence for the role of HuR in muscle wasting is the discovery of its regulation of the *iNOS* mRNA during the process [104]. It was shown that HuR translocates to the cytoplasm and stabilizes the *iNOS* mRNA by binding an ARE in its 3'UTR (Figure 1.7). This event selectively suppressed MyoD expression. In untreated C2C12 myotubes, HuR associates with *MyoD* mRNA, as reported previously [146, 183]. However, during TNFα and IFNγ treatment, which induces muscle wasting, HuR does

not associate with the *MyoD* mRNA, while it associates with the *iNOS* mRNA. Depletion of HuR in these cells resulted in a reduction of *iNOS* mRNA and protein expression, while the rescue of HuR expression by AP-GST-HuR rescues iNOS expression. Additionally, the activity of HuR itself was shown to be regulated by the AMPK activator AICAR. Treatment of cells with AICAR resulted in the decreased cytoplasmic localization of HuR and the inhibition of iNOS expression [340].

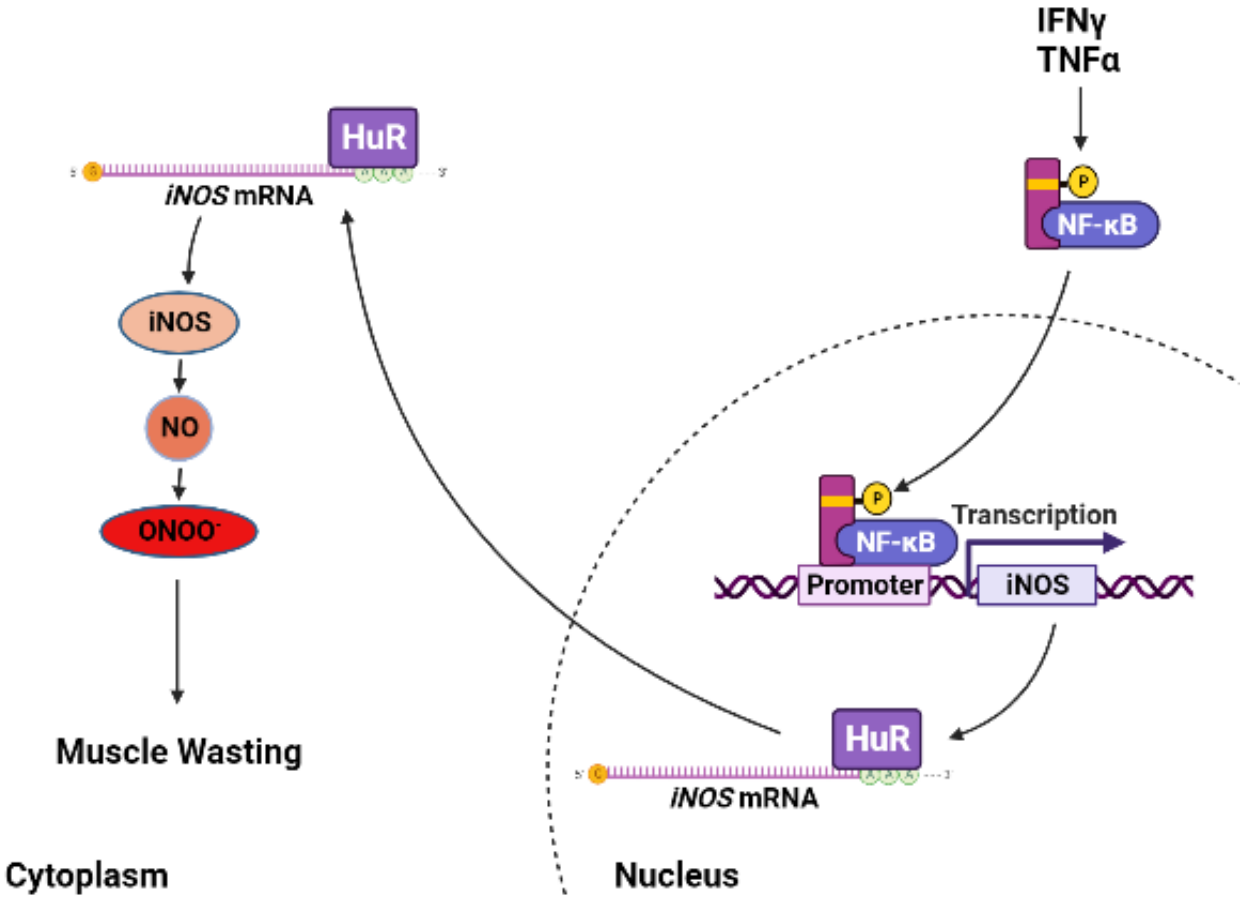


Figure 1.7. The role of HuR in regulating muscle wasting.

In response to cytokine treatment, the STAT3/ NFκB pathway is activated, leading to the increased transcription of iNOS [106]. HuR stabilizes the iNOS mRNA resulting in its increased expression, and the increased production of nitric oxide (NO) [104]. NO can react with reactive oxygen species (ROS) to form peroxynitrite (ONOO-), which triggers to muscle wasting.

Lately, more studies have demonstrated the implication of HuR in muscle-related diseases. In fact, HuR was shown to be involved in muscle wasting caused by the spinal muscular atrophy (SMA) disorder, which is caused by mutations or deletions in the survival of motor neuron 1 (*SMN1*) gene [341]. HuR was found to interact with SMN via the Tudor domain, which senses methylated arginine residues. Indeed, CARM1-mediated methylation of HuR was shown to be involved in this interaction. This pathway regulates the expression of HuR mRNA targets, namely *MyoD*, *myogenin*, *p21*, and *AChE* (acetylcholinesterase) and its receptor *AChR-β* (acetylcholine receptor-β subunit), in response to denervation *in vivo*. Consequently, in severe type 1 SMA, where the Tudor domain in SMN is mutated, the interaction of HuR and SMN is disrupted.

In another study, HuR was shown to be involved in oculopharyngeal muscular dystrophy (OPMD), which affects eyelid, pharynx, and proximal limb muscles [150]. Mutations in poly(A) binding protein, nuclear 1 (Pabpn1) causing it to sequester in toxic insoluble aggregates result in OPMD [342]. The lower basal level of Pabpn1 in skeletal muscle predisposes to be affected by these toxic aggregates compared to other tissues. HuR was identified as a negative regulator of the RNA and protein expression of Pabpn1 through its binding to an ARE, causing its lower expression in skeletal muscle [150].

Recently, it was shown that the lncRNA cachexia-related anti-adipogenesis lncRNA 1 (CAAlnc1) prevents HuR from regulating target messages during cancer-induced cachexia [145]. CAAlnc1 binds HuR through AREs and prevents it from binding target messages including the *C/EBPβ* and *PPARγ* mRNA, resulting in their decreased expression. These messages encode transcription factors that were previously shown to be implicated in adipogenesis [145, 343]. As such, the decreased binding of HuR to these mRNAs, due to CAAlnc1, decreased their expression resulting in a defective adipogenic phenotype.

1.5 The regulation of the function of HuR by posttranslational modifications

The mechanisms modulating the function of HuR in regulating opposite processes, including skeletal muscle formation and atrophy, remains elusive. Although numerous reports have shown that RBPs and miRNAs regulate the function of HuR in

skeletal muscle, the implication of posttranslational modifications might provide a potential explanation for the functional dichotomy of HuR. Several different modifications, including phosphorylation, methylation, cleavage (as described above), ubiquitination, and neddylation, have been shown to regulate the function of HuR [279]. These modifications of HuR have been shown to affect its protein levels as well as its localization and RNA binding activity in various cell models [209, 228, 230, 279, 341, 344, 345]. Of these modifications, the phosphorylation of HuR, on several residues (including tyrosine, serine, and threonine) has been extensively shown by several laboratories. For example, in human cervical carcinoma HeLa cells, phosphorylation of HuR by the G2-phase kinase CDK1 on the Ser202 residue promotes the interaction of HuR with 14-3-3, resulting in its nuclear localization [230]. Additionally, phosphorylation of HuR by PKC in renal mesangial cells allows it to localize to the cytoplasm and correlates with increased COX-2 mRNA stability [229]. As mentioned above, posttranslational modifications can also impact the interaction of HuR with target messages. For instance, in human colorectal cancer cells HCT116, phosphorylation of HuR by the cell cycle checkpoint kinase CHK2 upon IR treatment led to a global decrease in HuR association to mRNA [346]. Similarly, in HeLa cells, phosphorylation of HuR by CHK2 during hydrogen peroxide stress decreases its binding to the *Sirt1* mRNA resulting in the decay of the message [228].

Methylation has also been shown to modulate the function of HuR both during an inflammatory response and during muscle differentiation [323, 341]. Indeed, upon stimulation of macrophage cells with lipopolysaccharide (LPS), the coactivator-associated arginine methyltransferase 1 (CARM1) methylates HuR on the arginine 217 residue located in the hinge region. The methylation of HuR is suggested to regulate the ability of HuR to stabilize the *TNFα* mRNA [323]. In senescent human diploid fibroblasts (HDFs), CARM1-mediated methylation of HuR was also shown to promote the stabilization of *CCNA2, CCNB1, FOS*, and *Sirt1* target mRNAs [347]. Besides promoting the stabilization function of HuR, this methylation was shown to correlate with increased cytoplasmic accumulation of HuR in non-small cell lung carcinoma and was considered a prognostic marker [348]. In addition, as previously described, CARM1-mediated methylation of HuR was shown to be involved in its function in skeletal muscle

[341]. CARM1-mediated methylation of HuR on the arginine 217 residue regulates its levels and was required for the cytoplasmic localization of HuR in C2C12 cells. Mutating the arginine 217 residue to a lysine, such that HuR becomes non-methylatable, did not enhance myogenesis like the overexpression of WT HuR and constitutively methylated mutant (arginine to tryptophan) HuR did. These results suggest that methylation of HuR is involved in its pro-myogenic function. Methylation of HuR was also shown to be essential for the interaction of HuR with SMN, and in SMA, where this interaction is disrupted, there was an alteration of the expression of HuR mRNA targets, namely *MyoD, myogenin, p21*, and *AChE* and its receptor *AChR-β*, in response to denervation *in vivo*.

A more recently discovered modification of HuR named poly(ADP-ribose)ylation (PARylation) was also demonstrated to modulate the localization and the RNA-binding activity of HuR. In activated macrophages, poly(ADP-ribose) polymerase 1 (PARP1) PARylates HuR on the D226 residue and promotes its localization to the cytoplasm and its association to proinflammatory messages [349]. The PARylation of HuR was additionally shown to promote its oligomerization which promoted the dissociation of the RISC from its mRNA targets resulting in their increased stabilization [350].

1.5.1 PARylation

Although the role of the PARylation of HuR during muscle differentiation and wasting has never been investigated, the importance of this modification in skeletal muscle formation, function and wasting is underscored by the fact that this modification have been implicated in several muscle related diseases. PARylation is a dynamic posttranslational modification mediated by a large family of enzymes called poly(ADP-ribose) polymerases (PARPs). It is a reversible modification which can be hydrolyzed by poly(ADP-ribose) glycohydrolase (PARG), and other mono- and poly(ADP-ribose) hydrolases. It consists of a chain of ADP-ribose (ADPr) units. It was first described in 1963 by Paul Mendel as an adenine-containing RNA-like polymer which is the product of a nuclear enzymatic activity [351]. Later, it was discovered to be composed of an adenine diphosphate (ADP) molecule, linked to a ribose molecule. PARPs use NAD^+ as a substrate to transfer the ADP-ribose moiety into amino acid acceptors, and the

cleavage of NAD^+ during initiation is the rate limiting step of the reaction. There is a variety of amino acids that can be acceptors of pADPr modification, including glutamate, aspartate, lysine, arginine, and serine [352, 353]. Other amino acids including arginine, cysteine, threonine, histidine, tyrosine, and phospho-serine (through the phosphate group) have also been shown to be modified by PARylation. Therefore, it seems that PARylation or MARylation (mono(ADP-ribosyl)ation – modification with a single ADP-ribose moiety) do not have a preference for amino acids. It is rather the availability of a suitable amino acid that determines ADP-ribosylation [354].

PARylation can regulate the function of target proteins by changing their folding, consequently disrupting, or uncovering motifs, thereby preventing or promoting interactions (Figure 1.8 a). For example, the E3 ligase RNF146 contains a WWE (tryptophan, tryptophan, glutamate) PAR-binding motif, which when bound by PAR results in the conformational change of the RING domain. This modification thereby drastically increases its affinity to the ubiquitin-conjugating enzyme E2, from which it transfers ubiquitin to target proteins (Figure 1.8 b) [355, 356].

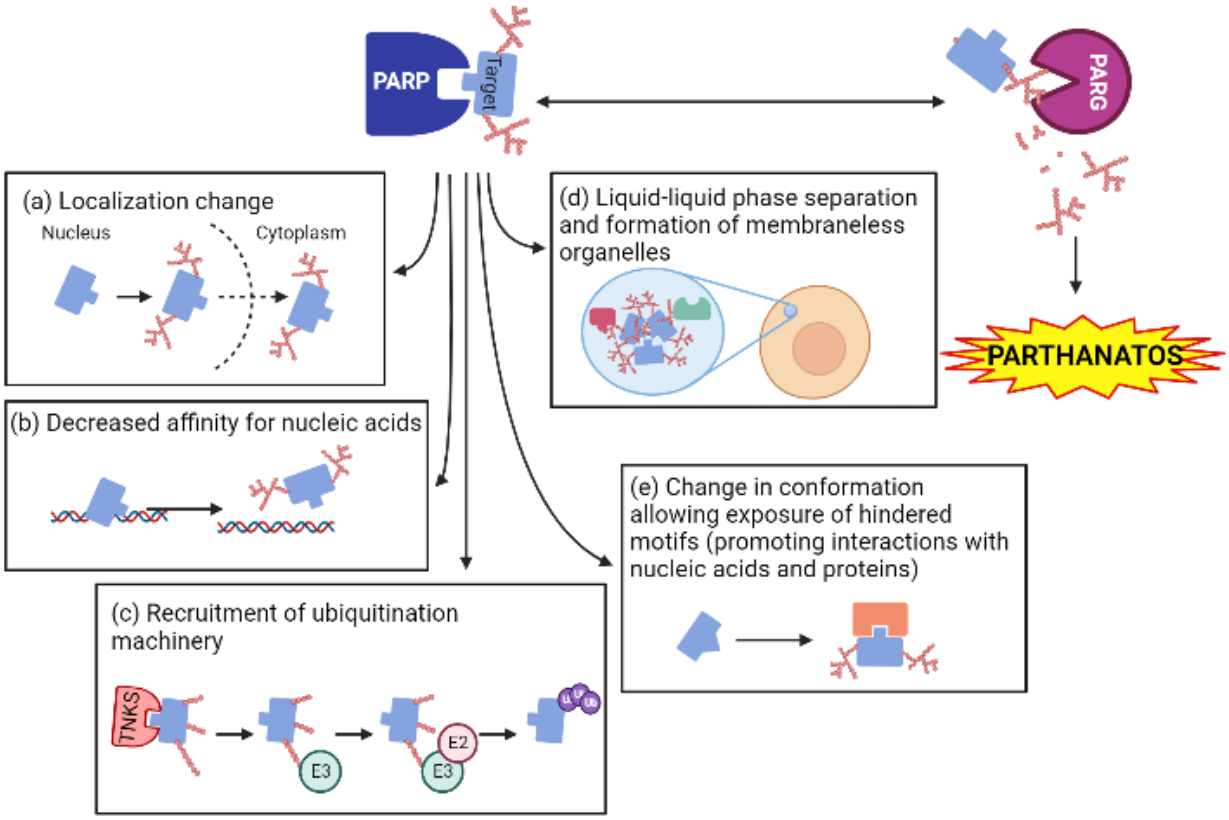


Figure 1.8. Mechanisms of PARylation.

PARP family members modify proteins through PARylation. PARylation can be reversed by PARG enzymes. During apoptotic stimuli, hydrolyzed pADPr acts as a death signal which shuttles from the nucleus to the mitochondria, where it activates the AIF-mediated cell death, and results in a process called parthanatos [357]. PARylation of proteins may (A) results in a conformational change which uncovers hindered functional motifs [355], (B) lead to their ubiquitination (specifically true for TNKS-mediated PARylation) [358], (C) decrease their affinity for nucleic acids [359, 360], (D) lead to a change in their subcellular localization [345, 361], and (E) result in their aggregation and their liquid-liquid phase separation to form membraneless organelles that are highly functional [362, 363], and.

The nucleotide-like composition of the pADPr molecule, and the negative charge conferred to it by its pyrophosphate group allows it to compete with nucleic acids for the binding of DNA- and RNA-binding proteins (Figure 1.8 c). As a result, PARylation has been shown to play a prominent role in regulating mRNA metabolism by modulating the RNA-binding function of RBPs [364]. For instance, PARP1-mediated PARylation resulted in the reduced binding of hnRNP A1 to the heat shock RNA ω (hsrω-RA)

transcript, thus affecting its splicing activity [365]. Additionally, during heat shock, PARP1-mediated PARylation of poly-A-polymerase (PAP) results in its dissociation from target mRNAs, consequently decreasing poly-adenylation, and thereby repressing mRNA synthesis [366].

PARPs have also been shown to modulate the function of RBPs by inducing change in their localization (Figire 1.8 d). For example, as mentioned above, PARP1-mediated PARylation of HuR affects its cytoplasmic localization in LPS-treated macrophages [349, 367]. Furthermore, PARylation was also shown to regulate the recruitment of hnRNP A1 to cytoplasmic entities named stress granules in response to oxidative stress. Stress granules are known to modulate the translation of mRNAs that are recruited to these entities. PARylation, therefore, by affecting the localization of hnRNP A1 to stress granules, affects the translation of its mRNA targets [363]. Interestingly, PARPs have been shown to modulate the PARylation of the RBP Ras-GTPase-activating protein (SH3 domain)-binding protein (G3BP), which was previously shown to be required for the formation/aggregation of stress granules (Figure 1.8 e) [367].

1.5.1.1 PARPs

Although there are 17 PARPs, all of which have the PARP catalytic domain, not all PARPs can form poly (ADP-ribose) (pADPr) polymers. Indeed, only PARP1, 2 as well as PARP5a (also known as Tankyrase-1, TNKS1) and PAPR5b (also known as Tankyrase-2, TNKS2) are known to have PARylation activity.

1.5.1.1.1 PARP1 and PARP2

The most extensively studied role of PARP1 is in DNA damage response and genomic maintenance. In fact, PARP1 activation is the earliest response to DNA damage, where it catalyzes pADPr and recruits DNA repair factors [368]. However, when the cells are subjected to high levels of DNA damage, PARP1 is overactivated and depletes NAD^+ and subsequently ATP levels, leading to necrosis, which is a process named parthanatos. To avoid the depletion of ATP levels which are used during apoptosis, PARP1 is cleaved by caspases-3 and 7, resulting in two inactive fragments. The smaller 24kDa fragment remains bound to the DNA and prevents the

activity of PARP1, while the bigger 89kDa fragment relocalizes to the cytoplasm [357]. This causes DNA fragmentation leading to apoptosis. Thus, PARP1 is widely used as a marker of apoptosis [369].

PARP1 is also known to function in cell differentiation, immune response, and transcriptional regulation [370, 371] [372]. Although the function of PARP2 is redundant to that of PARP1, it was shown to be significantly involved in lipid metabolism and in autophagosomes clearance in mice and/or in skeletal muscle cells, respectively [373, 374].

1.5.1.1.2 Tankyrase-1 and Tankyrase-2

PARP5a (or ARTD5), more commonly termed as tankyrase-1 (TNKS1) was first discovered in 1998, as part of the proteins bound to telomeres. It was identified in the proteomics analysis of proteins interacting with Telomeric repeat-binding factor 1 (TRF-1), which is a telomere-specific DNA-binding protein that blocks the access of telomerase to telomeres [375]. TNKS1 is mainly localized in the cytoplasm and has been recently shown to exert miRNA processing functions in the nucleus [376, 377]. TNKS2 has redundant functions to TNKS1, and their sequence and their catalytic domain are 80% and is 82% similar respectively [378]. TNKS1 and 2 double KO mice are embryonic lethal, underlining their cruciality [379]. In mice, deletion of TNKS1 causes increased energy expenditure and decreased adiposity. Deletion of TNKS2, however, results in growth retardation. The deletion of TNKS1 and 2, surprisingly, did not result in defects in telomere maintenance or telomere capping [380]. This is explained by the differing functions that tankyrases (TNKS) have in human and in mice [381]. TNKS1, nonetheless, was shown to regulates various processes, including DNA damage response, maintenance of telomere length, mitosis, Wnt- and Notch-mediated signalling transduction and insulin-mediated glucose uptake [376, 382-388]. One of the most studied functions of TNKS1 is its role in regulating the canonical Wnt/β-catenin signalling [384, 389-391]. This signalling pathway is crucial for the proper embryonic development and for post-natal processes such as maintenance of cell fate, tissue homeostasis, and regeneration.

TNKS comprise an ankyrin repeat region, a sterile alpha motif (SAM) fold, and a catalytic domain [392]. TNKS1 contains an additional low complexity sequence rich in histidine, proline, and serine (HPS) residues. Additionally, the catalytic domain of TNKS does not contain an autoinhibitory HD domain, which makes it largely accessible to bind NAD^+ substrate. Recently, it was shown that some TNKS1 ligands such as RNF146, desmin, and NELFE contain a TBM (TNKS1 binding motif dictated by the amino acid sequence RXXXXG) that mediates the binding of these proteins to TNKS1 [393]. Although the binding of TNKS1 to these proteins via this motif is required for its PARylation, several studies have shown that this interaction does not necessarily result in their PARylation [394].

TNKS-mediated PARylation can results in the change of the localization of target proteins. In fact, TNKS1 has been shown to be highly localized at the nuclear envelop, in the perinucleus (mitotic spindle and golgi), and at the lateral membrane, where the majority is located in the golgi region, linked to Glut4 vesicles [385, 395]. Studies have shown that TNKS1 associates to these vesicles through its association to IRAP (insulin regulated aminopeptidase), which is a sorting protein involved in their biogenesis. Then, TNKS1 mediates their transport to the plasma membrane upon insulin stimulation, to promote glucose intake [386].

TNKS-mediated PARylation is also involved in the ubiquitination of target proteins, which often, but not always leads to their degradation [358, 384, 385, 396, 397]. That is due to the recruitment of the E3 ubiquitin ligase RNF146, which contains a WWE and RING domain, which allows it to non-covalently bind pADPr. pADPr allosterically activates RNF146 by inducing conformational change to the RING domain, allowing it to bind stronger to ligands, including the ubiquitin-conjugating enzyme E2 [355]. RNF146, as a result, transfers ubiquitin from the E2 active site to a lysine residue of a substrate [356].

In addition to PARylating target proteins, TNKS were also shown to act as scaffolding proteins through their interaction with target proteins that bind ankyrin domains of TNKS multimers [398]. For instance, TNKS can bind PEX14 and ATG9A, which are two proteins involved in pexophagy [399]. Both proteins harbor TNKS-binding

motifs and are targets of TNKS, and can interact to induce pexophagy in a TNKS-dependant and PARylation-independent manner.

1.5.1.2 PARylation in skeletal muscle

Although the role of PARylation in myogenesis is not well established several PARPs, including PARP1 and TNKS have been shown to affect muscle formation/integrity. For example, PARP1 was recently suggested to regulate myogenesis by inhibiting the transcriptional activity of MyoD [400]. Although the activity of PARP1 was shown to be downregulated during muscle differentiation, it was shown, in myoblasts, to inhibit the activity of MyoD by binding to its regulatory regions within the chromatin, thus affecting the transcription of MyoD-targed genes namely *p57, p21, myogenin*, and *MEF2C*. Thereby, PARP1 prevents the recruitment of MyoD to the promoter of these genes thus inhibiting their transcription. This function of PARP1 in regulating MyoD, however, does not appear to require its catalytic activity.

The role of TNKS in embryonic myogenesis and in glucose uptake in skeletal muscle is also well established. Indeed, as previously mentioned, TNKS1 is known to regulate the canonical Wnt signalling pathway, which is heavily involved in embryonic and adult skeletal muscle formation [384, 389, 401-407]. In fact, KO mouse models of Wnt or Wnt signalling effectors display early embryonic lethality due to pronounced tissue damage and poor muscle development [408]. During adult myogenesis or regeneration, Wnt1 has been shown to induce the expression of the MRF Myf5. Wnt3, on the other hand, was shown to be involved in satellite cell differentiation [409, 410]. The activation of the Wnt/B-catenin pathway, furthermore, by podocan (a member of the small leucine-rich repeat (SLR) family of proteins) induces the differentiation of muscle cells *in vitro* and the regeneration of tibialis anterior muscle in response to injury *in vivo* [401]. These results, therefore, suggest that TNKS, including TNKS1 and 2, likely have a prominent role in regulating muscle fiber formation.

The rate of lipid oxidation, which is mainly a function of mitochondria, has been shown to be reduced in type 2 diabetes patients [411, 412]. PGC1-α co-activates transcription factors involved in mitochondrial biogenesis and oxidative metabolism in muscle and adipose tissues to promote gluconeogenesis in the liver [413-415]. PGC1-α

expression, however, is known to be repressed in muscle and adipose tissue of patients with obesity and diabetes [416, 417]. Interestingly, TNKS1 was shown to target PGC1-α for PARylation-mediated degradation. *in vivo* studies using an inhibitor of TNKS1 in animal models of obesity and diabetes, furthermore, demonstrated that targeting the function of TNKS1 ameliorates insulin resistance and diabetes, and that this effect was correlated with the increased expression of PGC1-α [418]. Consistent with these results, TNKS1 inhibition in diabetic mice resulted in increased muscle mitochondrial mass and increased fatty acid oxidation. These studies, therefore, suggest that TNKS1 plays an important role in regulating mitochondrial biogenesis by targeting PGC1-α.

Intriguingly, PARP2 has also been shown to have a role in obesity, in which levels of NAD^+ are significantly reduced [419, 420]. High fat diet-fed obese mice display increased PARylation levels mediated by PARP2, which itself is upregulated in these mice [420]. This increase in PARP2 expression and activity in these mice negatively regulates the expression and deacetylation function of NAD^+-dependent Sirt1 in the gastrocnemius muscle of obese mice. The PARP2-mediated inhibition of Sirt-1, therefore, results in the decreased deacetylation of PGC1-α thus affecting mitochondrial biogenesis [419].

	Myogenesis	**Muscle Wasting**
PARP1	Inhibition of MyoD transcriptional activity. [400] Inhibition of Sirt-1 pro-myogenic activity. [421-423]	Depletion of NAD^+, leading to mitochondrial dysfunction. [421] Inhibition leads to decreased inflammatory molecules [424].
PARP2	Inhibition of Sirt-1 expression and activity. [419, 420] Autophagosomes clearance. [374]	Not determined.
TNKS1	Glut4 vesicles-mediated glucose uptake. [385, 386, 395, 425] Wnt-signaling-mediated differentiation. [401, 402, 404]	Not determined.
TNKS2		Not determined.

Table 1. Brief description of the role of PARPs in skeletal muscle.

1.5.1.3 PARylation in Muscle Wasting

Recent reports indicate that PARylation is involved in muscle-related diseases and pathologies including cachexia, dystrophy, and sarcopenia. Indeed, PARylation activity was shown to be increased in skeletal muscle biopsies from third degree burn children [426]. One of the main reasons behind the negative impact of PARylation in muscle fibers is the consumption of NAD^+, which leads to mitochondrial dysfunction. There exists a positive relationship between mitochondrial biogenesis and NAD^+ metabolism [427]. Therefore, NAD^+ consumption by PARPs may affect muscle integrity by impacting cellular energy levels.

Studies performed by Chacon-Cabrera et al. investigated, *in vivo,* the role of PARPs in cachexia [422, 428]. Using a mouse model of cancer-induced muscle wasting they demonstrated that genetic ablation of PARP1 but not PARP2 in tumour-bearing mice decreased muscle mass and grip strength compared to their non-tumour bearing controls [428]. Importantly, they also demonstrated that wild-type mice bearing tumours exhibited increased PARylation levels compared to their controls suggesting that an increase in PARylation activity likely increases muscle wasting. They, furthermore, demonstrated, in these tumour-bearing mice, increased protein degradation in the diaphragm and gastrocnemius skeletal muscle (measured by the release of tyrosine amino acid), which is an important aspect of muscle atrophy. The increase in protein degradation in muscle, however, was evident in PARP2 but not PARP1 KO mice bearing tumours. Moreover, while the size of fibers was significantly reduced in cachectic wildtype mice compared to their non-cachectic controls, it did not significantly differ in cachectic PARP1 and PARP2 KO mice compared to their controls.

A more recent study showed that the PARP1 inhibitor BGP-15 has anti-cachectic properties [429]. Shen *et al.* used the irinotecan (IRI) chemotherapeutic agent (topoisomerase I inhibitor) to induce cachexia in mice. IRI induces cachexia by eliciting skeletal muscle toxicity by favoring protein degradation and mitochondrial dysfunction through which oxidative stress/damage is escalated. This treatment caused about 5% body mass loss. Indeed, this group observed that IRI treatment led to a decrease in the mass of the tibialis anterior (TA) mouse limb skeletal muscle, which was prevented by

the BGP-15 treatment. This observation was further supported by the decrease in cross-sectional area after the treatment with IRI, which was partially rescued by co-treatment with BGP-15. The ability of BGP-15 to prevent muscle wasting suggests that the loss of muscle observed during cachexia is mediated, in part, due to an increase in PARylation activity in skeletal muscle.

1.6 Thesis Rationale and Objectives

Skeletal muscle is an important tissue in the body whose integrity is vital for the survival and the quality of life of an organism. Skeletal muscle formation, a process called myogenesis, is tightly regulated to allow muscles to adapt to various insults, and disruption of this process leads to the development of various skeletal muscle disorders. Additionally, skeletal muscle can waste in response to chronic diseases that result in inflammatory conditions in this tissue. Currently, there exists no treatment for muscle-related diseases and syndromes, urging the necessity of understanding underlying regulatory mechanisms that may be used in therapy for such disorders. Our laboratory and others have previously shown that the RBP HuR is a master regulator of myogenesis through regulation of mRNAs that either inhibit cell cycle progression and/or promote the differentiation of muscle cells. Our work established that HuR promotes the decay of *NPM* mRNA, and the stability of *p21* mRNA to arrest the cell cycle, while it promotes the translation of HMGB1, and the stability of *MyoD* and *myogenin* mRNAs to promote muscle cell differentiation. Trans-acting factors of HuR in these functions have been identified. For example, KSRP was shown to form a complex with HuR and recruit the decay machinery to degrade the NPM message. Additionally, miR1192 was shown to compete with HuR for the inhibition of HMGB1 translation, whereas HuR promoted the translation of the message by preventing the recruitment of the RISC. Although the function of HuR in myogenesis is well established, regulatory mechanisms governing its ability to differentially regulate these messages at different posttranscriptional levels remain unclear. Therefore, the objective of my research in Chapter 2 was to identify a regulatory mechanism through which the pro-myogenic function of HuR is regulated. During these studies, we identified PARylation as a key posttranslational modification activated during the process, and which modifies HuR.

Recently, the importance of HuR in the function of muscle was confirmed in muscle-specific HuR knockout mice. Interestingly, knocking out HuR specifically in muscle was demonstrated to prevent cancer-induced muscle wasting, a syndrome called cachexia. While mechanisms through which HuR promotes muscle wasting remains elusive, it would not be surprising that HuR would have a more significant role in this process since HuR was previously shown to have dual and opposite functions in cell fate (survival or apoptosis). Therefore, in Chapter 3, we aimed to further characterize the pro-cachectic function of HuR and identify the network of mRNA targets through which it promotes muscle wasting. Towards this end we identified the key pro-cachectic transcription factor, STAT3 as a novel mRNA target of HuR during muscle wasting and demonstrate that HuR promotes the translation of the *STAT3* mRNA by negating the action of miR-330.

By determining the regulatory mechanisms of the function of HuR in myogenesis, we further identify ways to target this function of HuR without having to use HuR inhibitors to avoid their toxicity. Additionally, by identifying new pro-cachectic genes regulated by HuR, we further demonstrate that HuR plays dual opposite functions in skeletal muscle depending on the conditions to which the tissue is subjected. These studies shed light on the importance of posttranslational modifications in skeletal muscle and more importantly, they highlight new roles of TNKS1 and HuR in skeletal muscle, which can widen our understanding of how TNKS1 inhibitors can be repurposed in therapy and what systemic impacts the overexpression of HuR in cancer may be having.

Chapter 2 | Investigating the role of the Posttranslational Modification PARylation in the pro-myogenic Function of HuR

2.1 Preface

While the function of HuR in muscle fiber formation is well-established, regulatory mechanisms governing the function of HuR are not fully understood in this process. HuR has been shown to be modified by several modifications, including methylation and phosphorylation. We tested the impact of these modifications on the pro-myogenic function of HuR and observed no effect (unpublished data), therefore we decided to investigate if other posttranslational modifications may be implicated. A new posttranslational modification was recently shown to modify the RNA-binding activity, stabilization function, and localization of HuR, which are key functions of HuR in the myogenic process. Thus, we were interested in determining whether this modification regulates the function of HuR in myogenesis. To this end, in this chapter, we investigate the importance of PARylation in myogenesis and the significance of this modification in the function of HuR in this process.

These findings and discussions are part of a manuscript that is under review.

Mubaid S, Adjibade PM, Hall DT, Lian XJ, Brusque S, Ashour K, Carlile G, Gagné J-P, Di Marco S, Thomas DY, Poirier GG, Gallouzi IE. “Tankyrase-1 regulates RBP-mediated mRNA turnover to promote muscle fiber formation” *Manuscript to be submitted shortly*.

2.2 Abstract

Poly(ADP-ribosylation) (PARylation) is an ubiquitous posttranslational modification mediated by a subset of ADP-ribosyl transferases (ARTs). Although PARylation-inhibition based therapies is being considered as an avenue to combat debilitating diseases such as cancer, diabetes and some myopathies, the role of this posttranslational modification in physiological processes such as cell differentiation is still unclear. Here we show that Tankyrase1 (TNKS1), one of the PARylating ARTs, plays a major role in myogenesis, a vital process known to drive muscle fiber formation and regeneration both *in vitro* and *in vivo*. Even though all *bona fide* PARPs (*i.e.* PARP1, PARP2, TNKS1 and TKNS2) are highly expressed in muscle cells, experiments using siRNA-mediated knockdown or pharmacological inhibition show that TKNS1 is the main enzyme responsible of catalyzing PARylation during muscle cell differentiation. Via this activity, TKNS1 controls the turnover and the expression of mRNAs encoding myogenic regulatory factors such as *nucleophosmin* (NPM) and *myogenin*. TKNS1 mediates these effects by targeting key promyogenic RNA-binding proteins (RBPs) such as the Human Antigen R (HuR). HuR harbors a conserved TNKS-binding motif (TBM), the mutation of which not only prevents the association of HuR with TKNS1 and its PARylation, but also precludes HuR from regulating the turnover of the *NPM* and *myogenin* mRNAs as well as from promoting myogenesis. Therefore, our data uncover a new role for TNKS1 as a key modulator of RBP-mediated post-transcriptional events required for vital processes such as myogenesis.

2.3 Introduction

Covalent addition of functional groups is one of the well characterized posttranslational modifications used by the cell to modulate and expand the function of its protein network. There are more than 400 different types of posttranslational modifications affecting many aspects of protein functions. During the last few decades, the list of posttranslational modifications have expanded to include phosphorylation, glycosylation, ubiquitination, SUMOylation, nitrosylation, methylation, acetylation, lipidation, as well as poly(ADP-ribosylation) (PARylation) [430-439]. The majority of these posttranslational modifications have been associated with numerous physiological and pathological phenotypes. Yet, our understanding of the role and impact of PARylation in cell homeostasis and physiology is quite limited [438, 439]. The importance of PARylation is underscored however, by the fact that it is ubiquitous in nature as well as by numerous *in vitro* and *in vivo* studies highlighting the benefit of PARylation inhibition for the treatment of diseases such as cancer, muscle myopathies and some metabolic disorders [438-440]. Recent clinical trials using these inhibitors nonetheless showed limited successes in combatting diseases such as diabetes as well as prostate, and colon cancers [438-440]. Therefore, to design wider and efficient PARylation-inhibition-based therapies, a better understanding of the role of PARylation in vital processes such as cell homeostasis, metabolism, and differentiation is needed.

PARylation is a reversible posttranslational modification that is mediated by the hydrolysis of NAD^+ and the transfer of ADP-ribose moieties to protein acceptors [351, 441]. This catalytic reaction is mediated by a subset of enzymes called poly(ADP-ribose) polymerases (PARPs) which are part of the ADP-ribosyl transferases (ARTDs) family of proteins [442, 443]. Although the majority of these enzymes transfer a single ADP-ribose (ADPr) moiety (*i.e.* mono(ADP-ribosyl) transferases or MARTs) or are catalytically inactive [444, 445] four members, PARP1 (ARTD1), PARP2 (ARTD2) as well as PARP5a (ARTD5) and PARP5b (ARTD6) (also called tankyrase-1 (TNKS1) and -2 (TNKS2) respectively) exhibit PARylation activity by adding a polymer composed of up to 200 ADP-ribose subunits (termed poly(ADP-ribose); pADPr) to target proteins. These four PARPs are designated as ‘*’bona fide* PARPs’’ since, as their name suggests, they synthesize polymers of ADPr [380, 444, 446-449]. pADPr was initially

characterized as a nucleic-acid-like molecule that is a product of a nuclear enzymatic activity [351]. However, it was later discovered that PARylation is a posttranslational modification capable of reprogramming the biochemical properties and activities of target proteins. It has been shown to regulate the interaction of target proteins with nucleic acids [354, 361, 450] and protein ligands [350, 367, 450-452] (by affecting their ubiquitination [355, 358, 393, 452] or their cellular localization [363, 367]).

The function of PARPs is essential since the double knockout of PARP1/PARP2 or TNKS1/TNKS2 in mice is embryonically lethal [379, 453]. They are known to play a role in several cellular processes including genomic maintenance, DNA damage response, transcription, and inflammation [354, 450, 454-457]. While PARP1 is generally associated with actively transcribed genes [458, 459], binding to nucleosomes and the DNA-damage response, PARP2 was shown to be involved, among other processes, in lipid metabolism and in autophagosomes clearance [373, 374, 451, 460]. TNKS1 and TNKS2, on the other hand, have a well-established role in regulating telomere maintenance, canonical Wnt-signaling pathway, as well as the vesicular transport signaling pathway [384-386, 394, 395, 403, 461, 462]. Importantly, large-scale identification of PARylation targets through transcriptomic and proteomic analyses revealed that these PARPs modulate RNA metabolism through the modification of RNA binding proteins involved in various levels of post-transcriptional regulation [463-465].

Recently, several reports have shown that PARylation activity is linked to the outcome of several muscle-related diseases including cancer-induced muscle wasting (cachexia), dystrophy, and sarcopenia [386, 418, 423, 426, 428, 429, 466]. This is due, in part, to changes in mitochondrial biogenesis and production of pro-inflammatory cytokines (such as IL-6 and TNFα) [386, 423, 466-469]. While these and other observations suggest that PARylation could play an important role in the physiology of skeletal muscle tissue and its ability to adapt to internal and external assaults, this possibility has not been fully explored. Indeed, although synthesis of PAR was suggested to be correlated with the differentiation of limb mesodermal cells into muscle cells [470], we do not know the PARPs involved in this process and whether/how PARylation could impact myogenesis, a process that drives muscle fiber formation

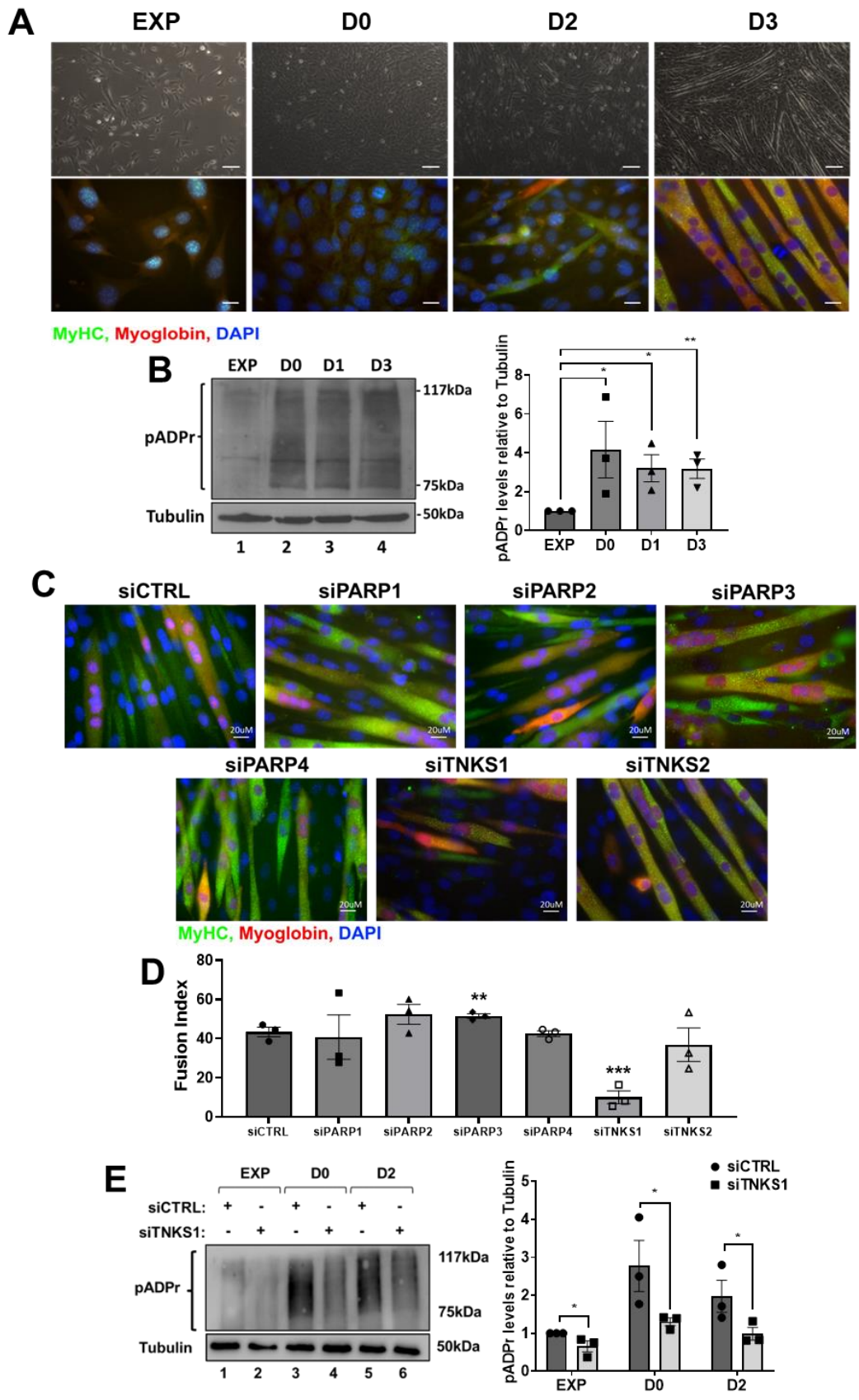
during development as well as in response to injuries [471]. Myogenesis is a multi-stage process through which mono-nucleated muscle precursor cells, called myoblasts, fuse to form multi-nucleated myotubes. This process is mediated through the controlled expression of pro- and anti-myogenic factors, including nucleophosmin (NPM) and myogenin that collaborate together to ensure the commitment of myoblasts to the myogenic program [2, 5, 471]. Therefore, uncovering the mechanisms by which PARylation impacts muscle fiber formation is an essential step towards our understanding of the physiological role of this important posttranslational modification.

In this study, we provide strong evidence that TNKS1-dependent PARylation is required for proper muscle fiber formation. TNKS1 achieves this effect by targeting promyogenic RNA binding proteins (RBPs), such as HuR, to modulate post-transcriptional events involved in the expression of key myogenic regulatory factors. HuR harbors a conserved TNKS1-binding motif that is essential for its PARylation as well as its promyogenic function. Together, our data uncover that TNKS1-mediated PARylation of RBPs could be a general mechanism required for proper muscle fiber formation.

2.4 Results

2.4.1 TNKS1-mediated PARylation activity is required fo.r myogenesis

In order to determine the role of PARPs during myogenesis we assessed, as a first step, PARylation activity during the differentiation of C2C12 muscle cells [472] (**Figure 2.1a**). Using western blot experiments with an anti-pADPr antibody, which is one of the gold standard methods used to identify PARylated proteins in extracts [15], we observed a steady increase of PARylation activity during the differentiation of myoblasts into myotubes (**Figure 2.1b**). To investigate the importance of PARylation during myogenesis, we depleted the main PARP enzymes: PARP1, PARP2, TNKS1 or TNKS2 **(Figure 2.2a)** in C2C12 cells that were subsequently induced for differentiation. The impact of depleting PARP3 and PARP4, which modify proteins through mono(ADP-ribosyl)ation rather than PARylation [438, 439] was included as controls. The depletion of TNKS1 but not the other PARPs prevented the commitment of C2C12 cells to the myogenic process (**Figure 2.1c-d)**. Of note, although TNKS2 has redundant functions with TNKS1 [379], its depletion did not affect the formation of myotubes (**Figure 2.1c)**. We next determined if TNKS1 is responsible for the observed increase in the PARylation activity during myogenesis (**Figure 2.1b**). Towards this end we knocked down TNKS1 and assessed levels of PARylated proteins during myogenesis. In the absence of TNKS1 the level of PARylated proteins in differentiating muscle cell was reduced by ~2 fold (**Figure 2.1e**). These observations were confirmed using a well-known specific TNKS1 inhibitor, XAV939 [384, 473, 474]. XAV939 but not DMSO (used as a negative control) significantly decreased the levels of PARylated proteins during myogenesis as well as reduced the formation of myotubes by ~40% (**Figure 2.3**). Collectively, these observations indicate that TNKS1 is not only the main enzyme responsible for the PARylation activity during myogenesis, but it is also required for proper muscle fiber formation.


A
EXP
D0
D2
D3
MyHC, Myoglobin, DAPI
B
EXP D0 D1 D3
117kDa
pADPr
75kDa
Tubulin
50kDa
1 2 3 4
pADPr levels relative to Tubulin
EXP D0 D1 D3
C
siCTRL
siPARP1
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E
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siCTRL:
siTNKS1:
117kDa
pADPr
75kDa
Tubulin
50kDa
1 2 3 4 5 6
pADPr levels relative to Tubulin
siCTRL
siTNKS1
EXP D0 D2

Figure 2.1. Tnks1 is required for muscle cell differentiation.

(A) Phase Contrast (upper panels) and immunofluorescence (lower panels) images demonstrating exponentially growing (EXP) and differentiating (D0, D2, D3) C2C12 muscle myoblasts. (Scale bars, 50µm for phase contrast images). Immunofluorescence staining was performed with antibodies (anti-MyHC, anti-myoglobin) against known markers of muscle fibers. DAPI was used to stain nuclei. Images of a single representative field are shown and are representation of three independent experiments. (Scale bars, 20µm.) **B)** Total cell extracts were prepared from exponential and differentiating C2C12 myoblasts (EXP, D0, D1, D3). (Left panel) Western blot experiments were performed using antibodies against pADPr or α-tubulin (loading control). (Right panel) Histogram representation of the quantification of the western blot. Values were quantified using ImageJ and normalized to tubulin. **C)** Immunofluorescence experiments demonstrating the differentiation of C2C12 myoblasts treated with scrambled control (siCTRL), or specific siRNAs against PARP1, PARP2, PARP3, PARP4, TNKS1 or TNKS2. Immunofluorescence staining was performed with antibodies against known markers of muscle fibers (anti-MyHC, anti-myoglobin). DAPI was used to stain nuclei. Images of a single representative field are shown and are representation of three independent experiments. (Scale bars, 20µm.) **D)** Fusion index quantifying the nuclei in myotubes relative to the total number of nuclei. **E)** Total extracts were prepared from C2C12 cells that were transfected with scrambled control (siCTRL) or siRNA against TNKS1 (siTNKS1) and collected from exponentially growing (EXP) and differentiating C2C12 myoblasts (Day 0 and Day 2). (Left panel) The extracts were subjected to western blot analysis using antibodies against pADPr or α-tubulin (loading control). (Right panel) Histogram representation of the quantification of the western blot in the left panel. Values were quantified using ImageJ, normalized to tubulin and shown relative to the EXP siCTRL treated condition. Data are presented +/- the s.e.m. of 3 independent experiments with *P <0.05, **P <0.01, ***P <0.001 by unpaired *t*-test.

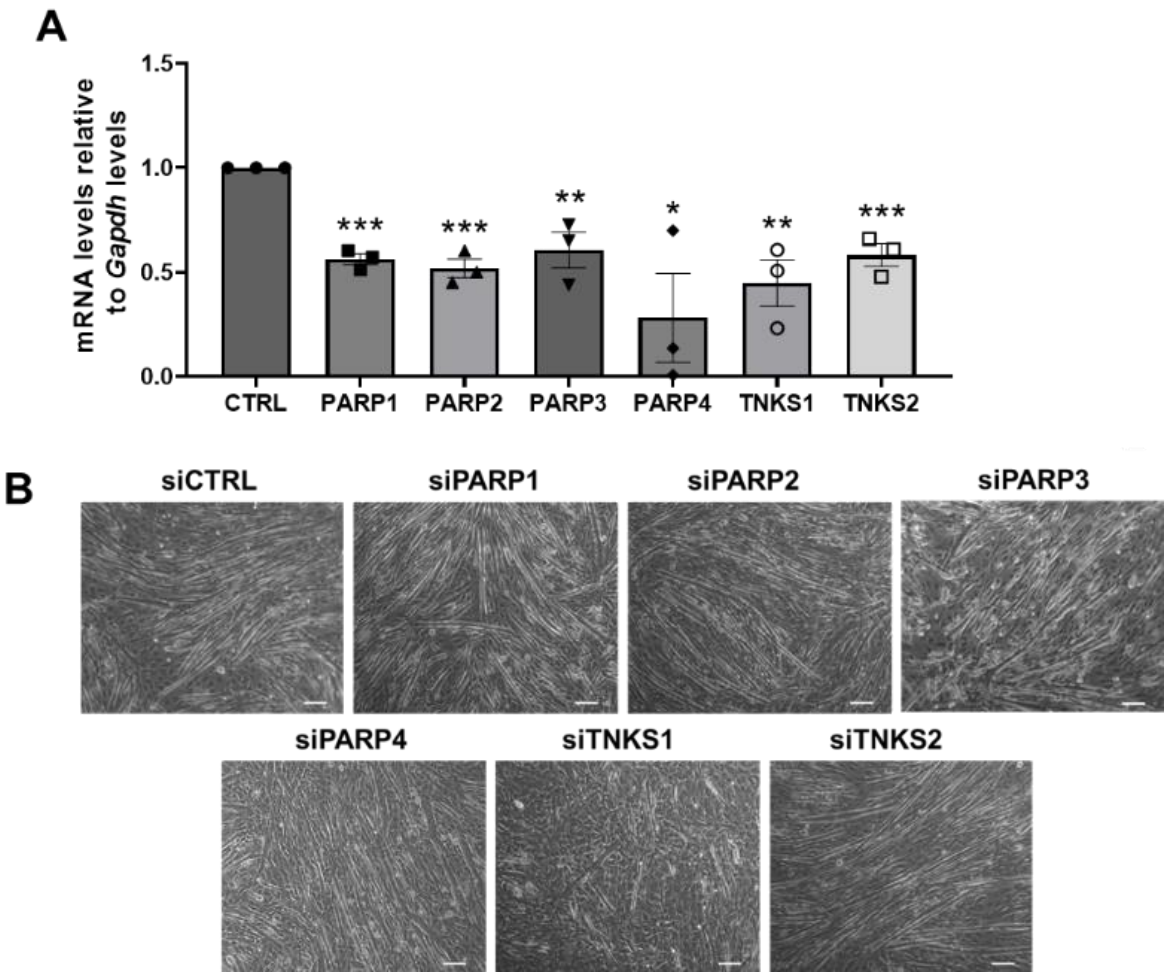


Figure 2.2. A-B) C2C12 muscle cells were transfected with scramble control (siCTRL) or with siRNAs specific for the different PARPs (1 to 5b). **A)** Total RNA was isolated from myotubes on Day 3 post-induction of differentiation and *PARP1*, *PARP2*, *PARP3*, *PARP4*, *TNKS1*, and *TNKS2* mRNA levels were determined by RT-qPCR. Levels were standardized against *GAPDH* mRNA and expressed relative to siCtrl conditions. **B)** Phase contrast images were taken of cells treated with the siRNAs described above. Images of a single representative field are shown and are representation of three independent experiments. (Scale bars, 50µm.)

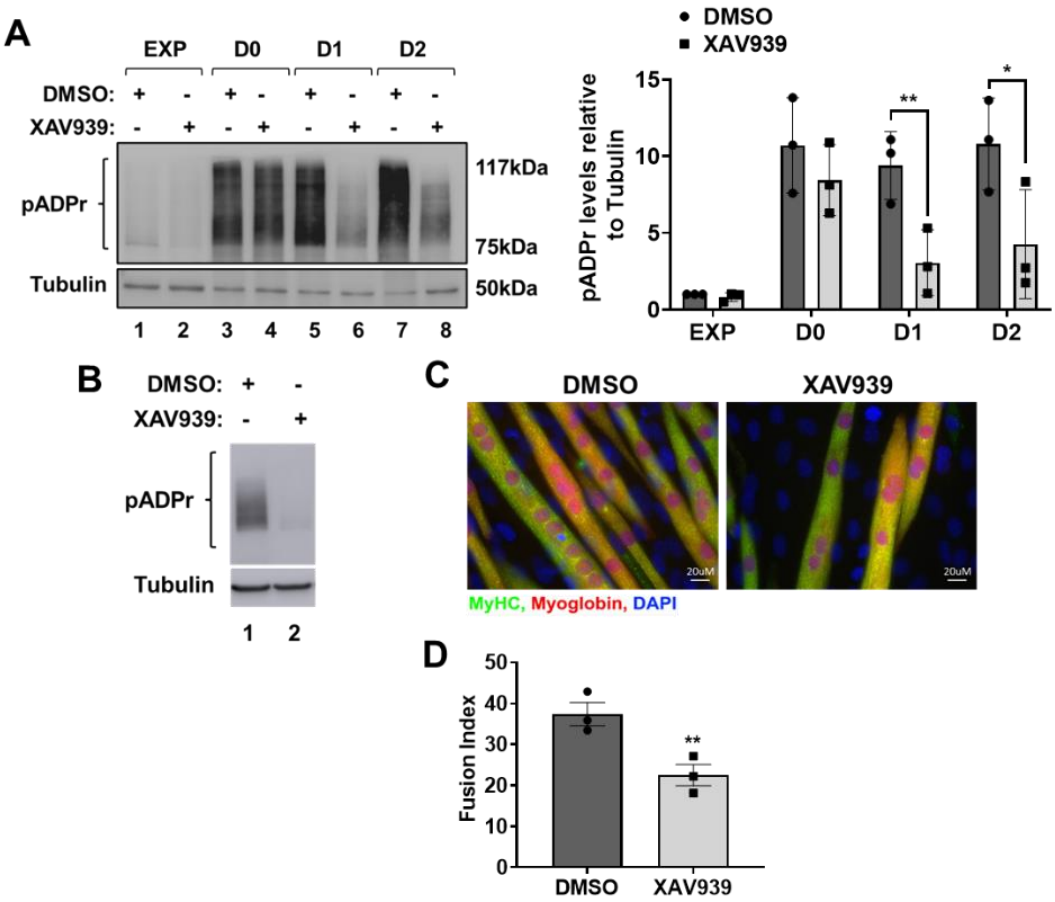


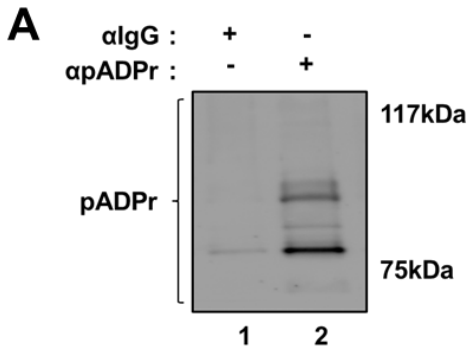
Figure 2.3. A) Total cell extracts were prepared from C2C12 cells that were treated with 10uM XAV939 or DMSO (used as a control) and collected from exponentially growing (EXP) (24h treatment) and differentiating C2C12 myoblasts (Day 0, Day 1, and Day 2) (24h, 48h, and 72h post-induction of differentiation). These extracts were used for western blot analysis with antibodies against pADPr or α-tubulin (loading control) (Left panel). Histogram representation of the quantification of the western blot (right panel). Values were quantified using ImageJ and normalized to tubulin and shown relative to DMSO treated control condition. **B)** Total cell extracts were prepared from C2C12 cells treated with 10uM XAV939 or DMSO (used as a control) and collected on Day 3 post-induction of differentiation. These extracts were used for western blot analysis using antibodies against pADPr or α-tubulin. **C)** Immunofluorescence experiments on differentiating C2C12 myoblasts treated with 10uM XAV939 or DMSO. Staining was performed with antibodies against known markers of muscle fibers formation, MyHC and myoglobin. DAPI was used to stain nuclei. Images of a single representative field are shown and are representation of three independent experiments. (Scale bars, 20µm.) **D)** Fusion index showing quantification of nuclei in myotubes relative to the total number of nuclei counted in H. Data shown are presented +/- the s.e.m. of 3 independent experiments with *P <0.05, **P <0.01, ****P <0.0001 by unpaired *t*-test.

2.4.2 TNKS1 promotes myogenesis by PARylating key promyogenic RNA-binding Proteins

To decipher the mechanism through which TNKS1 regulates myogenesis, we began by identifying the network of proteins that are PARylated by this enzyme in muscle fibers. To this end, we performed mass spectrometry analysis on pellets obtained from an immunoprecipitation experiment using anti-pADPr or anti-IgG (negative control) antibodies and lysates from differentiated C2C12 myotubes (**Figure 2.4a**). We identified 204 proteins that were selected by counting unique peptides and by eliminating proteins that were bound to the IgG control. Classification of these proteins, based on known molecular function, using the Panther software, revealed that 87 of them belong to the family of RNA binding proteins (RBPs) (**Figure 2.4b**). Among these, 7 proteins (**Figure 2.4c**) have been previously associated with muscle function/integrity [471, 475-480]. From this short list, HuR (ELAL1) is the only RBP that has been extensively characterized as one of the key posttranscriptional regulators of muscle fiber formation and function both *in vitro* and *in vivo* [149, 183, 481-483]. Therefore, as a proof of concept for the role of PARylation in muscle fiber formation, in this study, we chose to delineate the role of TNKS1 in the promyogenic function of HuR. First, we confirmed the PARylation of HuR during myogenesis by repeating the immunoprecipitation experiment described above (with anti-pADPr or anti-IgG antibodies) followed by western blot analysis using anti- HuR and -KSRP (as negative control) antibodies [367, 484]. We observed that anti-pADPr antibody immunoprecipitated HuR but not KSRP (**Figure 2.4d**). Moreover, TNKS1 knockdown significantly reduced the level of PARylated HuR (**Figure 2.4e**). Of note, since the level of HuR in muscle cells was not affected in the absence of TNKS1 (**Figure 2.5a**), we concluded that the observed decrease in pADPr-HuR association is not due to an effect on HuR expression.

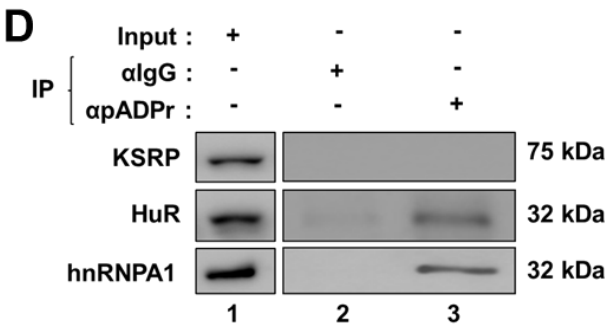
While these results clearly indicate that in differentiating muscle cells, TNKS1 is responsible for HuR PARylation, they do not provide any information on whether this effect is due to a direct or indirect interaction between HuR and TNKS1. To address this, we performed an *in vitro* PARylation assays (**Figure 2.4f**) [349] using recombinant TNKS1 and HuR. PARP1 and PARP3 were also included in the assay as a positive

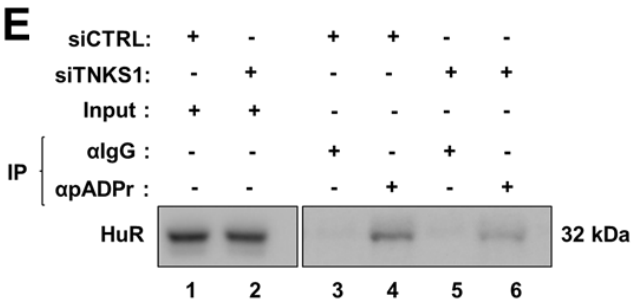
and negative control respectively since PARP1 was previously shown to PARylate HuR in macrophage cells [349] and PARP3 is a known as a mono(ADP-ribose) transferase [485]. Similarly, to PARP1, TNKS1 but not PARP3 PARylated HuR *in vitro* (**Figure 2.4g**). Additionally, XAV939 but not DMSO drastically reduced HuR PARylation in differentiated muscle cells (**Figure 2.5b**). Together, these results, suggest that during muscle cell differentiation TNKS1 is the main enzyme responsible for the PARylation of promyogenic RBPs such as HuR.

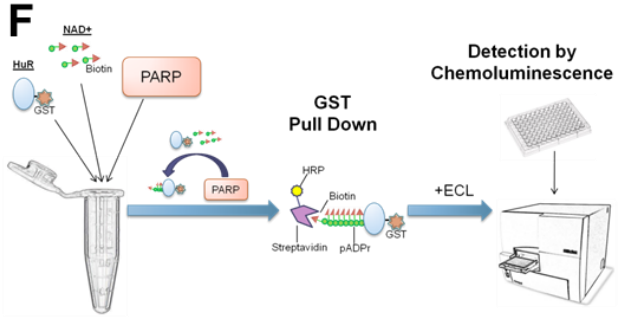


C

RBPs involved in Myogenesis
DDX3X
ELAVL1 (HuR)
G3BP1
HNRNPA1
MATRIN-3
PCBP1
YBX1







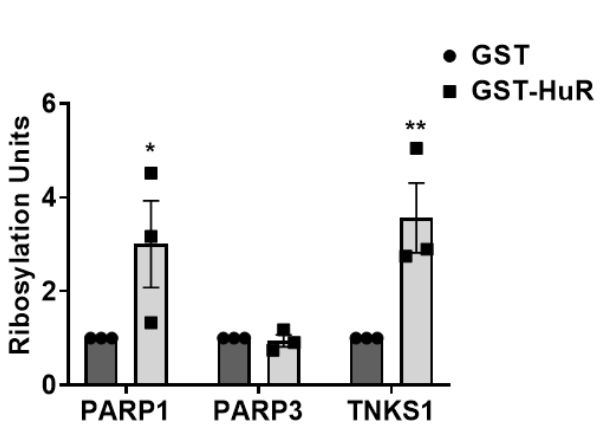


Figure 2.4. TNKS1 PARylates promyogenic RNA-Binding Proteins such as HuR.

A) Total cell extracts isolated from differentiating C2C12 cells (Day 2) were used for immunoprecipitation experiments with pADPr or IgG antibodies and were analyzed by mass spectrometry. The immunoprecipitation of pADPr was validated by western blot analysis using a pADPr antibody. The blot shown is a representation of three independent experiments. **B)** The identified proteins were classified according to their gene ontology molecular function, and the percentage of proteins identified in each molecular function is represented in a pie chart. **C)** A list of RNA-binding proteins identified in B that have been previously associated with myogenesis. **D)** Total extracts isolated from confluent C2C12 myoblasts (D0) were used for immunoprecipitation experiments with pADPr or IgG antibodies. Association of RNA-binding proteins such as HuR and KSRP to pADPr was determined by western blot analysis. The blot shown is a representation of three independent experiments. **E)** Immunoprecipitation experiments using pADPr or IgG antibodies were performed with extracts from confluent (D0) C2C12 cell transfected with scrambled control (siCTRL) or siRNA against TNKS1 (siTNKS1). Association of HuR to pADPr was determined by western blot analysis. The blot shown is a representation of three independent experiments. **F)** Schematic representation of the *in vitro* ribosylation assay procedure. GST-HuR and biotinylated NAD^+ were incubated with recombinant PARP1, PARP3 or TNKS1 enzymes. HRP-conjugated streptavidin was added to the reaction and the signal was measured as arbitrary units by chemiluminescence, by detection with luminol. **G)** Quantification of Ribosylation units demonstrating the PARylation of GST-HuR by PARP1 and TNKS1, but not PARP3, *in vitro.* Levels are normalized to GST control. Data shown in Figure 2 are presented +/- the s.e.m. of 3 independent experiments with *P <0.05, **P <0.01 by unpaired *t*-test.

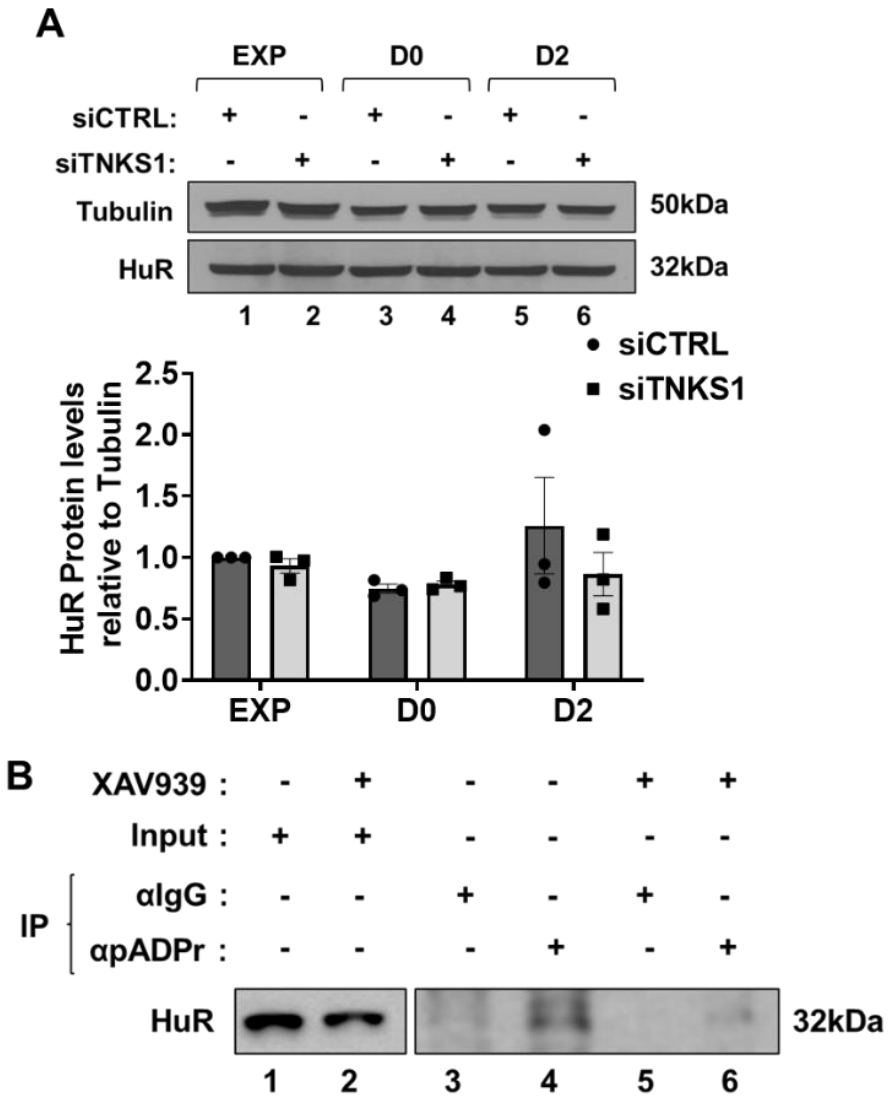


Figure 2.5. A) Total cell extracts were prepared from C2C12 cells that were transfected with scrambled control (siCTRL) or siRNA against TNKS1 (siTNKS1) and collected from exponentially growing (EXP) and differentiating C2C12 myoblasts (Day 0 and Day 2). (Top panel) Extracts were used for western blot analysis with antibodies against HuR or α-tubulin (loading control). (Lower panel) Quantification of the western blot. Values were quantified using ImageJ and normalized to tubulin quantification and shown relative to the EXP siCTRL treated condition. Data shown are presented +/- the s.e.m. of 3 independent experiments. **B)** Immunoprecipitation experiments using pADPr or IgG antibodies were performed with extracts from confluent (D0) C2C12 cell treated with 10uM XAV939 or DMSO treated control. Association of HuR to pADPr was determined by western blot analysis. The blot shown is a representation of three independent experiments.

2.4.3 TNKS1-meedaited PARylation is required for HuR function during myogenesis

One of the main features of the promyogenic function of HuR is its functional dichotomy. Indeed, others and we have demonstrated that to promote myogenesis HuR simultaneously exercises two opposite functions on some of its target mRNAs: the decay of *NPM* and the stability of *myogenin* [183, 319, 401, 471, 482, 486]. Therefore, we investigated the impact of TNKS1-mediated PARylation on these two opposite but complementary functions of HuR during myogenesis. Our data show that the depletion of TNKS1 in C2C12 cells, similarly to what was observed for the knockdown of HuR [152, 183, 482], differentially impacted the expression levels of NPM (increase) and Myogenin (decrease) mRNAs and proteins (**Figures 2.6a-c**). Next, we performed immunoprecipitation experiments with the anti-HuR antibody on extracts from differentiating C2C12 cells depleted or not of TNKS1, and the presence of *NPM* and *myogenin* mRNAs was assessed by RT-qPCR analysis. Our data show that TNKS1 depletion significantly reduced (by >3 fold) the association of HuR to both *NPM* and *myogenin* mRNAs (**Figure 2.6d-e**), effects that were also reproduced using XAV939 (**Figure 2.7a-e**). Next, actinomycin D pulse-chase experiments [152, 319, 482] were used to determine the impact of TNKS1 on the half-lives of *NPM* and *myogenin* mRNAs. The knockdown of TNKS1 in C2C12 cells, similarly to HuR depletion [152, 183, 482], increased the half-life of *NPM* mRNA, while at the same time significantly decreased the stability of *myogenin* mRNA (**Figures 2.6f-g**). Therefore, together, these observations clearly establish that the TNKS1-mediated PARylation of RBPs such as HuR is required for the posttranscriptional regulation of key promyogenic factors such as NPM and myogenin.

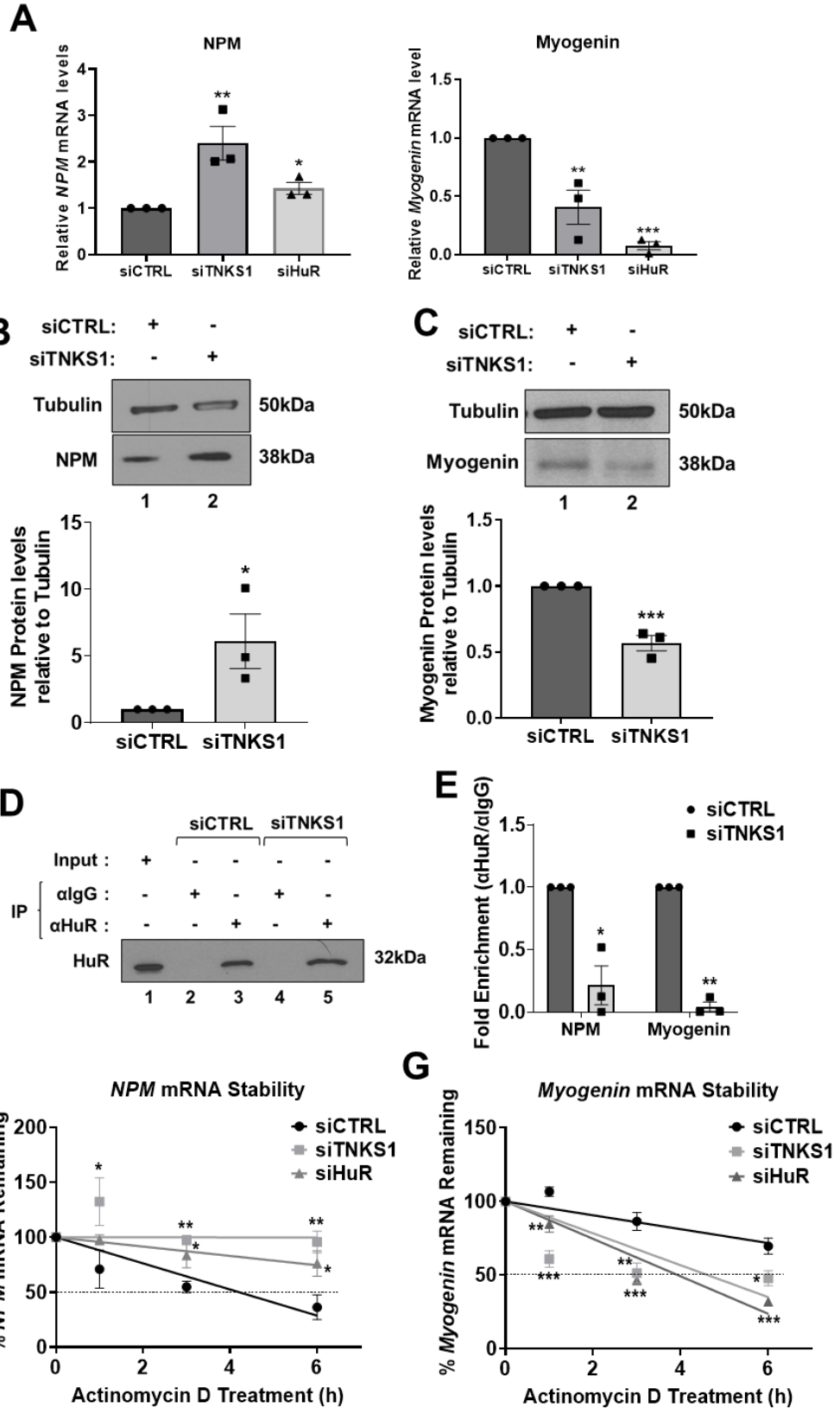

A
NPM
Relative NPM mRNA levels
siCTRL
siTNKS1
siHuR
Myogenin
Relative Myogenin mRNA level
B
siCTRL:
siTNKS1:
Tubulin
50kDa
NPM
38kDa
NPM Protein levels relative to Tubulin
C
Myogenin
Myogenin Protein levels relative to Tubulin
D
Input :
IP
αIgG :
αHuR :
HuR
32kDa
E
Fold Enrichment (αHuR/αIgG)
F
NPM mRNA Stability
% NPM mRNA Remaining
Actinomycin D Treatment (h)
G
Myogenin mRNA Stability
% Myogenin mRNA Remaining

Figure 2.6. TNKS1-mediates binding of HuR to myogenic mRNA targets during myogenesis.

A) Total RNA was isolated from lysates treated with siRNA against TNKS1 or HuR (siTNKS1 and siHuR) or scrambled control (siCTRL), and *NPM* (Right panel) and *myogenin* (Right panel), mRNA levels were determined by RT-qPCR, standardized against *GAPDH* mRNA, and expressed relative to siCTRL conditions. **B-C)** Total extracts from C2C12 myoblasts transfected at the exponential growth phase with scrambled control (siCTRL) or siRNA against TNKS1 (siTNKS1) and collected 48h later (D2), were used for western blot analysis (Top panels) to determine NPM (B) and myogenin (C) protein levels. (Lower panels) Histogram representation of the quantification of the western blot. Values were quantified using ImageJ and normalized to α-tubulin and shown relative to the EXP siCTRL treated condition. **D-E)** RNA-Immunoprecipitation coupled to RT-qPCR experiments was performed using anti-HuR (3A2) and anti-IgG antibodies on total extracts from differentiating C2C12 cells treated with scrambled control (siCTRL) or siRNA against TNKS1 (siTNKS1). (D) Western blot assessing the immunoprecipitation of HuR. (E) *NPM* and *myogenin* mRNA levels in the immunoprecipitates were normalized to the corresponding IgG sample. The levels of *NPM* and *myogenin* mRNA in siTNKS1 conditions were plotted relative to siCTRL conditions. . **F-G)** Actinomycin D (Act. D) pulse-chase assays were performed using C2C12 myoblasts transfected with scrambled control (siCTRL) or siRNA against TNKS1 (siTNKS1). 48h after differentiation the cells were treated for various periods of time with Actinomycin D to assess the stability of *NPM* (F) and *myogenin* (G) mRNAs. Data shown in Figure 3 are presented +/- the s.e.m. of 3 independent experiments with *P <0.05, **P <0.01, ***P <0.001 by unpaired *t*-test.

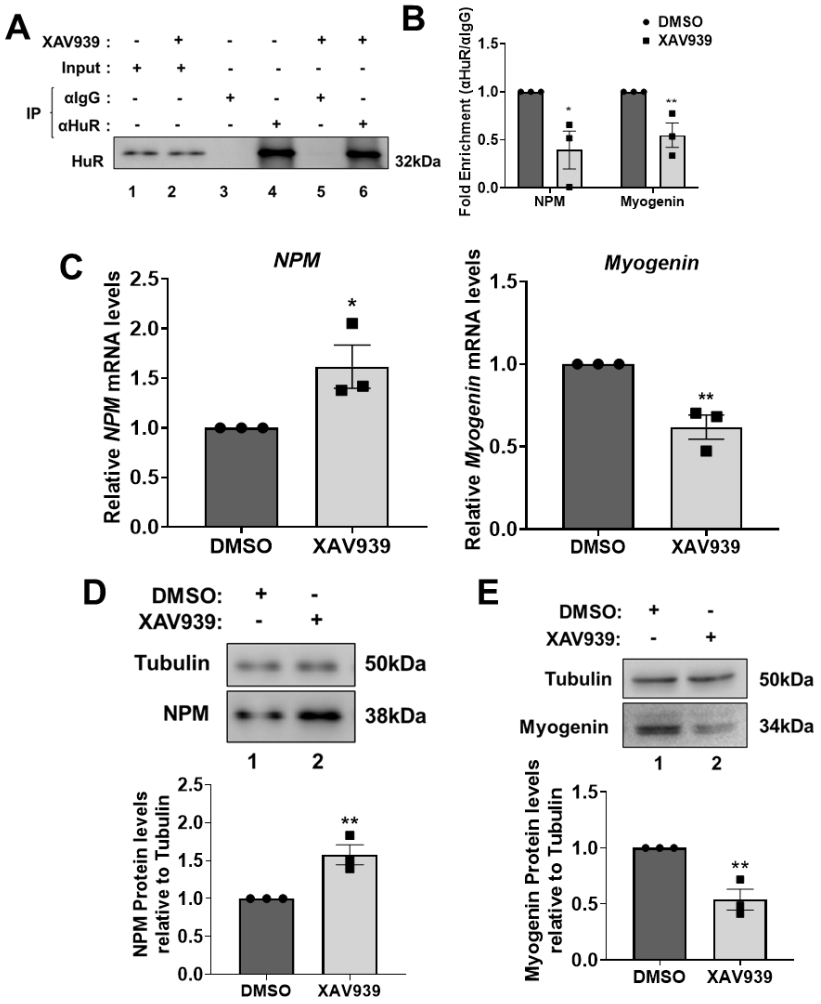


Figure 2.7. A-B) RNA-Immunoprecipitation coupled to RT-qPCR experiments was performed using anti-HuR (3A2) or anti-IgG antibodies on total extract from differentiating C2C12 cells that were treated with 10uM XAV939 or DMSO as a control. **A)** Western blot assessing the immunoprecipitation of HuR. **B)** The levels of *NPM* and *myogenin* mRNAs in the immunoprecipitates were normalized to the corresponding IgG sample. The *NPM* and *myogenin* mRNA levels in XAV939-treated conditions were plotted relative to DMSO conditions. **C)** Total RNA was isolated from differentiating C2C12 cells (Day 2) that were treated with 10uM XAV939 or DMSO as a control. The levels of *NPM* and *myogenin* mRNAs were determined by RT-qPCR, standardized against *GAPDH* mRNA, and expressed relative to siCTRL conditions. **D-E)** Lysates were prepared from C2C12 cells as described in A and used for western blot analysis (Top panels) using antibodies against NPM, myogenin, or α-tubulin (Loading control). (Lower panels) Quantification of the western blots. Values were quantified using ImageJ and normalized to tubulin, then to the DMSO condition. Data shown are presented +/- the s.e.m. of 3 independent experiments with *P <0.05, **P <0.01, ***P <0.001 by unpaired *t*-test.

We have previously shown that the cytoplasmic translocation of HuR is essential for its pro-myogenic function [471, 481]. In fact, during muscle cell differentiation, about 10-15% of HuR move to the cytoplasm where they are cleaved by caspase-3/7 generating two cleavage products: HuR-CP1 (24kD) and HuR-CP2 (8kD) [471, 481]. We also showed that HuR-CP1 competes with HuR for the binding to transportin-2 (TRN2), an import factor responsible of the movement of HuR from the cytoplasm to the nucleus [471, 481]. Consequentially, HuR-CP1 competes with the remaining non-cleaved HuR for its association with TRN2, causing the cytoplasmic accumulation of HuR [471, 481]. Therefore, as a next step, we determined the impact of depleting TNKS1 on the localization and the cleavage of HuR during myogenesis. Immunofluorescence experiments assessing the localization of HuR in differentiating muscle cells showed that, while as expected [319], HuR partially accumulates in the cytoplasm in control conditions, HuR is completely sequestered in the nucleus upon TNKS1 knockdown (**Figure 2.8a**). Similarly, treating cells with XAV939 also resulted in the nuclear accumulation of HuR (**Figure 2.9**). We then verified if this nuclear accumulation is the result of an inhibition of HuR export from the nucleus, or rather an increased import of HuR from the cytoplasm. Immunoprecipitation assays using anti-TRN2 or anti-IgG antibodies indicated that in muscle cells depleted of TNKS1, HuR associates more to TRN2, suggesting an increase in TRN2-mediated HuR import (**Figure 2.8b**). Additionally, the observed nuclear accumulation of HuR should in principle correlate with a decrease in its caspase-mediated cleavage. This was indeed the case since the depletion of TNKS1 in C2C12 resulted in a significant decrease in the levels of HuR-CP1 (**Figure 2.8c**). As such, our results show that TNKS1-mediated PARylation promotes the pro-myogenic function of HuR not only by enabling its interaction with target messages, but also by promoting its cytoplasmic accumulation and caspase-mediated cleavage.

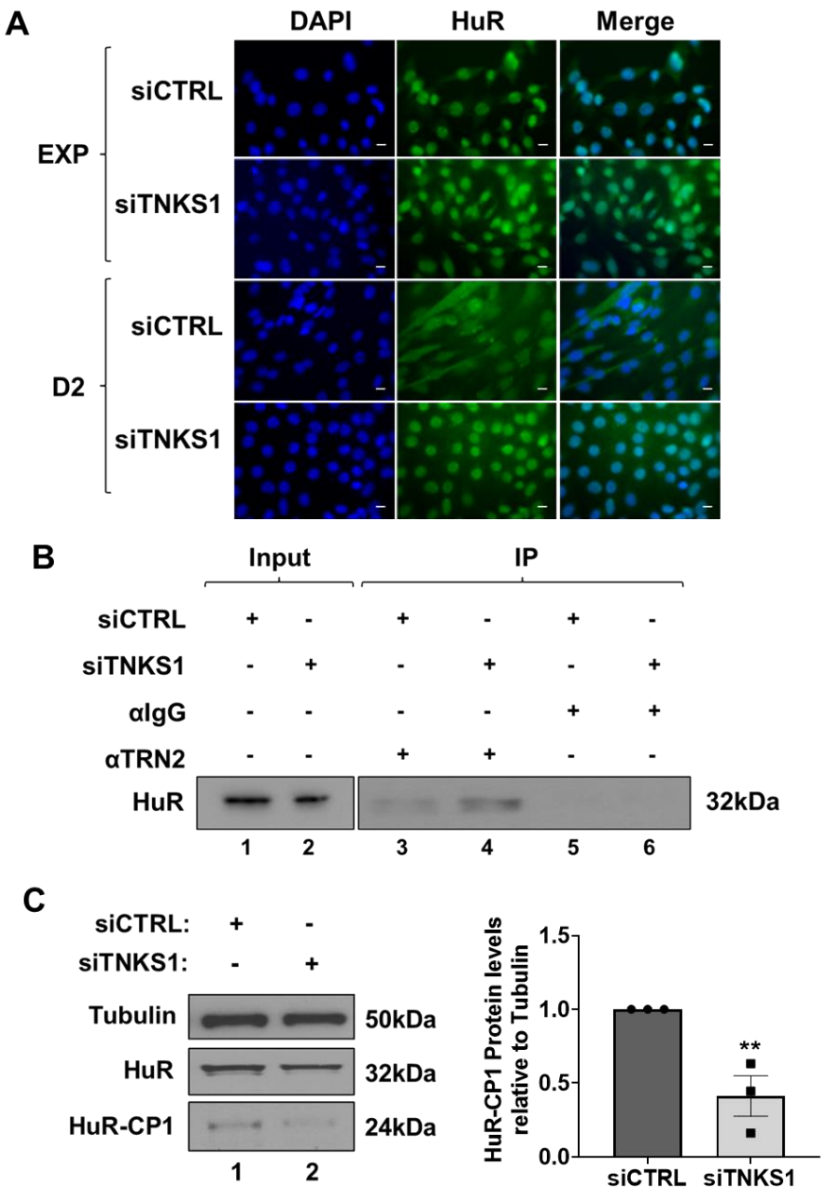


Figure 2.4. TNKS1-mediated PARylation of HuR regulates its cellular movement.

A) Immunofluorescence images showing the localization of HuR in exponentially growing (EXP) and differentiating (Day 2) muscle myoblasts transfected with scramble control (siCTRL) or siRNA against TNKS1 (siTNKS1). Immunofluorescence staining was performed with an antibody against HuR. DAPI was used to stain nuclei. Images of a single representative field are shown and are representation of three independent experiments. (Scale bars, 20µm.) **B)** Immunoprecipitation experiments using TRN2 or IgG antibodies were performed with extracts from differentiating (D2) C2C12 cells transfected with scrambled control (siCTRL) or siRNA against TNKS1 (siTNKS1). Association of HuR to TRN2 was determined by western blot analysis. The blot shown is a representation of three independent experiments. **C)** Total extracts from C2C12 myoblasts transfected at the exponential growth phase with scrambled control (siCTRL) or siRNA against TNKS1 (siTNKS1) and collected 48h later (D2), were used for western blot analysis (Left panel) to determine HuR-CP1 protein levels. (Right panel) Histogram representation of the quantification of the western blot. Values were quantified using ImageJ and normalized to a-tubulin and shown relative to the siCTRL treated condition. Data shown are presented +/- the s.e.m. of 3 independent experiments with **P <0.01 by unpaired *t*-test.

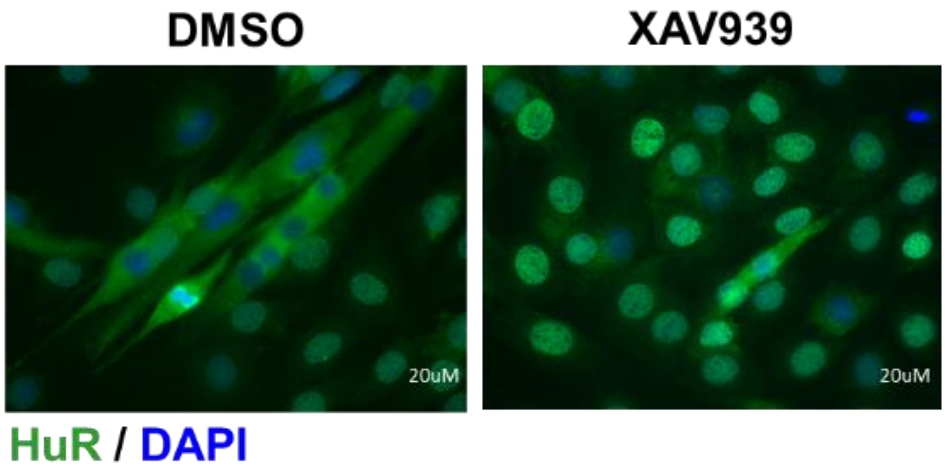


Figure 2.9. Immunofluorescence images showing the localization of HuR in differentiating (Day 2) muscle myoblasts treated with 10uM XAV939 or DMSO (used as a control). Immunofluorescence staining was performed with an antibody against HuR. DAPI was used to stain nuclei. Images of a single representative field are shown and are representation of three independent experiments. (Scale bars, 20µm.)

2.4.4 TNKS1 associates with HuR via a conserved Tankyrase-binding motif

It is well established that TNKS1-mediated enzymatic activity requires the direct association of TNKS1 to its target proteins via a consensus motif known as the tankyrase binding motif (TBM) [383, 487-489]. TBM consists of six residues (RXXPXG) with arginine and glycine being the most critical for binding [383, 487-490]. Interestingly, scanning the primary sequence of HuR we identified a potential TBM in its C-terminal region (^{219}**R**FS**P**M**G**^{224}) (that we dub HuR-TBM) that is conserved across different species such as human, rat, mouse, and *xenopus* (**Figure 2.10a**). It is well-established that mutating the glycine residue in the TBM of a given protein completely abolishes its ability to bind TNKS1 [490]. Hence, to assess if the HuR-TBM is required for the interaction of HuR with TNKS1 in myotubes, we generated constructs expressing GFP-HuR wild-type (GFP-HuR^{WT}) or GFP-HuR containing a glycine (G) → aspartate (D) mutation at the 224 position (GFP-HuR^{G224D}) (**Figure 2.10b**). Our data show that the G→D mutation at the 224 residue abolished the interaction of HuR with TNKS1 (**Figure 2.10c**). Subsequently, we conducted an *in vitro* PARylation assay using GST-HuR^{WT} or GST-HuR^{G224D}, and GST alone as a negative control to assess the importance of the

HuR-TBM for the TNKS1-mediated PARylation of HuR (**Figure 2.10d** and **Figure 2.11**). We observed a substantial decrease in the PARylation levels of GFP-HuR^{G224D} when compared to its wild-type counterpart (**Figure 2.10d**), suggesting that an intact HuR-TBM is required for the TNKS1-mediated PARylation of HuR *in vitro*. To determine the importance of this motif in HuR PARylation in myotubes, we performed immunoprecipitation experiments with anti-pADPr or anti-IgG antibodies on myotube extracts expressing the two HuR forms. Consistent with the above-mentioned results, the PARylation level of GFP-HuR^{G224D} was substantially reduced when compared to that of GFP-HuR^{WT} (**Figure 2.10e**). Rescue experiments in HuR-depleted muscle cells showed that GFP-HuR^{WT} but not its mutant counterpart was able to re-establish the expression of HuR mRNA targets *NPM* and *myogenin* (**Figure 2.12a**) as well as the ability of these cells to enter myogenesis (**Figure 2.12b** and **Figure 2.13**). In addition, RNA-immunoprecipitation experiments with anti-GFP antibody on extracts from cells expressing the two HuR isoforms, showed that these effects on myogenesis and the expression of *NPM* and *myognin* mRNAs are due to the inability of GFP-HuR^{G224D} to associate with these messages (**Figure 2.12c-d**). Together, these observations demonstrate that HuR harbors a *bonafide* TBM that is required for its PARylation by TNKS1 and that this posttranslational modification is essential for its promyogenic function.

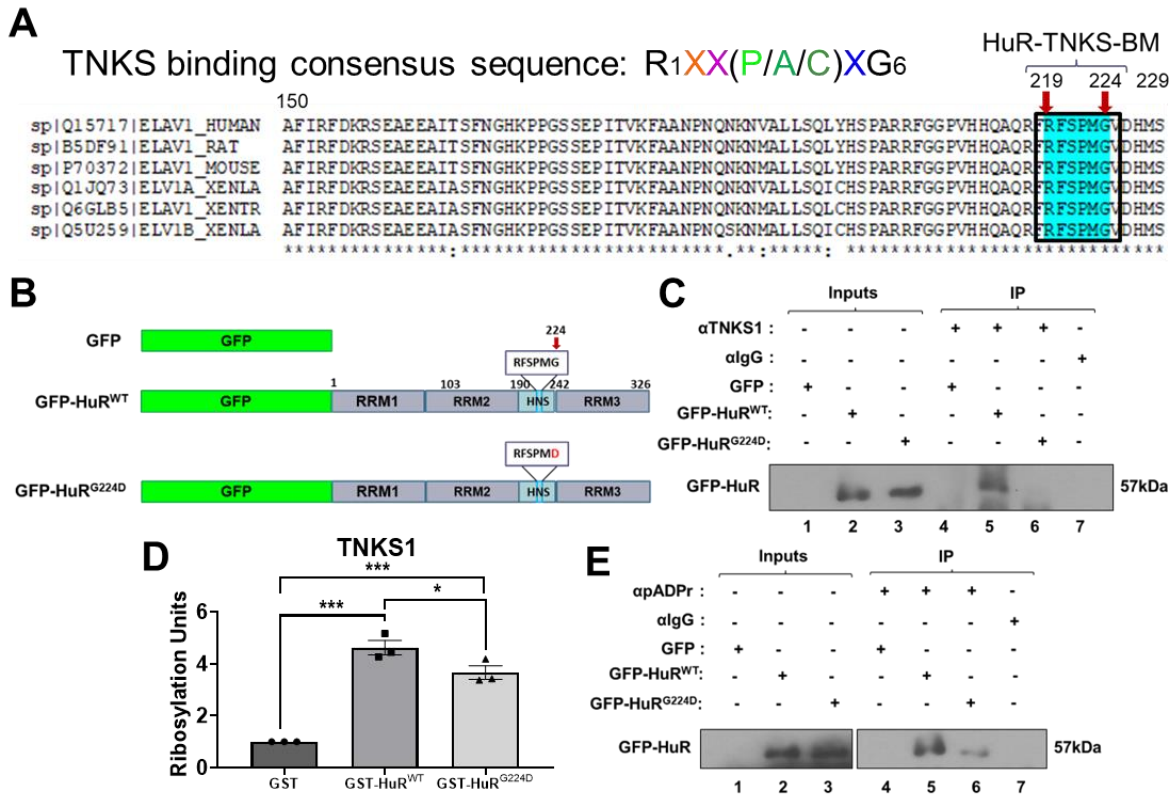


Figure 2.10. HuR contains a TNKS1 consensus binding motif.

A) Swiss-Prot Database (www.expasy.ch/prosite) blast of the putative TNKS1-binding motif identified in HuR. **B)** Schematic of the GFP, GFP-HuR^{WT} and GFP-HuR^{G224D} constructs. **C)** Immunoprecipitation experiment using differentiating C2C12 cells expressing GFP, GFP-HuR^{WT} and GFP-HuR^{TBM} were performed using anti-IgG or anti-TNKS1 antibodies to assess the association of the exogenous proteins to TNKS1 by western blot analysis. The data are representative of three independent experiments. **D)** *In vitro* PARylation assay: Quantification of Ribosylation units showing the PARylation of GST-HuR^{WT} and GST-HuR^{G224D} by recombinant TNKS1 *in vitro*. Levels are normalized to GST control. Data shown are presented +/- the s.e.m. of 3 independent experiments with *P <0.05, **P <0.01, ***P <0.001 by unpaired *t*-test. **E)** Immunoprecipitation experiment using differentiating C2C12 cells expressing GFP, GFP-HuR^{WT} or GFP-HuR^{G224D}, were performed with anti-IgG and anti-pADPr antibodies to assess the PARylation by western blot analysis. The blot is representative of three independent experiments.

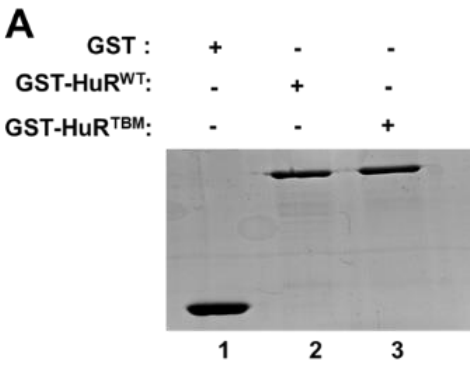


Figure 2.11. Coomassie staining of GST, GST-HuR^{WT} and GST-HuR^{G224D} used in Ribosylation assay in Figure 4D.

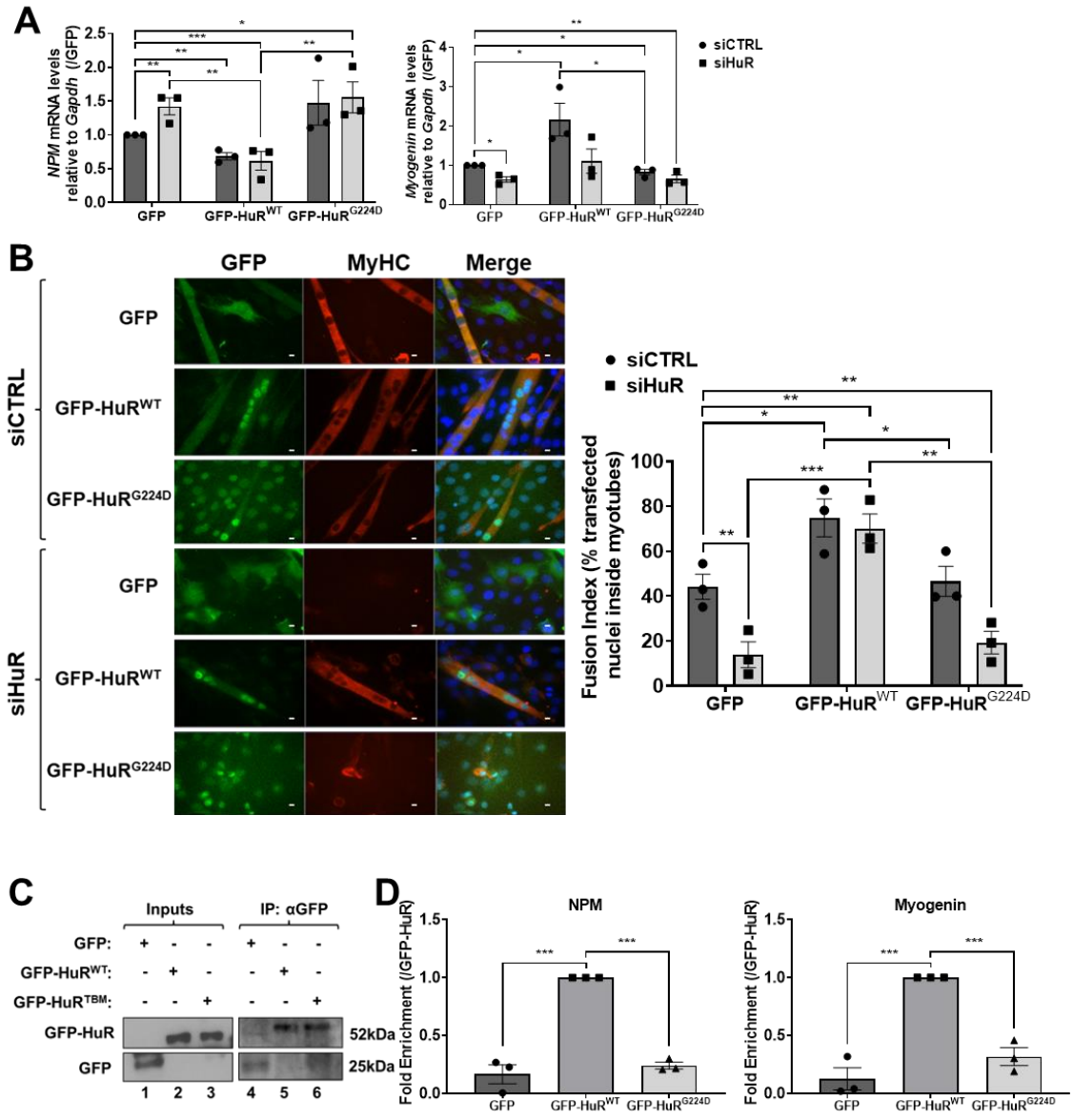


Figure 2.12. TNKS1 mediated PARylation of HuR is required for its pro-myogenic function.

C2C12 cells were transfected with scrambled control (siCTRL) or siRNA against HuR (siHuR), and then constructs expressing GFP, GFP-HuR^{WT} or GFP-HuR^{G224D}. **A)** Total RNA was isolated from these C2C12 and the level of *NPM* (Right graph) and *myogeninc* (Left graph) mRNAs was assessed by RT-qPCR, standardized against *GAPDH* mRNA, and expressed relative to siCtrl+GFP conditions. Values Data represented as mean ± SEM (*n* = 3) with *P <0.05, **P <0.01, ***P <0.001 by unpaired *t*-test. **(B)** (Right panel) immunofluorescence (staining with anti-MyHC and anti-GFP antibodies, as well as with DAPI to stain nuclei) images of cells described in A assessing rescue of the myogenic phenotype in HuR knockdown cells. (Scale bars, 50µm and 20µm respectively). (Left Panel) Histogram representation of the fusion index of immunofluorescence shown in C. Data represented as mean ± SEM (*n* = 3) with *P <0.05, **P <0.01, ***P <0.001 by unpaired *t*-test. **(C-D)** RNA-Immunoprecipitation experiment using differentiating C2C12 cells expressing GFP, GFP-HuR^{WT} and GFP-HuR^{G224D} were performed using anti-GFP antibodies. (C) Western blot confirming the immunoprecipitation of GFP-conjugated HuR isoforms. The blot is representative of three independent experiments. (D) Association of GFP, GFP-HuR^{WT} and GFP-HuR^{G224D} to *NPM* (Right panel) *or myogenin* (Left panel) was determined by RT-qPCR analysis.

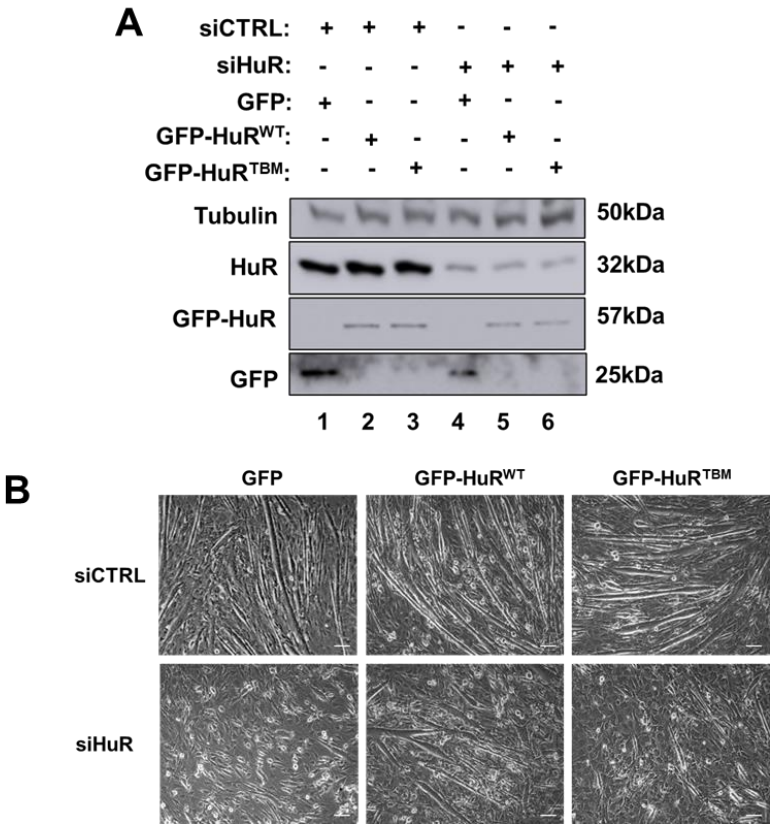


Figure 2.13. A) Knockdown of HuR and overexpression of GFP, GFP-HuR^{WT} and GFP-HuR^{G224D} were assessed by western blot analysis using antibodies against HuR, GFP, or α-tubulin (loading control). The blot is representative of three independent experiments. **(B)** Phase contrast images of cells described in A assessing rescue of the myogenic phenotype in HuR knockdown cells. (Scale bars, 50µm and 20µm respectively)

2.5 Discussion

Although PARylation has been shown to be implicated in the onset of several skeletal muscle pathologies [423, 424, 426, 428, 466, 467], its role in the induction of muscle cell differentiation remains elusive. In this study we investigated the importance of this posttranslational modification in the myogenic process. We showed that TNKS1-mediated PARylation is required for muscle cell differentiation. Our data demonstrate that TNKS1 is essential for this process since its depletion or chemical inhibition reduced muscle fiber formation. We show that these effects are due to TNKS1-mediated PARylation of the RBP HuR. TNKS1 modulates the cytoplasmic accumulation of HuR, as well as the binding of HuR to its mRNA targets, such as *NPM and myogenin* resulting in the regulation of their turnover. We, furthermore, showed that TNKS1 binds HuR via a conserved consensus motif (HuR-TBM). Mutating the TBM of HuR prevented the interaction of HuR with target messages and the rescue of their expression in HuR depleted conditions. More importantly, the mutant could not rescue the myogenic phenotype when overexpressed in cells depleted of HuR, a condition known to impair muscle fiber formation [483]. Thus, our work reveals a new mechanism where the TNKS1-mediated PARylation of HuR is a key requisite for the induction of muscle cell differentiation (**Figure 2.14**).

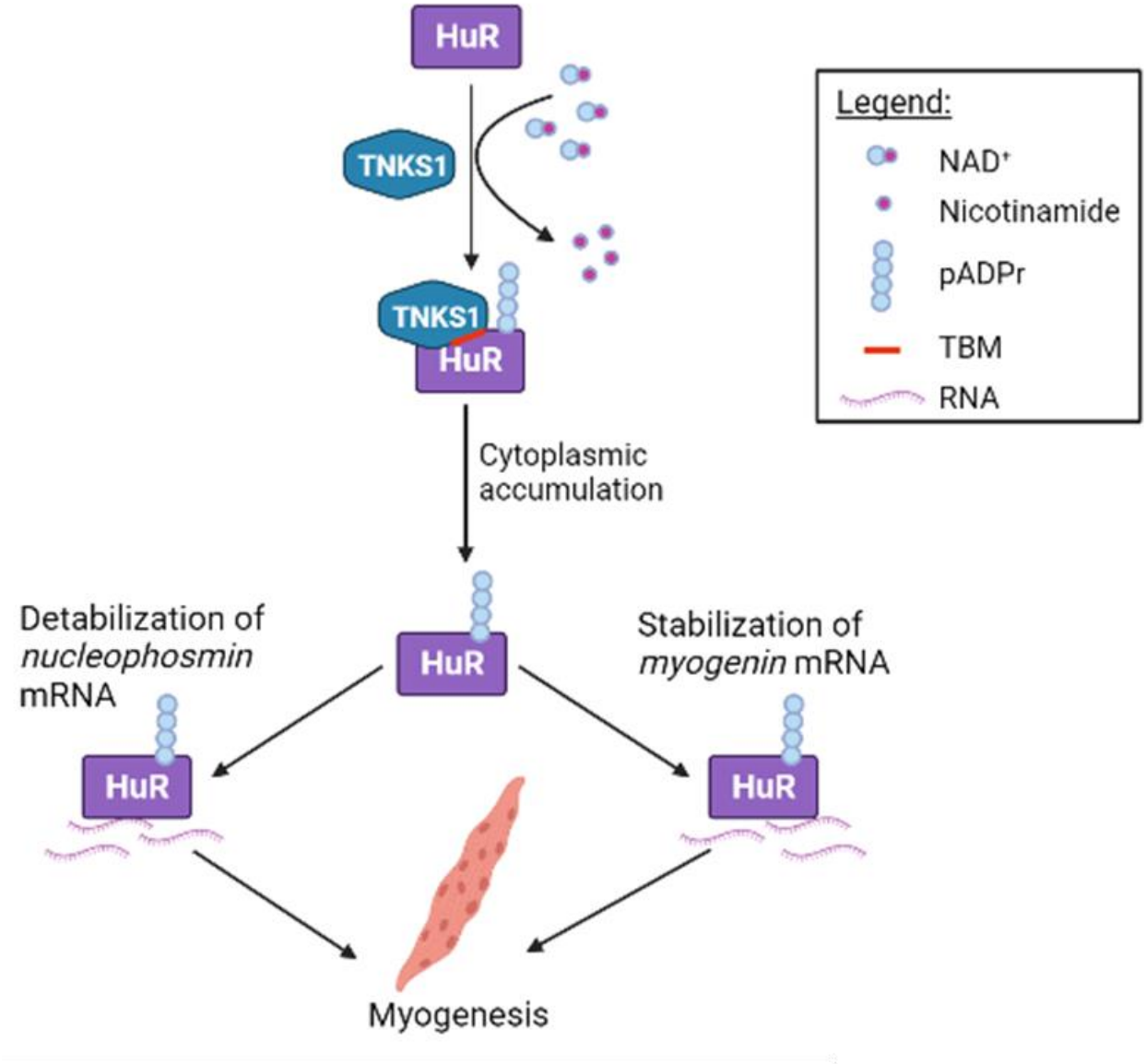


Figure 2.14. ***Model depicting the mechanism by which TNKS1-mediated PARylation of the promyogenic RBP HuR impact myogenesis.***

TKNS1-mediated PARylation promotes the cytoplasmic translocation of 10 to 15% of total HuR. In the cytoplasm, PARylated HuR undergoes caspase-mediated cleavage generating HuR-CP1 that, in turn, binds with TRN2 to prevent the import to the nucleus of the remaining non-cleaved fraction of HuR [481]. The combination of our published data [481] and the observation reported in this study suggest that by retaining its PARylation status, this cytoplasmic fraction of HuR promotes myogenesis by differentially regulating the turnover and expression of its target mRNA *NPM* (decay) and *myogenin* (stability).

Compounds that inhibit PARP1/2-mediated PARylation activity have been shown to ameliorate muscle performance/function in muscle related diseases such as sarcopenia, Duchene muscle dystrophy, and cachexia [421, 424, 426, 428, 429, 466]. The onset of these diseases, therefore, seems to correlate with an increase in the activity of PARP1/2. It is not surprising, however, that TNKS1-mediated PARylation beneficially impacts muscle cell differentiation. Our data indicate that PARP1 is not involved in myogenesis, and that it is rather TNKS1 that is involved in the process. Although both PARP1 and TNKS1 mediate PARylation of proteins, both have unique and specific roles in various cellular processes. Thus, the fate of muscle (whether it is formed or wasted) may depend on the specific PARP which is expressed and active under these conditions. Interestingly, our results suggest that TNKS1-mediated PARylation activity beneficially modulates the formation of skeletal muscle. In agreement with this, TNKS1 is known to regulate the canonical Wnt signalling pathway which was previously shown to be involved in embryonic and adult skeletal muscle formation [384, 389, 401-407]. Knockout mouse models of Wnt or Wnt signalling effectors display early embryonic lethality due to pronounced tissue damage and poor muscle development [408]. During adult myogenesis or regeneration, Wnt1 has been shown to induce the expression of the MRF Myf5, whereas Wnt3 is involved in satellite cell differentiation [410, 491]. Additionally, both Wnt1 and Wnt3 are heavily involved in somitic myogenesis [492]. Interestingly, others have also shown that XAV939 treatment decreases PARylation in rat L6 skeletal muscle cells [386]. Therefore, the PARylation of RBPs such as HuR by TNKS1, in addition to the activation of the Wnt signalling pathway, may explain the importance of TNKS1 in modulating the myogenic process.

Posttranslational modifications of HuR have been previously shown to play an important role in regulating its function. For example, phosphorylation of HuR by the G2-phase kinase CDK1 on the Ser202 residue promotes the interaction of HuR with 14-3-3, resulting in its nuclear localization [230]. The caspase-mediated cleavage of HuR on the D226 residue is another modification that modulates the localization of HuR during apoptosis and myogenesis leading respectively to the stabilization and expression of pro-apoptotic and pro-myogenic messages [481, 493]. Posttranslational modifications can also impact the interaction of HuR with target messages. For instance,

phosphorylation of HuR by the cell cycle checkpoint kinase CHK2 upon IR treatment led to a global decrease in HuR association to mRNA [346]. More recently, HuR was shown to be modified by PARP1—mediated PARylation under inflammatory conditions and that this modification impacted the localization and the RNA-binding activity of HuR [349]. Indeed, in activated macrophages, PARP1 PARylates HuR on the D226 residue and promotes its association to proinflammatory messages. Our work uncovers that PARylation is also important for the promyogenic function of HuR. However, it is TNKS1 but not PARP1 that is responsible for the PARylation of HuR during myogenesis. TNKS1-mediated PARylation of HuR promotes its mRNA binding ability and function during myogenesis. Additionally, in this work, we show that TNKS1-mediated PARylation is essential for the cytoplasmic accumulation of HuR and its cleavage during myogenesis. Importantly, the cytoplasmic accumulation of HuR was shown to be a crucial event for the myogenic process and is associated to the stabilization function of HuR [183, 319, 471, 483].

Posttranslational modification of proteins is known to play an important role in mediating protein-protein interactions. In some instances, it has also been shown to regulate the interactions of RBPs with other proteins during myogenesis [148, 288, 494-498]. Indeed, HuR is known to collaborate or compete with other RBPs to regulate the stability of target messages [85-87]. As such, since one of the impacts of PARylation is to modulate protein-protein interactions, it is possible that TNKS1-mediated PARylation of HuR during myogenesis modulates its interactions with protein partners to differentially regulate the expression/stability of its mRNA targets. In fact, our group has previously shown that during the early steps of the myogenic process, HuR forms a complex with KSRP to promote the degradation of the *NPM* mRNA and that this event is required for the commitment of muscle cells to the differentiation process [39]. The fact TNKS1-mediated PARylation is also important for this event, raises the possibility that this modification is also important for the association of HuR with protein ligands such as KSRP.

Our study, therefore, uncovers the importance of TNKS1-mediated PARylation as a key determinant of skeletal muscle formation. This outcome occurs, in part, due to the PARylation of RBPs, such as HuR, that regulate the expression of mRNAs encoding factors that control the fate of skeletal muscle.

2.6 Materials and Methods

Cell Culture. C2C12 muscle cells (ATCC, Manassas, VA, USA) were grown and maintained in 20% fetal bovine serum (Sigma) and 1% penicillin/streptomycin antibiotics in DMEM (Dulbecco's modified Eagle medium) (ThermoFisher). Cells were grown in a humidified incubator at 37°C, 5% CO_2. To induce muscle differentiation, cells were switched to differentiation media (DMEM containing 2% horse serum (ThermoFisher) and 1% penicillin/streptomycin antibiotics) when they reached 100% confluency.

XAV939 Treatment. C2C12 cells were treated with 10 μM XAV939 or DMSO as a negative control during the exponential phase and, additionally, upon induction of muscle cell differentiation.

Plasmids and GST-tagged protein expression. The pGEX-6P1 plasmids containing the full length HuR were generated as previously described [171]. The GST-HuR^{G224D} plasmid was generated by Norclone Biotech Laboratories. BL21 bacteria were transformed with either GST or the GST-HuR constructs described above. The expression of the proteins was induced by IPTG (0.5mM for 4hours at 37°C) in a 1-liter culture. The bacteria were collected and lysed. The GST proteins were pulled down using Glutathione Sepharose beads.

Transfection. Transfections with siRNAs were performed when cells reached 50-60% confluency using Jetprime (Polyplus Transfection) for 48 hours according to the manufacturer's instructions. For DNA plasmid, cells were transfected at 70% confluency with 1 μg/ml of plasmid DNA for 24 hours using Jetprime as well. Cells were then switched to differentiation media when 100% confluency is reached and collected as indicated at various time points after the induction of differentiation. siRNAs used are listed in Suppl. Table 3. The GFP and GFP-HuR plasmids were generated as described in [493] while the GFP-HuR^{G224D} construct was generated by Norclone Biotech Laboratories. For the rescue experiments, subconfluent C2C12 cells were transfected with siRNA against HuR which specifically targets the 3'UTR of the mRNA to avoid targeting of the exogenous GFP-HuR constructs that were transfected the next day. On the following day, a second hit of transfection was performed for 4 hours, followed by a transfection using GFP, GFP-HuR, and GFP-HuR^{G224D} plasmids.

Immunofluorescence. Cells were fixed in 3% paraformaldehyde (Sigma) for 20 minutes. They were then permeabilized with a solution containing 0.5% Triton X-100 and 1% goat serum in phosphate-buffered saline (PBS) with agitation for 15 minutes. After washing with 1% goat serum in PBS, cells were incubated with primary antibodies against myosin heavy chain (MF-20, developmental studies Hybridoma Bank, 1:250), myoglobin (Abcam, 1:500) diluted in 1% goat serum in PBS for one hour at room temperature. Following further washing, cells were incubated with appropriately labeled Alexa Fluor® (Invitrogen) secondary antibodies (1:1000) for an additional hour at room temperature. 4',6-diamidino-2-phenylindole (DAPI) staining was used to visualize nuclei. Cells were visualized using a Zeiss Axio Observer.Z1 inverted microscope with a 40X oil objective, and images were obtained using an AxioCam MRm digital camera.

Fusion index. The fusion index was used to determine the efficiency of C2C12 differentiation. It was quantified by calculating the ratio of the number of nuclei in myotubes versus the total number of nuclei counted in the same field.

Protein Extraction and immunoblotting. Total cell extracts were prepared by lysing cells with mammalian lysis buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 10% glycerol, 1% Triton, 10 mM pyrophosphate sodium, 100 mM NaF, 1 mM EGTA, 1.5mM $MgCl_2$, 1 X protease inhibitor (Roche) and 0.1 M orthovanadate) for 15 minutes on ice, with vortexing every 5 minutes. Lysates were collected after centrifugation for 15 minutes at 12000rpm. The lysates were run on SDS-PAGE and transferred on nitrocellulose membranes (Bio-Rad), and then analysed by western blotting using antibodies against HuR (3A2), pADPr (96-10), TNKS1, KSRP, myogenin and NPM. Quantifications were performed using ImageJ and normalized to tubulin. Antibodies used are listed in Suppl. Table 4.

In vitro Ribosylation Assay. The PARP Universal Chemilumunescent assay kit (Trevigen #4676-096K) was used to test the PARylation of recombinant HuR by PARP1 (Enzo Life Sciences Enzo Life Sciences # ALX-201-063-C020), PARP3 (Enzo Life Sciences Enzo Life Sciences # 201-170-C020), and TNKS1 (BPS Bioscience # 80504) (Figures 2 F-G). The TNKS1 Histone Ribosylation assay kit (Biotin-labeled NAD+) (BPS Bioscience # 80573) was used to compare the TNKS1-mediated PARylation of

recombinant HuR and HuR^{G224D}, since this kit is optimized for TNKS1-mediated PARylation. The assays were performed as per the manufacturer's protocol, except that 1.5 mL tubes were used rather than the 96-well plate provided. Briefly, PARP enzymes, biotinylated NAD+, and the PARP buffer were incubated with GST or the GST-HuR isoforms for one hour at room temperature. GST-Sepharose beads (GE Healthcare) were added to the tubes, which were rotated for 30 minutes. The samples were washed with PBS. HRP-conjugated Streptavidin provided in the kits was added to the beads, which were rotated for 40 minutes at room temperature. The beads were washed again and transferred to 96 well plates where luminol (ECL) was added for chemiluminescence measurement by a Synergy Mx Multimode Plate Reader using the Gen5 Data Analysis software.

RNA-Immunoprecipitation: Cells were lysed in lysis buffer (50mM Tris-HCl pH 8.0, 0.5% triton 100X, 150mM NaCl, 100mM NaF, 1X protease inhibitors (Roche)). Pre-washed Protein A beads were incubated with the antibodies for 4 hours, rotating at 4°C. The beads were washed three times with low salt buffer (50 mM Tris pH 8, 0.5% Triton X-100, 150 mM NaCl, 1 X protease inhibitors). 800 ug of total cell extracts were added and the samples were rotated overnight at 4°C. The next day, samples were washed three times with low salt buffer and the co-immunoprecipitated RNA was purified and resuspended in 10 µl of nuclease-free water. 4 µl of the RNA was used for RT-qPCR analysis.

Immunoprecipitation: Lysates were incubated with 5 µg antibodies overnight, rotating at 4°C. The following day, protein A/G magnetic beads (GE healthcare - 17152104011150) were added to the lysates and rotated for an hour at room temperature. The beads were washed three times with washing buffer (25mM Tris-HCl pH 8.0, 650mM NaCl, 0.05% Tween-20, 100mM NaF, 1X protease inhibitors) by placing the tubes in the magnetic stand and removing the washing buffer. The beads were washed once with water. Lamelli dye was added to the tubes and the tubes were rotated for 10 minutes. The supernatant was analyzed by western blot.

Actinomycin D pluse-chase experiments. Cells were transfected with scrambled control or siRNAs against TNKS1 or HuR. Two days after induction of differentiation the

cells were treated for 0h, 1h, 3h, and 6h with 2.5ug/ml of the RNA polymerase II inhibitor, actinomycin D (Act. D) (Sigma - A1410) to assess the stability of *NPM* and *myogenin,* mRNAs. RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's protocol. The level of *myogenin* and *NPM* mRNAs were determined by RT-qPCR and normalized to Gapdh mRNA levels in each sample. The stability was assessed by plotting the mRNA levels relative to the abundance of the messages at 0h of Act. D treatment considered as 100%.

Quantitative RT-PCR. One microgram of total RNA or four microliters of immunoprecipitated RNA was reverse transcribed using the 5X iScript reagent (Bio-Rad) according to the manufacturer's protocol. qPCR was done using 20 folds dilutions of the cDNA using SsoFast EvaGreen Supermix (Bio-Rad). RNA levels of the genes of interest were normalized to Gapdh mRNA levels by calculating the $2^{-\Delta\Delta C_T}$ values, in which $\Delta\Delta C_T$ is the difference in C_T between the gene of interest and the house keeping gene (Gapdh).

Mass Spectrometry. Sample preparation: Following immunoprecipitation, pellets were washed three times with PBS. The pellets were then sent to Southern Alberta Mass Spectrometry Facility for preparation and analysis by mass spectrometry. 583 proteins were identified by selecting for unique count peptides, which are peptides that are identified in unique samples and not in all samples as they are considered background. By eliminating the proteins that were bound to the IgG control, 204 proteins remained (**Suppl. Table 1**). The list of proteins was subjected to PANTHER classification system (http://www.pantherdb.org/), selecting for classification by the Gene Ontology Molecular Function type of characterization.

Statistical Analyses. All values are reported as mean ± standard error of the mean (S.E.M). Significance of the difference between two group means was assessed by 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 (***).

2.7 Author Contributions

SM contributed to conceptualization, conducted the investigation and validation of experimental findings, wrote the original draft, and performed the formal analysis and visualization of experimental findings. DTH contributed to the conceptualization and conducted the preliminary investigation and validation of experimental findings. PMA contributed to the investigation and validations of the experiments involving the XAV939 inhibitor. XJL and helped by performing some of the RNA-Ips, Western Blot and qPCR experiments. SDM assisted with conceptualization, data analysis, and helped edit and review the manuscript. GC helped in the conceptualization, conduction, and analysis of the in vitro ribosylation assays used. DYT provided the technical and experimental expertise with the in vitro PARylation assay and helped with data analysis and interpretation. J-PG provided materials and technical assistant in the execution and trouble shoot the *in vitro* PARylation assays GGP provided provided guidance with all the in vito data as well as helped with data interpretation. I-EG conceptualized, established, and directed the execution of the project, interpreted the data, reviewed, and edited the manuscript.

2.8 Conflict of interest

The authors declare that they have no conflict of interest.

2.9 Acknowledgements

This work was funded by a CIHR operating grant (MOP-142399) and a CIHR project grant (PJT-159618) to IEG. SM was funded by three scholarships received from the Faculty at Medicine of McGill University.

Chapter 3 | Identification of STAT3 as a target of HuR-mediated regulation in Cachexia

3.1 Preface

Recently we showed, using muscle-specific HuR KO mice, that HuR is involved in promoting, *in vivo*, cancer-cachexia-induced muscle wasting [149]. This accentuates an earlier study in our lab that shows the implication of HuR in promoting inflammation-induced muscle wasting through the stabilization of the mRNA encoding the pro-cachectic effector, iNOS [104]. Although very little is known about the function of HuR in this process, the phenotypic evidence *in vivo* calls for further characterization of mechanisms governing the pro-cachectic function of HuR. Therefore, in this chapter, we sought to identify the network of mRNAs targeted by HuR in cytokine-treated muscle cells, which is a cell model of muscle wasting. Our microarray analysis identified the key pro-cachectic factor, STAT3, as a novel target of HuR. To this end, we aimed to decipher the mechanism through which HuR regulates posttranscriptionally the STAT3 mRNA during inflammation-induced muscle wasting.

These findings and discussions were originally published in PNAS in the following manuscript:

Mubaid S, Ma JF, Omer A, Ashour K, Lian XJ, Sanchez BJ, Robinson S, Cammas A, Dormoy-Raclet V, Di Marco S, Chittur SV, Tenenbaum SA, Gallouzi IE. “HuR counteracts miR-330 to promote STAT3 translation during inflammation-induced muscle wasting.” *PNAS* August 2019; 10.1073/pnas.1905172116.

3.2 Abstract

Debilitating cancer-induced muscle wasting, a syndrome known as cachexia, is lethal. Here we report a novel posttranscriptional pathway involving the RNA binding protein HuR as a key player in the onset of this syndrome. Under these conditions, HuR switches its function from a promoter of muscle fiber formation to become an inducer of muscle loss. HuR binds to the *STAT3* mRNA, which encodes one of the main effectors of this condition, promoting its expression both *in vitro and in vivo*. While HuR does not affect the stability and the cellular movement of this transcript, HuR promotes the translation of the STAT3 mRNA by preventing miR-330-mediated translation inhibition. To achieve this effect, HuR directly binds to a U-rich element in the *STAT3 mRNA-3'untranslated region (UTR*) located within the vicinity of the miR-330 seed element. Even though the binding sites of HuR and miR-330 do not overlap, the recruitment of either one of them to the *STAT3-3'UTR* negatively impacts the binding and the function of the other factor. Therefore, together our data establish the competitive interplay between HuR and miR-330 as a novel mechanism via which muscle fibers modulate, in part, STAT3 expression to determine their fate in response to promoters of muscles wasting.

3.3 Significance

Pro-inflammatory diseases, such as cancer, AIDS, and COPD, are often associated with a progressive loss of skeletal muscle tissue, a syndrome also known as cachexia. Cytokines trigger muscle loss by activating downstream effector pathways including the ones driven by STAT3 protein. Although high levels of STAT3 protein is required for the onset of muscle wasting, the mechanisms modulating STAT3 expression in cachectic muscles remain elusive. Here we identify the RNA binding protein HuR and its ability to interfere with miR-330 action as a key promoter of *STAT3* mRNA translation. Our work identifies the competition between HuR and miR-330 as a novel mechanism that could be targeted to design novel anti-cachexia therapies.

3.4 Introduction

Skeletal muscle is the largest organ in the body, accounting for at least 40% of the total body mass of healthy individuals [499]. The maintenance of skeletal muscle mass requires a balance between protein synthesis and protein degradation to ensure continuous renewal of muscle proteins [67, 500]. Chronic diseases such as cancer, AIDS, and COPD (chronic obstructive pulmonary diseases) can disrupt this balance to favor protein degradation, leading to the onset of cachexia, a syndrome characterized by rapid muscle deterioration and wasting [51]. Patients that develop muscle wasting experience weakness, a lower quality of life, a decreased response to therapy, and reduced survival rates [40, 501]. Despite its deleterious effects, there are currently no effective treatment options available for muscle wasting, highlighting the need to better understand the molecular mechanisms mediating this deadly syndrome, which will help identify novel targets for therapy.

It is well-accepted that one of the most common promoters of cachexia-induced muscle wasting is the excessive production of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα, interferon gamma (IFNγ, and interleukin 6 (IL-6) that is triggered by the underlying disease [501]. One way by which these cytokines promote muscle loss is by activating downstream effectors in the targeted skeletal muscle, such as the transcription factors NF-κB and STAT3 (Signal Transducer and Activator of Transcription 3) [502-504]. For example, IL-6 triggers the phosphorylation of STAT3 on Tyrosine-705 (Y705) residue leading to its activation, which in turn promotes the transcription of a STAT3-dependent network of pro-cachectic genes [502, 505]. On the other hand, cytokines such as IFNγ and TNFα promote muscle loss by activating the NF-κB pathway, leading to the transcription of numerous effector genes such as *Atrogin1, MuRF1* and *iNOS* (inducible nitric oxide synthase) [122, 502, 504, 506]. Recent observations have uncovered that IFNγ and TNFα promote muscle loss by also activating the STAT3 pathway in a mechanism that, while independent of IL-6, involves the collaboration of STAT3 with NF-κB [502]. Consistent with previous findings [507], the activation of STAT3 under these conditions is always accompanied by a significant increase in the expression levels of STAT3 protein in the targeted muscles [502]. In keeping with this, increased levels in total STAT3 have been shown to enhance STAT3

activity and also correlate with poor prognosis of cancer patients [508, 509]. Therefore, these observations establish that the increase in the expression levels of promoters of muscle wasting, such as STAT3, is an important molecular event behind the progression of this deadly syndrome. However, the mechanisms controlling the expression of this and other pro-cachectic factors in muscles undergoing wasting are still poorly understood.

The control of gene expression can occur at numerous levels including transcriptionally and post-transcriptionally. While the role of transcriptional events in the onset of muscle atrophy is well-established, the implication of posttranscriptional regulators, such as RNA Binding Proteins (RBPs) and micro-RNAs (miRNAs), in this process has also been reported [506, 510, 511]. Several studies have associated muscle atrophy with a change in the expression levels or the functional properties of many RBPs. For example, the genetic ablation of the zinc-finger RBP Zfp106, in mice, triggers an ataxia-like syndrome that is associated with severe muscle loss, leading to the death of these animals within the first 6 months of their birth [510]. Additionally, a change in the expression levels of the ELAV family members of RBPs HuD and HuR or in their ability to interact with target mRNAs has been associated with the onset of muscle atrophy and loss triggered by underlying diseases such as spinal muscular atrophy (SMA), amyotrophic lateral sclerosis (ALS) and cancer [506, 512-514]. Hence, these and other findings clearly demonstrate the importance of RBP-driven posttranscriptional events in regulating the expression of key promoters of disease-induced muscle atrophy and degeneration.

HuR and its role in both muscle fiber formation and muscle loss represents one of the best examples illustrating how an RBP could play a dual and opposite functions in the same tissue [471]. Indeed, HuR affects the fate of muscle fibers by modulating the stability and translation of mRNAs that either promote or hinder muscle differentiation and integrity [471, 481-483, 506, 515]. For example, during the early steps of muscle fiber formation, a process also known as myogenesis, HuR promotes the expression of the alarmin HMGB1 by preventing miR-1192-mediated inhibition of *HMGB1* translation [515]. The promyogenic function of HuR also involves the HuR-mediated stabilization of

mRNA encoding key myogenic regulatory factors (MRFs) such as MyoD and myogenin [481, 516]. In muscle fibers exposed to IFNγ and TNFα however, HuR loses its ability to associate with the *MyoD* mRNA, yet, under these conditions, HuR binds the *iNOS* mRNA to promote its expression leading to the activation of the iNOS/NO pathway [506]. Collectively, these results suggest that by switching its network of mRNA targets in response to cachectic conditions, HuR changes its function from a promoter of muscle fiber formation to become a key player in the onset of muscle wasting. However, the network of pro-cachectic mRNA targets of HuR and the way by which HuR affects their expression during muscle wasting remain elusive.

In this study we identified *STAT3* mRNA as a novel HuR target during the onset of muscle wasting both *in vitro* and *in vivo*. We showed that while HuR does not affect the half-life of *STAT3* mRNA, HuR promotes STAT3 translation by binding to a U-rich element in the 3'UTR to prevent miR-330-mediated translation inhibition. Our findings, therefore, clearly establish both HuR and miRNA-330 as key regulatory factors that modulate both STAT3 expression and STAT3-induced muscle wasting.

3.5 Results

3.5.1 *STAT3* is a novel HuR mRNA target in myotubes undergoing wasting

To identify the network of mRNAs that associate with HuR during muscle wasting, we used C2C12 myotubes treated with or without IFNγ/TNFα to perform RNA Immunoprecipitation (RIP) coupled to cDNA microarray experiments using an anti-HuR monoclonal antibody (3A2) [482, 506, 515, 517]. This *in vitro* cell model of muscle wasting is routinely used to mimic the effects of cytokines on muscle fibers, as seen during cachectic conditions [502, 506, 518, 519] (**Figure 3.1*A***). Previous observations have indicated that the expression of promoters of muscle wasting in myotubes treated with IFNγ and TNFα as well as other cytokines is usually initiated as early as 12h posttreatment [506]. Therefore, the RIP-cDNA microarray experiments mentioned above was performed on C2C12 myotubes exposed to IFNγ and TNFα for 12h. We identified 74 mRNAs that were associated with HuR two-fold or more, when compared to the messages immunoprecipitated with IgG (*Dataset 1*). The Panther classification software analysis (http://www.pantherdb.org) [520] revealed that under these conditions, HuR associates with mRNA encoding members of several signaling pathways, the most relevant of which to cytokine-induced muscle wasting being the JAK/STAT pathway [40, 502] (**Figure 3.2**). A heatmap of the identified messages indicated that the *STAT3* mRNA, one of the drivers of this pathway [502], associates with HuR ~3.5 fold-more when compared to its association with the IgG control (**Figure 3.3**). Next, by repeating the RIP experiment followed by RT-qPCR we validated the cDNA array data and confirmed that HuR associates with *STAT3* mRNA not only in both IFNγ/TNFα-treated C2C12 myoblasts and myotubes but also, albeit to a lesser extent, in their untreated counterparts (**Figure 3.1B** and **Figure 3.4**). These experiments show that the *STAT3* message is a novel HuR mRNA target in muscle fibers undergoing wasting.

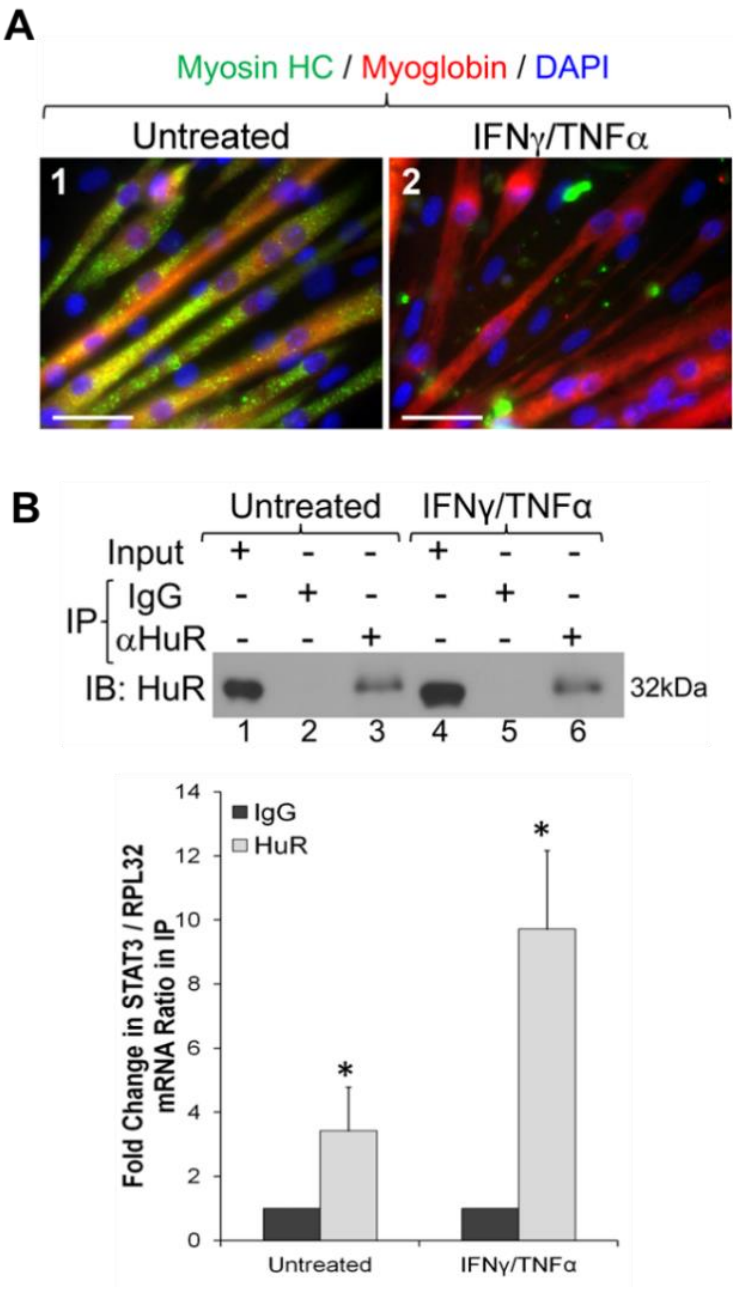


Figure 3.1. HuR associates with *STAT3* mRNA in C2C12 myotubes during muscle wasting.

(A) C2C12 myotubes were treated with or without IFNγ/TNFα for 72h, fixed and stained with antibodies against Myosin Heavy Chain, Myoglobin and DAPI. Images are representative of 3 independent experiments. Scale bar = 50 μm. ***(B)*** Lysates obtained from C2C12 myotubes treated with or without IFNγ/TNFα for 24h were used for immunoprecipitation experiments using antibodies against HuR or IgG as a negative control. Western blot experiments demonstrating immunoprecipitated HuR **(top panel)** and analysis by RT-qPCR of STAT3 mRNA associated to HuR **(lower panel)** are shown. Levels of *STAT3* in **(lower panel)** were standardized to RPL32 mRNA levels. Data are representative of three independent experiments (n=3), and error bars represent the SEM. Significance P-values were calculated using the unpaired T-test. *P<0.05 from equivalent IgG samples.

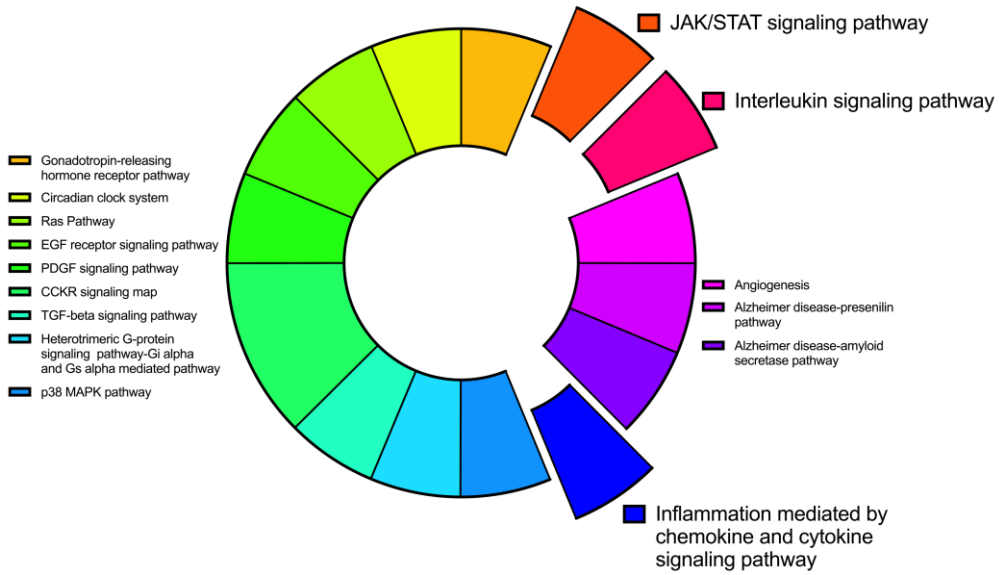


Figure 3.2. Panther analysis of HuR mRNA ligands. RNA-Immunoprecipitation (RIP)-coupled to DNA microarray experiments were performed to identify the network of mRNAs that associate with HuR in C2C12 myotubes treated with IFNγ/TNFα for 12h. The HuR mRNA targets were analysed using Panther classification software analysis (http://www.pantherdb.org) [520] to determine the signaling pathways they belong to.

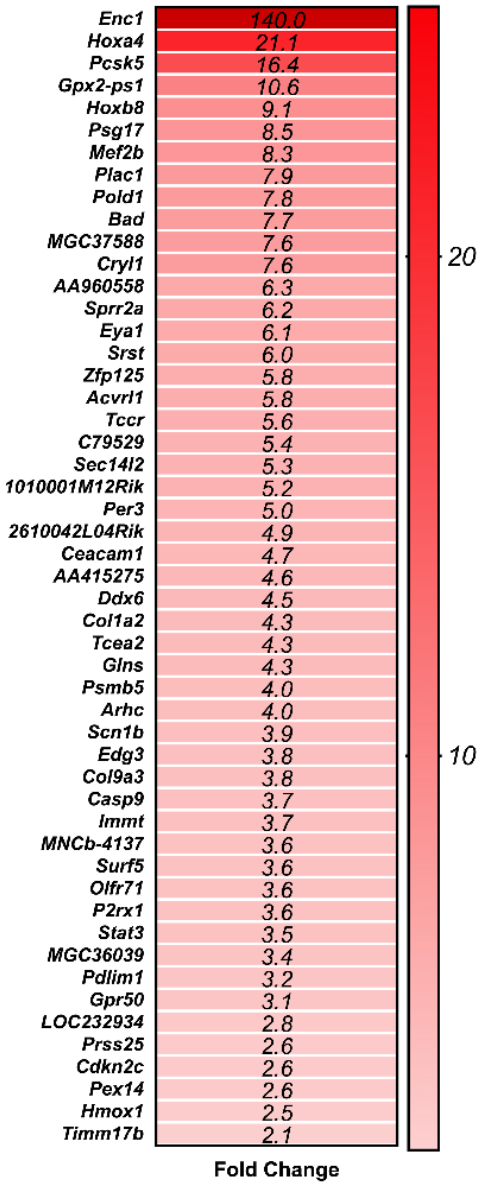
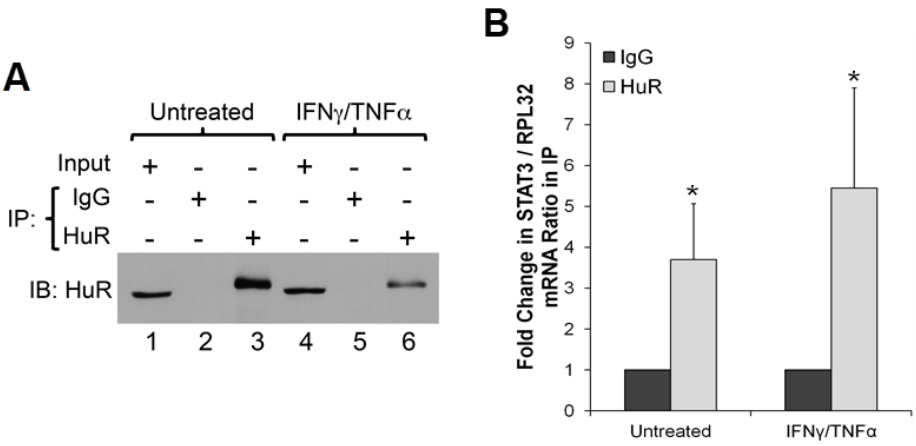


Figure 3.3. Heat map of the HuR mRNA targets. A heat map was generated using the HuR mRNA targets list obtained from the (RIP)-coupled to DNA microarray experiments described in Figure 3.2. Shown is the fold change of the binding of mRNA with HuR compared to IgG control.

Figure 3.4. HuR associates with the *STAT3* mRNA in muscle cells treated with or without IFNγ/TNFα.

(A, B) Immunoprecipitation experiments were performed using antibodies against HuR or IgG as a negative control with lysates obtained from C2C12 myoblasts treated with or without IFNγ/TNFα for 24h. Western blot experiments demonstrating immunoprecipitated HuR *(A)* and analysis by RT-qPCR of STAT3 mRNA associated to HuR *(B)* are shown. Levels were standardized to RPL32 mRNA levels. Quantifications are of three independent experiments (n=3), and error bars represent the SEM. Significance P-value was calculated using the unpaired T-test. *P<0.05 from equivalent IgG samples.



3.5.2 HuR promotes STAT3 expression during muscle wasting both *in vitro* and *in vivo.*

Previous studies have shown that, during muscle wasting, phosphorylation-mediated activation of STAT3 is concomitant with a substantial increase in its expression levels [502, 507]. We confirmed these observations and showed that the increase in expression levels of STAT3 protein in myotubes exposed to IFNγ/TNFα was detected as early as 4h posttreatment and that this effect is not associated with an increase in the levels of HuR (**Figure 3.5A** and *SI Appendix* Fig. S4). Knocking down HuR however, significantly reduced the levels of STAT3 protein in both untreated and treated muscle cells without affecting the steady state levels of *STAT3* mRNA (**Figures 3.**5*B-C*). Of note, the basic level of STAT3 in untreated cells was very low compared to the treated ones, yet the absence of HuR further reduced STAT3 expression in these cells (**Figure 3.5B**). We next confirmed these effects *in vivo* using the *Elavl1 (HuR)* muscle-specific knockout (muHuR-KO) mice that we recently generated [521]. These muHuR-KO mice appear healthy and do not differ in size when compared to their control counterparts (**Figure 3.5D**). Using the Lewis Lung Carcinoma model of cancer inflammation induced muscle wasting [49, 519], we observed that the genetic ablation of HuR protected muHuR-KO mice from the LLC tumour-induced muscle loss that is normally observed in the control mice (**Figures. 3.5E-F**). Interestingly, the protection from muscle wasting in the muHuR-KO mice correlated with a significant decrease in the expression levels of STAT3 protein when compared to its levels in control mice (**Figure 3.5G**). Of note, similar to the *in vitro* data, depleting HuR in skeletal muscles reduced the basic expression levels in untreated muHuR-KO muscle (**Figure 3.5G**). These results, therefore, demonstrate that HuR plays a key role in promoting STAT3 expression both *in vitro* and *in vivo* in muscle fibers undergoing wasting.

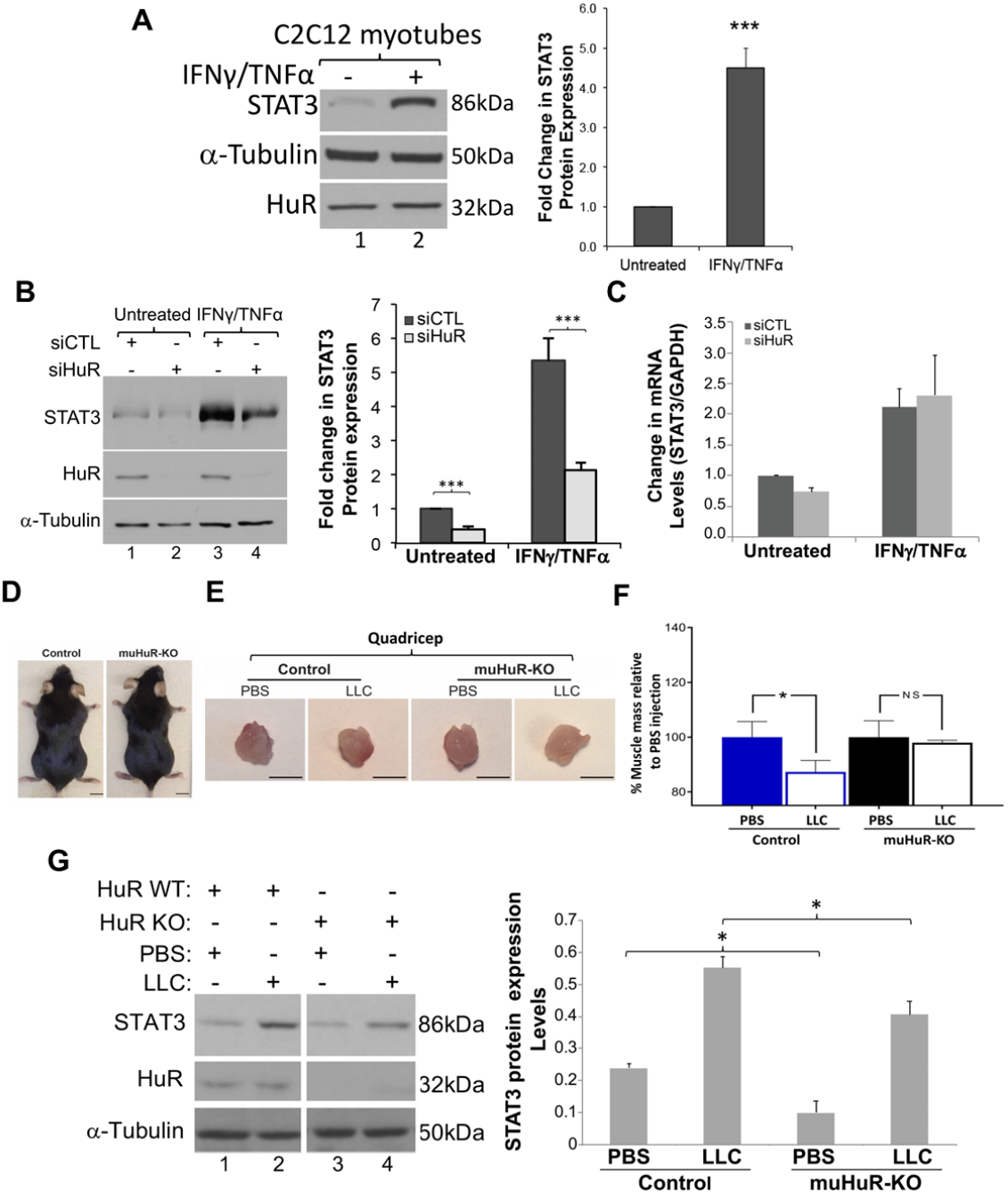

A
C2C12 myotubes
IFNγ/TNFα - +
STAT3 86kDa
α-Tubulin 50kDa
HuR 32kDa
1 2
Fold Change in STAT3 Protein Expression
***
Untreated IFNγ/TNFα
B
Untreated IFNγ/TNFα
siCTL + - + -
siHuR - + - +
STAT3
HuR
α-Tubulin
1 2 3 4
Fold change in STAT3 Protein expression
siCTL
siHuR
***
***
Untreated IFNγ/TNFα
C
Change in mRNA Levels (STAT3/GAPDH)
siCTL
siHuR
Untreated IFNγ/TNFα
D
Control muHuR-KO
E
Quadricep
Control muHuR-KO
PBS LLC PBS LLC
F
% Muscle mass relative to PBS injection
*
NS
PBS LLC PBS LLC
Control muHuR-KO
G
HuR WT: + + - -
HuR KO: - - + +
PBS: + - + -
LLC: - + - +
STAT3 86kDa
HuR 32kDa
α-Tubulin 50kDa
1 2 3 4
STAT3 protein expression Levels
* *
PBS LLC PBS LLC
Control muHuR-KO

Figure 3.5. HuR regulates the expression of STAT3 both in vitro and in vivo during cancer inflammation-induced muscle wasting.

(A) Lysates obtained from C2C12 myotubes treated with or without IFNγ/TNFα for 24h were used for western blot analysis **(left panel)** with antibodies against STAT3 and α-tubulin. **(right panel)** Densitometric quantification of STAT3 signal in the western blot relative to α-tubulin signal. ***(B)*** Total cell lysate from C2C12 cells depleted or not of HuR treated with or without IFNγ/TNFα for 24h were used for Western blot analysis **(left panel)** with antibodies against STAT3, HuR, and α-tubulin. **(right panel)** Densitometric quantification of STAT3 signal relative to α-tubulin signal. ***(C)*** Total RNA extracted from C2C12 cells treated as described in *(B)* were analyzed by RT-qPCR using primers specific for STAT3 and GAPDH cDNAs. Quantification of STAT3 mRNA levels relative to GAPDH levels is shown. ***(D-G)*** Control or muHuR KO male mice (8 to 10 weeks old) were injected with PBS or LLC cells to induce muscle wasting. *(D)* Photographs of muHuR-KO and control male mice. Scale bars=1 cm. *(E)* Photographs of quadriceps collected from mice described above. Scale bars=1 cm. *(F)* Weight of the quadriceps described in *(E)*. Levels are shown as the percentage of weight remaining when compared to the PBS-treated control mice (shown as 100%). Quantifications are of 4 mice (n=4). *(G)* STAT3 protein levels in quadriceps muscle obtained from mice described above were assessed by Western blot **(left panel)** using antibodies against STAT3, α-tubulin (loading control) and HuR. **(right panel)** Densitometric quantification of STAT3 signal relative to α-tubulin. All quantifications are of three independent experiments (n=3) for 2A-C and of quadricep muscles from 4 different mice for 2G (n=4). Error bars of all quantifications represent the SEM. Significance P-values were calculated using the unpaired T-test. *P<0.05, ***P<0.001 from untreated sample in *(A)*, siCTL untreated samples in *(B)*, from PBS treated control mice samples in *(F)* and untreated as well as LLC-treated control mice in *(G)*.

3.5.3 HuR promotes *STAT3* translation via a U-rich element in the *STAT3-3'UTR*

HuR is known to influence gene expression by modulating the stability, export, and/or the translation of its target mRNAs [192, 471, 482, 515]. Our Actinomycin D pulse-chase and experiments [522] and *in situ* hybridization experiments indicated that the depletion of HuR did not affect the half-live nor the of the cellular movement of the *STAT3* mRNA (**Figure 3.6**). We then assessed whether HuR affects the translation of the *STAT3* mRNA under muscle wasting conditions. Using sucrose fractionation experiments we followed the distribution of *STAT3* mRNA in polysome (P) and non-polysome (NP) fractions in muscle cells depleted or not of HuR and treated with or without IFNγ/TNFα. Neither the knockdown of HuR nor the treatment of muscle cells

with cytokines affected general translation as determined by the profile of the polysome (P) and non-polysome (NP) fractions (**Figure 3.7A**). Consistent with an increase in STAT3 protein levels, as shown above (**Figure 3.5A** and **Figure 3.8A**), the levels of *STAT3* mRNA recruited to polysomes dramatically increased in muscle cells treated with IFNγ/TNFα **(Figure 3.7B**). The depletion of HuR in both untreated and IFNγ/TNFα-treated cells however, prevented the recruitment of *STAT3* mRNA to polysomes (**Figure 3.7B**). Together, these data clearly show that HuR promotes the translation of the *STAT3* mRNA in muscle cells exposed to wasting conditions.

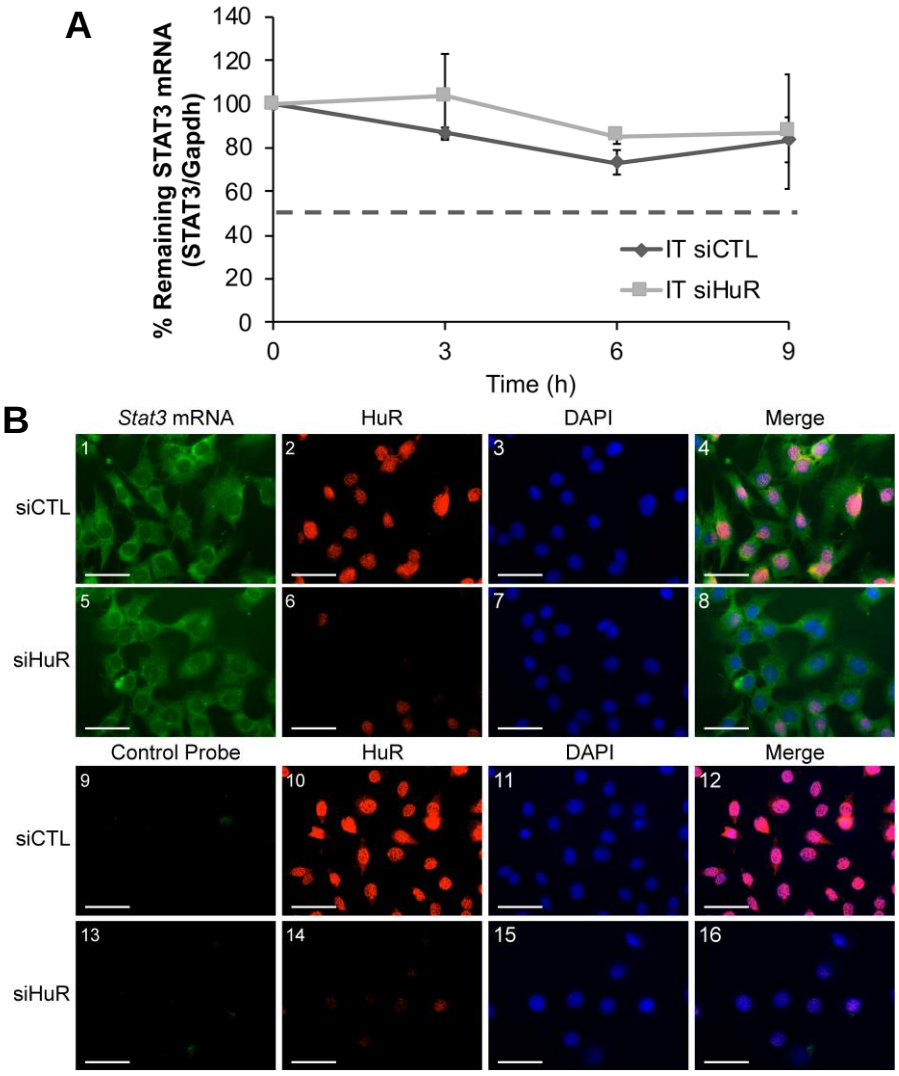


Figure 3.6. HuR does not affect the stability or the cellular movement of the *STAT3* mRNA. *(A)* The stability of the STAT3 mRNA was assessed by performing Actinomycin D (AcD) experiments. C2C12 myoblasts transfected with either a control siRNA (siCTL) or a siRNA specific for HuR were treated with IFNγ/TNFα for 24h. Cells were then incubated with ActD for the indicated period of time. Total RNA was extracted and used for RT-qPCR analysis using STAT3 and GAPDH primers to determine *STAT3* mRNA expression levels at time points post AcD treatment. Levels are shown as the percentage of mRNA remaining when compared to levels at 0 hour time point (shown as 100%). The dashed line indicates time point where 50% of mRNA is remaining. Quantifications are of three independent experiments (n=3), and error bars represent the SEM. ***(B)*** *In situ* hybridization experiments were performed using C2C12 myoblasts depleted or not of HuR. Cells were fixed and then stained with the anti-sense or sense probe for *STAT3* mRNA, HuR protein, and DAPI. Images are representative of three independent experiments (n=3) experiments. Scale bar = 50 μm.

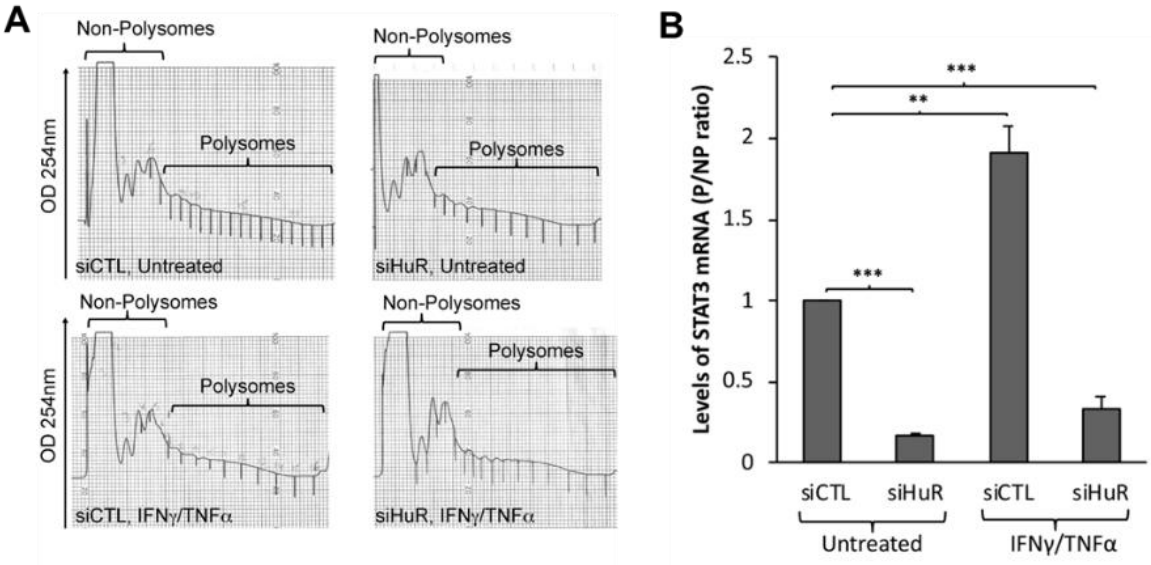


Figure 3.7. HuR promotes the translation of STAT3 mRNA.

(A) Polysome profile of extracts obtained from C2C12 cells depleted of HuR or not and treated with or without IFNγ/TNFα for 24h. ***(B)*** RNA was extracted from each fraction and RT-qPCR analysis was performed using primers for *STAT3* and *5.8S* mRNA. The levels of STAT3 relative to 5.8S were graphed as the Polysomal (P) to Non-polysomal (NP) ratio. Quantifications are of three independent experiments (n=3), and error bars represent the SEM. Significance P-values were calculated using the unpaired T-test. **P<0.01, ***P<0.001 from untreated siCTL samples.

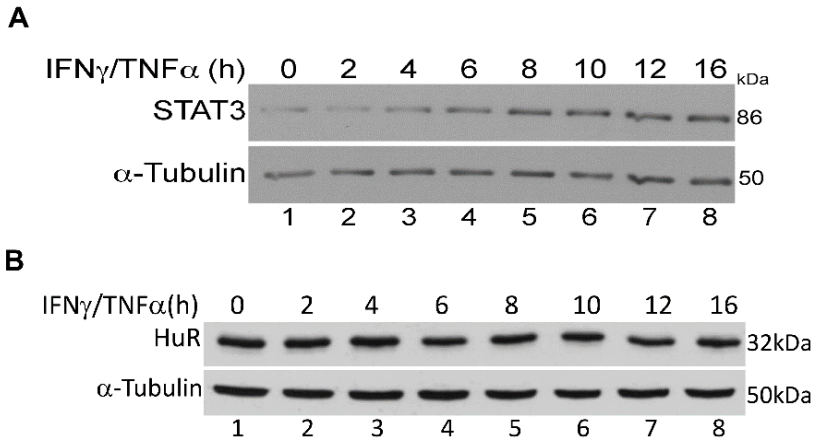


Figure 3.8. The expression of STAT3 protein, but not HuR, increases over time in myotubes treated with or without IFNγ/TNFα.

Total lysates obtained from myotubes treated for the indicated period of time with IFNγ/TNFα were used in western blot experiments using antibodies against STAT3 ***(A)***, HuR ***(B)*** and α-tubulin (assessed as a loading control).

It is well-established that HuR modulates the expression of its mRNA targets by directly binding to U/AU-rich elements in their 3'UTRs [192, 471, 482, 515]. Hence, to delineate the mechanism by which HuR regulates STAT3 translation, we first identified the exact *cis*-element through which HuR binds to the *STAT3-3'UTR*. Sequence analysis of the *STAT3*-3'UTR revealed both U- and AU-rich elements to which HuR could potentially bind (**Figure 3.9**). To determine whether HuR directly binds to the *STAT3* mRNA through any of these elements, we performed RNA electrophoretic mobility shift assays (REMSA) [482, 515] using purified recombinant GST-HuR and sixteen radiolabelled RNA probes that span the entire *STAT3*-3' UTR (**Figure 3.10A** and *SI Appendix* Table 1). We found that HuR forms a complex with only five of these RNA probes (P2, P6, P13, P14 and P16) (**Figure 3.10B**). Next, the strength of HuR binding to these probes was tested by introducing RNase T1 treatment into the REMSA. In this assay, HuR-mRNA complexes are formed prior to digestion with RNase T1, which cleaves specifically after G residues [523]. It is well-accepted that resistance to RNAse T1 treatment will only occur as a result of a direct binding of HuR to the RNA probe [523]. We observed that P2, and to a lesser extend the P16 probe, exhibited resistance to RNAse T1 treatment (**Figure 3.11**). These data show that both P2 and P16 elements resist RNase T1 while directly bound to HuR. These data, however, do not inform on the strength/affinity (K_d) of HuR binding to these sequences. Therefore, we performed REMSA [524] with increasing concentrations of GST-HuR to determine the binding affinity (K_d) of the HuR to the P2, P6, P13, P14 and P16 probes. Each one of these probes was incubated with increasing amounts of GST-HuR and the complexes were identified using REMSA as described [524] (**Figure 3.10C,** left panel). When measuring the fraction of free probe bound to HuR, we found that HuR associated with P2 with a high affinity (K_d ~20.5 nM). P16 as well as the other probes (P6,P13 and P14) however, did not exhibit a measurable binding affinity at the concentrations of the recombinant HuR used (**Figure 3.10C,** right panel). Next, we determined the minimum HuR binding site within P2. To do this, we divided this probe into four smaller fragments, P2-A, -B, -C, and -D (**Fig. 3.10D**, upper panel). Based on the fact that the P2B-HuR complex generated the strongest signal in the REMSA

experiment (**Figure 3.10D**, lower panel), we decided to further investigate the role of this *cis*-element in HuR-mediated regulation of STAT3 translation.

MOUSE_STAT33UTR

GGAGCTGAAACCAGAAGCTGCAGAGACGTGACTTGAGACACCTGCCCCGTGCTCCACCCCTAAGCAGCCGAACCCCA

TATCGTCTGAAACTCCTAACTTTGT**GGTTCCAGATTTTTTTTTTTAATTTCCTACTTCTG**CTATCTTTGGGCAATCT

HuRBS-P2B

GGGCACTTTTTAAAATAGAGAAATGAGTGAGTGTGGGTGATAAACTGTTATGTAAAGAGGAGAGCACCTCTGAGTCT

GGGGATGGGGCTGAGAGCAGAAGGGAGCAAGGGGAACACCTCCTGTCCTGCCCGCCTGCCCTCCTTTTTCAGCAGCT

CGGGGTTGGTTGTTAGACAAGTGCCTCCTGGTGCCCATGGCATCCTGTTGCCCCACTCTGTGAGCTGATACCCCAGG

CTGGGAACTCCTGGCTCTGCACTTTCAACCTTGCTAATATCCACATAGA**AGCTAGGACTAAGCCCAGAGG**TTCCTCT

miR330BS

TTAAATTAAAAAAAAAAAAAATAAGAATTAAAGGGCAAAACACACTGACACAGCATAGCCTTTCCATATCAAGGAAT

ACTCAGTTAACAGCCTCTCCAGCGCTGTCTTCAGGCTGATCATCTATATAAACCCTGGAATGGTTGCAGATCAAATC

TGTAAAAGAGATCCGAGAGCTGTGGCTTGGCCTCTGGTTCAAACACAAAGGCTAGAGAGAACCTAGATATCCCTGGG

TTTTGTTTACCCAGTATGCTTGTCGGTTGGAGGTGTGAGGTAGGCCAAGGGCACTGGAAAGCCTTTGTCATCACCCT

ACTCCCTCCCCAACCCAGACTCCAGACCCTGTTTCAGGGTCAGCCTGCCCTGTGGGTGCCTTACTGGGCCTAGGGTC

AACCTGCCTTCCTTTCCCACTTGACCTTGCTGGTAGTATGTCCCCTTCCCATGTCCAAAGGCCCTCTGTCCTGCTTC

TATTGGGAATCCCTGCCTCAGGACCTTGTGTCGAGAGGGATTGCCTTACAGGTTTGAACCTGCCTCAGACTACAGGC

CCTCAGCAAAGCTCAGGGAGTATGGTCCTTATTCTATGCGCTTGGTTCCCAGGGATATCTGTAACCACAGGGCAAAA

GCTGACATATACTCCAGGTCTGCCCTCATATGAGTGGTGTATTCTTGGCCTCCCCTGAGACTGGCAACTGTCTGCTC

CCCATTGGGTCTCCCAGGTGAGGTGGAACACAGTTCCTGCACCTACTGTGGCCTCCATGTCGCTTGCTTGCTTCGCT

CACTCAGCTTACTGGAACACTGAGTGTTCAAGGCAAGCCTTTCCTGACAGAGGCATGGCTAGATTCAGTGACTCAAA

GCCACCTCATTCAGCTGATCAGTGTCTGTGGAATTGTTTCCTTCCAGTTAACCAGTGTCTGAATTAAGGGCAGTGAG

GACATTGTCTCCAAGACGAACTGCTTGCCTTGACCACCCCAGCCTTCTGCTTCGAGACAGTTACTGCTCTCCCACCC

CATCAATGTTCTTTAGTTATACAATAAGCTGAACTTATAAACTGAAAGGGTATTTAGGAAGGCAAGGCTTGGGCATT

TTTATGGCTTTCAATCCTGGGGACCCAGGAACAAGGTGAGGGCTTCTCTGGGGCTGGTGTTGTACCTCAGGGGCTCT

GGGAAGTCTGTGTGCCTGGGTTAACCACCCATAGTGAGCCCCTGGAACTGCCCACTTTCCCTCTCCTTGGCCCCACT

TGGCCCCAGCCTCACCCAGCCTGCAGACTGCTTAGCCTTTCAGTGC*'AGTGGCTTGTGTTCTGGCCACTGCACTCAG*

ATTCCAATGTAAACTTTCTAGTGTAAAAATTTATATTATTGTGGGTTGTTTTTTGTTGTTGTTTGTTTTTGTATATT

GCTGTAACTACTTTAACTTCCAGAAATAAAGATTATATAGGAACTGTC

Figure 3.9. Sequence of the *STAT3* mRNA 3'untranslated region.

The sequence of the HuR binding site P2B and the miR-330 seed element are respectively shown in blue and red.

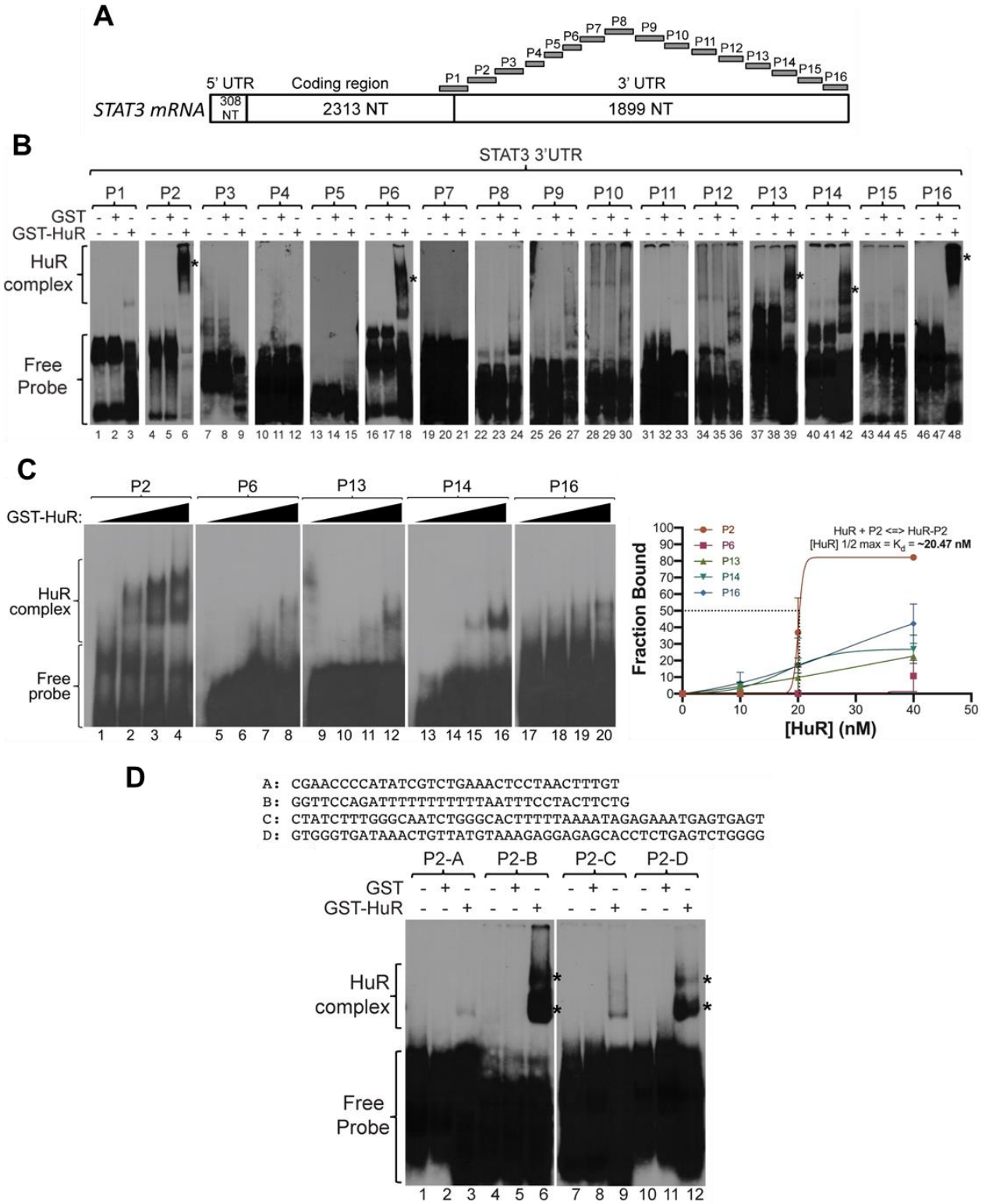

A
5' UTR
Coding region
3' UTR
STAT3 mRNA
308 NT
2313 NT
1899 NT
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14 P15 P16
B
STAT3 3'UTR
GST
GST-HuR
HuR complex
Free Probe
C
GST-HuR:
HuR complex
Free probe
HuR + P2 <=> HuR-P2
[HuR] 1/2 max = Kd = ~20.47 nM
Fraction Bound
[HuR] (nM)
D
A: CGAACCCCATATCGTCTGAAACTCCTAACTTTGT
B: GGTTCCAGATTTTTTTTTTTAATTTCCTACTTCTG
C: CTATCTTTGGGCAATCTGGGCACTTTTTAAAATAGAGAAATGAGTGAGT
D: GTGGGTGATAAACTGTTATGTAAAGAGGAGAGCACCTCTGAGTCTGGGG
P2-A P2-B P2-C P2-D
GST
GST-HuR
HuR complex
Free Probe

Figure 3.10. HuR directly binds to a U-rich element in the 3'UTR of STAT3 mRNA.

(A) Schematic representation of the *STAT3* mRNA sequence. The location of the cRNA probes covering the 3'UTR of *STAT3* mRNA used for RNA electrophoretic mobility shift (REMSA) assays are indicated (P1 to P16). ***(B)*** Gel shift assays was performed using recombinant GST-HuR protein or GST as a control incubated with radiolabelled cRNA probes as indicated in (A). Representative gels of each probe from n=3 experiments are shown. HuR/cRNA complex are indicated with an asterisk (*). ***(C)*** **(left panel)** REMSA was used to determine the dissociation constant (K_d) of HuR binding to P2, P6, P13, P14 and P16 probes. Increasing amounts of GST-HuR protein (0, 10, 20 and 40 nM) were incubated with P2, P6, P13, P14 or P16 radiolabeled probes and complexes were resolved using REMSA as described above. **(right panel)** The binding of GST-HuR with the various probes was plotted as fraction of the associated RNA against nM of GST-HuR to determine the K_d. ***(D)*** REMSA with radiolabelled probes spanning P2 region (P2-A, P2-B, P2-C, and P2-D) were performed to further delineate the HuR binding site. Upper panel indicates the nucleotide sequence of each probe used for gel shift in panel D.

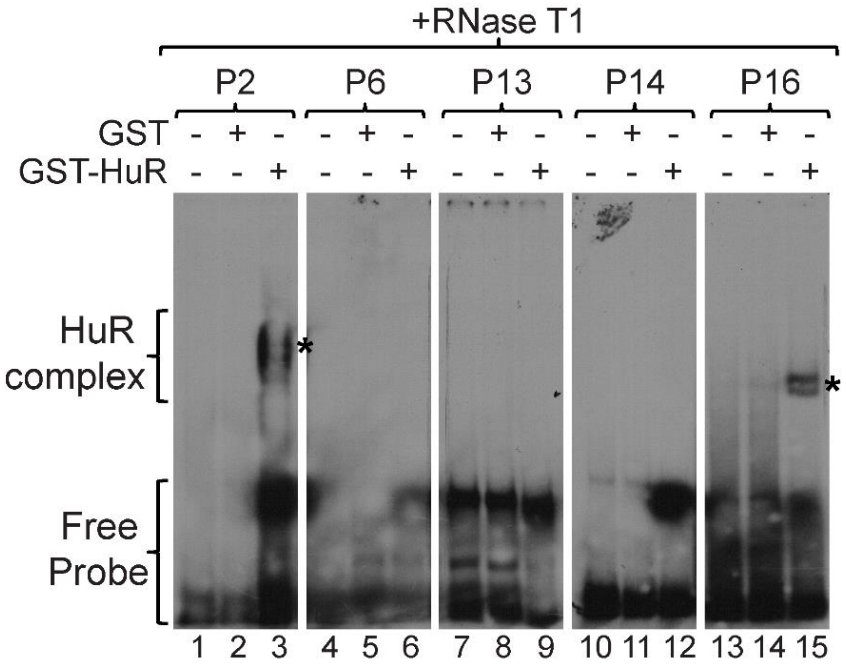


Figure 3.11. P2 and P16 exhibit resistance to RNase T1 treatment.

Representative gels of REMSA assays performed with radiolabelled cRNA probes (P2, P6, P13, P14, and P16) incubated with purified GST or GST-HuR prior to treatment with RNaseT1 to assess strength of complex formation seen in in Fig. 4B.

To achieve this, we generated Renilla-luciferase (R-*Luc*) constructs that expresses wild type *STAT3*-3'UTR (R-*Luc*-3'STAT3) or the *STAT3*-3'UTR from which the P2B (R-*Luc*-3'STAT3-ΔP2B) or the P16 (R-*Luc*-3'STAT3-ΔP16) elements were deleted (**Figure 3.12A** and **Figure 3.13A**). These reporter constructs were subsequently expressed in C2C12 cells and used to determine the impact of deleting P2B or P16 on the luciferase activity. We observed that the deletion of P2B but not that of P16 significantly reduced luciferase activity (**Figure 3.13B**). Moreover, while both R-*Luc*-3'STAT3 and R-*Luc*-3'STAT3-ΔP2B mRNAs were expressed to the same levels (**Figure 3.12B**), the significant reduction in the luciferase activity of R-*Luc*-3'STAT3-ΔP2B construct (**Figure 3.12C**) correlated with the inability of its mRNA to associate with HuR (**Figure 3.12D**). Therefore, these *in vitro* and *ex-vivo* experiments demonstrate that HuR promotes *STAT3* mRNA translation and that this effect requires the binding of HuR to the *STAT3*-3'UTR via the U-rich P2B *cis*-element.

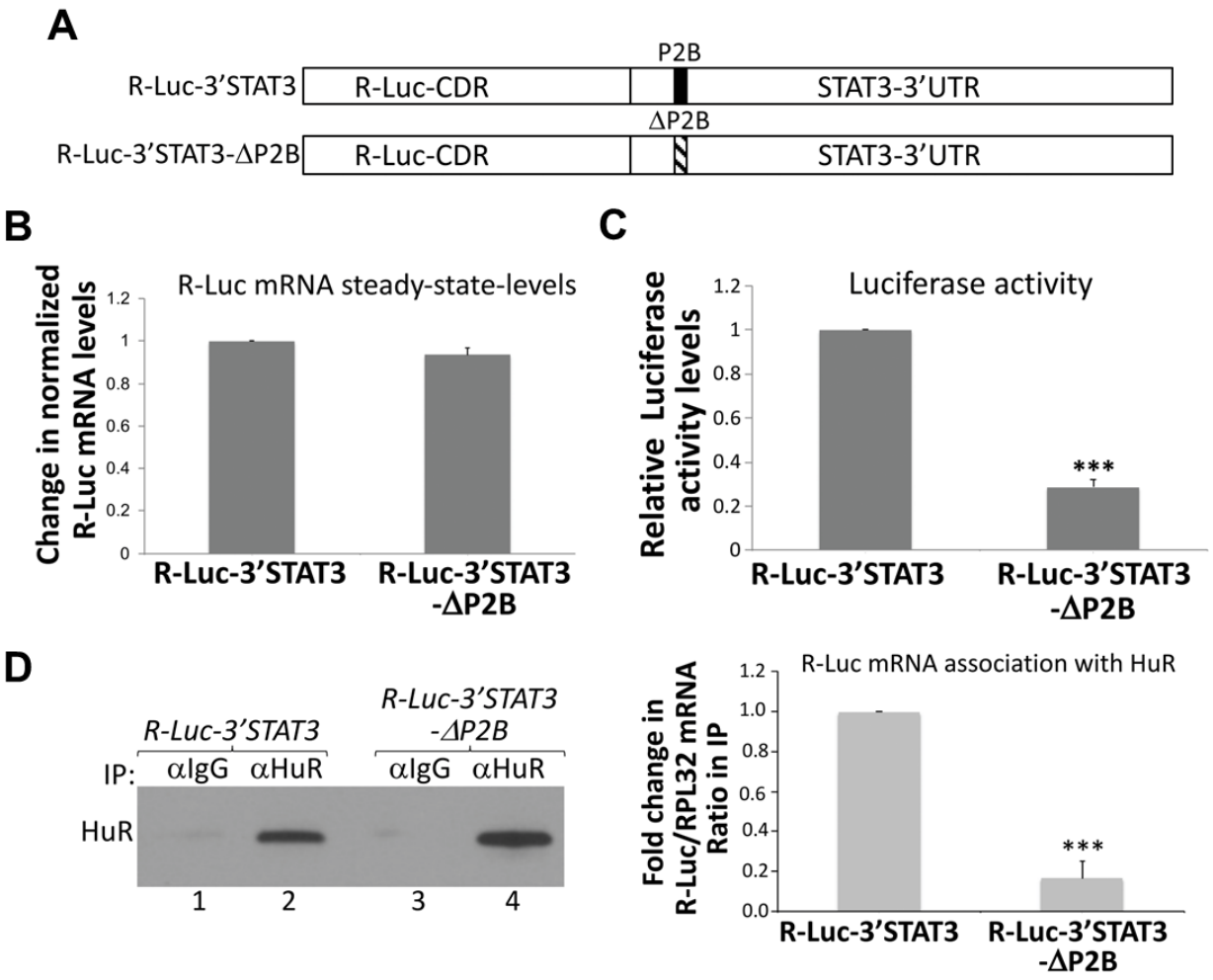


Fig. 3.12. The P2B element is required for the STAT3-3UTR-mediated translation regulation and association with HuR.

(A) Schematic demonstrating the Renilla-luciferase (R-*Luc*) constructs with the *STAT3*-3'UTR (R-*Luc*-3'STAT3) or the *STAT3*-3'UTR mutant in which the P2B element was deleted (R-*Luc*-3'STAT3-ΔP2B). **(*B-D*)** The reporter constructs described in *(A)* were transfected in C2C12 cells. R-Luc reporter mRNA steady state levels *(B)* as well as Luciferase activity *(C)* was determined for each construct. mRNA levels and luciferase activity for the R-*Luc*-3'STAT3-ΔP2B mRNA is shown relative to those obtained with the R-*Luc*-3'STAT3 construct. *(D)* **(left panel)** Western blot showing the immunoprecipitation of HuR from cell extracts expressing R-*Luc*-3'STAT3 or R-*Luc*-3'STAT3-ΔP2B mRNAs. The blot was probed with the monoclonal anti-HuR antibody. **(right panel)** Total RNA from HuR immunoprecipitate was prepared and the association of R-*Luc*-3'STAT3 or R-*Luc*-3'STAT3-ΔP2B mRNAs was determined by RT-qPCR. Levels were standardized to RPL32 mRNA levels. Quantifications for *(B-D)* are of three independent experiments (n=3), and error bars represent the SEM. Significance P-value in (C,D) was calculated using the unpaired T-test. ***P<0.001 from R-*Luc*-3'STAT3 samples.

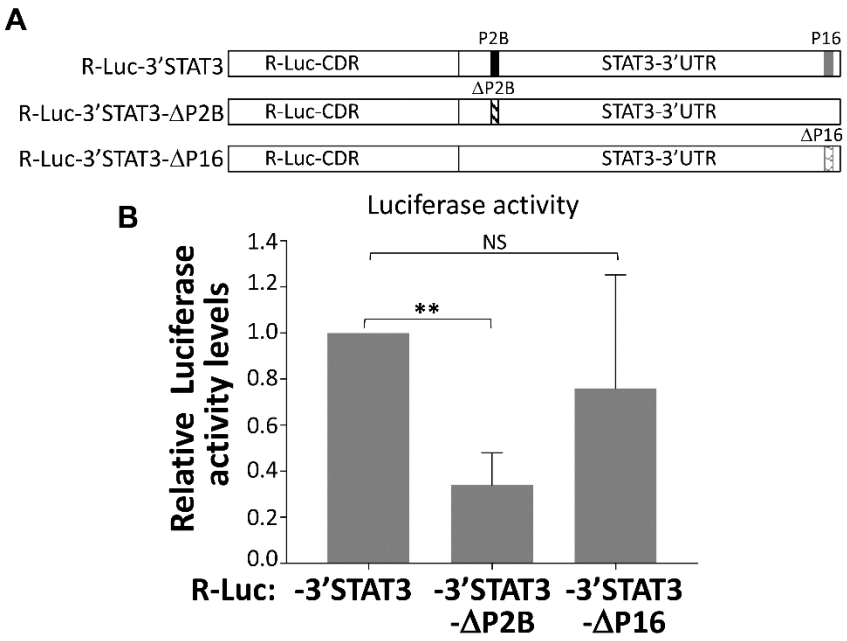


Figure 3.13. Unlike P2, the P16 element is not required for the STAT3-3'UTR mediated translation regulation.

(A) Schematic demonstrating the Renilla-luciferase (R-*Luc*) constructs with the *STAT3*-3'UTR (R-*Luc*-3'STAT3) or the *STAT3*-3'UTR mutant in which the P2B (R-*Luc*-3'STAT3-ΔP2B) or the P16 (R-*Luc*-3'STAT3-ΔP16) element were deleted. **(*B*)** The reporter constructs described in *(A)* were transfected in C2C12 cells. Luciferase activity was determined for each construct. Luciferase activity for the R-*Luc*-3'STAT3-ΔP2B and R-*Luc*-3'STAT3-ΔP16 mRNAs is shown relative to those obtained with the R-*Luc*-3'STAT3 construct.

3.5.4 HuR prevents miR-330-mediated translation inhibition of STAT3

It is well established that one way by which HuR modulates the translation of some of its mRNA targets is by competing or collaborating with microRNAs (miRNAs) [515, 525]. Therefore, we investigated whether this could also be the case for the HuR-mediated translation regulation of the *STAT3* mRNA. As a first step, we identified miRNA(s) that could form a complex with HuR in C2C12 myotubes by performing a RIP experiment coupled to miRNA sequencing analysis using antibodies against HuR or IgG as a negative control. Although 15 miRNAs were co-immunoprecipitated with HuR in C2C12 myotubes, only one of them, miR-330, is predicted by three online prediction programs (TargetScan, miRBase, and microrna.org) to target the *STAT3* mRNA.

Scanning the primary sequence of the *STAT3 mRNA-3'UTR* revealed that the predicted seed element of miR-330 is located 297nt away from the HuR binding site (HuRBS) P2B (**Figure 3.9**, and **Figure 3.14A**). Although the HuRBS P2B and miR-330 binding sites could appear far apart from one another, previous observations have shown that HuR is able to interfere with miRNA-mediated translation repression even when its binding site is positioned at a considerable distance from the miRNA seed element [526]. By repeating the RIP experiment followed by RT-qPCR, we validated the association of HuR with miR-330 in both untreated and IFNγ/TNFα-treated C2C12 myotubes (**Figure 3.14B**) as well as in myoblasts (**Figure 3.15**). The observed HuR-miR-330 association is not due to an increase in the expression levels of HuR or miR-330 in response to IFNγ and TNFα (**Figure 3.8B and 3.16**). However, although the HuR-miR-330 complex was totally disrupted in muscle cells depleted of STAT3, the association between HuR and miR-330 was re-established by introducing back, in these *STAT3* knockdown muscle cells, the R-*Luc*-3'STAT3 construct expressing an intact *STAT3*-3'UTR (**Figure 3.14C-D**). Together, these data clearly show that the association of HuR with miR-330 is indirect and occurs via the *STAT3*-3'UTR. It is well-established that recruiting AGO2, one of the main components of the RNA-induced silencing complex (RISC), represents one of the key steps used by miRNAs to inhibit the translation of their target mRNA [263]. Interestingly, our data show that the depletion of HuR increases the association of AGO2 with both *STAT3* mRNA and miR-330 (**Figure 3.14E-H**), suggesting that one way by which HuR interferes with miR-330 mediated inhibition of the translation of the *STAT3* mRNA is by preventing the recruitment of AGO2.

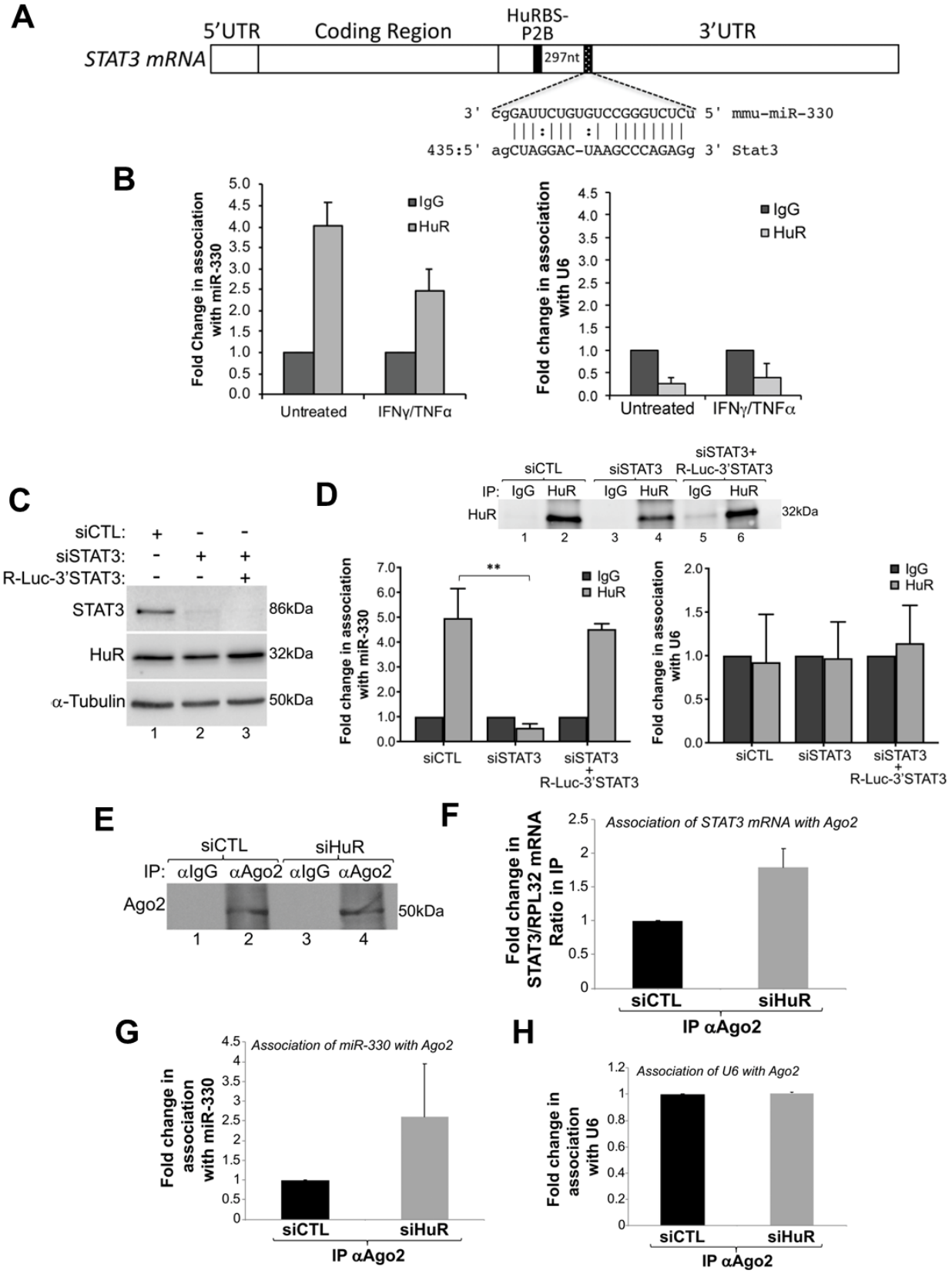

A
5'UTR
Coding Region
HuRBS-P2B
3'UTR
STAT3 mRNA
297nt
3' cgGAUUCUGUGUCCGGGUCUCu 5' mmu-miR-330
435:5' agCUAGGAC-UAAGCCCAGAGg 3' Stat3
B
Fold Change in association with miR-330
IgG
HuR
Untreated
IFNγ/TNFα
Fold change in association with U6
C
siCTL:
siSTAT3:
R-Luc-3'STAT3:
STAT3
HuR
α-Tubulin
86kDa
32kDa
50kDa
D
siCTL
siSTAT3
siSTAT3+ R-Luc-3'STAT3
IP:
32kDa
**
E
siCTL
siHuR
IP: αIgG αAgo2 αIgG αAgo2
Ago2
50kDa
F
Association of STAT3 mRNA with Ago2
Fold change in STAT3/RPL32 mRNA Ratio in IP
siCTL
siHuR
IP αAgo2
G
Association of miR-330 with Ago2
Fold change in association with miR-330
H
Association of U6 with Ago2
Fold change in association with U6

Fig. 3.14. HuR interacts with miR-330 in a STAT3-dependant manner.

(A) Schematic demonstrating the alignment between the sequence of mmu-miR-330 with the predicted seed element in the 3' UTR of mouse *STAT3* mRNA. ***(B)*** Lysates obtained from C2C12 myotubes treated with or without IFNγ/TNFα for 24h were used for immunoprecipitation experiment using antibodies against HuR or IgG as a negative control. Analysis of miRNA isolated from the immunoprecipitate was performed by RT-qPCR using primers specific for miR-330 **(left panel)** or for U6 **(right panel)** as a negative control. Quantifications are of four independent experiments (n=4). ***(C-D)*** Lysates obtained from IFNγ/TNFα treated C2C12 myoblasts expressing or not R-*Luc*-3'STAT3 transfected with siCTL or a siRNA (siSTAT3) specifically targeting STAT3 were used for immunoprecipitation experiment using antibodies against HuR or IgG as a negative control. *(C)* Western blot analysis with antibodies against STAT3, α-tubulin, and HuR validating the knockdown of STAT3. *(D)* **(upper panel)** Immunoprecipitated samples from C2C12 muscle cells, treated with sCTL, siSTAT3 or both siSTAT3 and expressing the R-*Luc*-3'STAT3 mRNA, were used for western blot analysis using antibodies against HuR. **(lower panel)** Analysis of miRNA isolated from the immunoprecipitate obtained using the anti-HuR antibody was performed by RT-qPCR using primers specific for miR-330 (**left panel**) or for U6 (**right panel**) as a negative control. Quantifications for *(B-D)* are of three independent experiments (n=3), and error bars represent the SEM. Significance P-value in *(D)* was calculated using the unpaired T-test. **P<0.01 from siCTL samples. ***(E)*** Total extracts from C2C12 muscle cells expressing (siCTL) or not HuR (siHuR) were used for immunoprecipitation experiments with a rabbit monoclonal AGO2 antibody, or anti-IgG antibody as a control. AGO2 immunoprecipitation was then determined by western blot using an anti-AGO2 antibody. The western blot shown is representative of 3 independent experiments. ***(F-H)*** Associated RNAs were isolated from the AGO2 immunoprecipitate (IP), and quantitative RT-qPCR was performed using primers specific to *STAT3* and *RPL32* mRNAs *(F)*, miR-330 *(G)* and U6 *(H)*. The levels of *STAT3* mRNA in each IP, relative to those in the IgG IP, were normalized against the *RPL32* mRNA. Error bars represent S.E.M of two independent experiments.

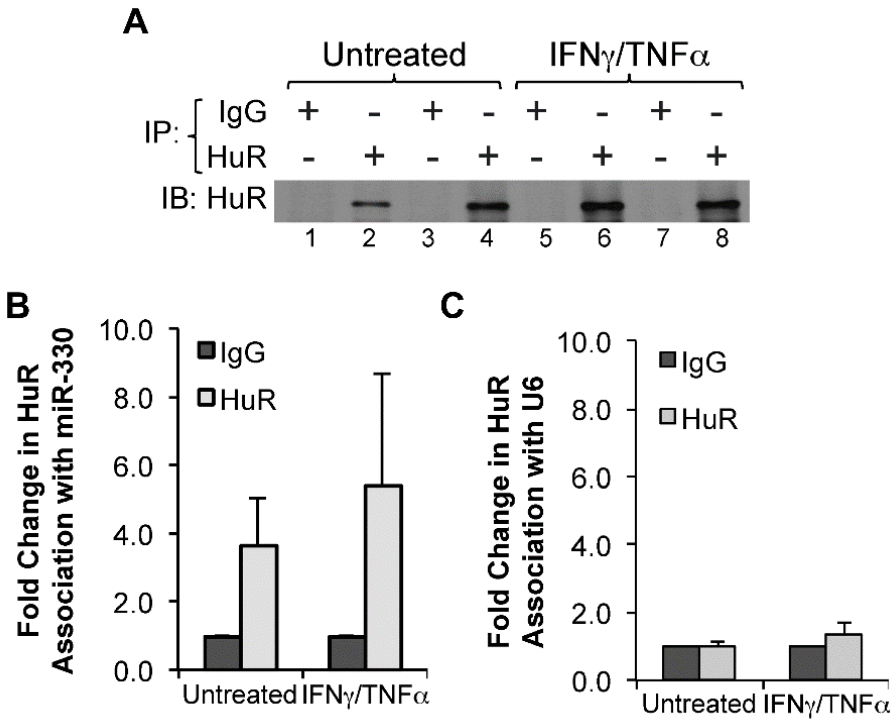


Figure 3.15. HuR associates with miR-330 in myoblasts treated with or without IFNγ/TNFα.

(A) Lysates from C2C12 muscle cells treated with or without IFNγ/TNFα for 24h were used for immunoprecipitation experiment using antibodies against HuR or IgG as a negative control. Analysis of miRNA isolated from the immunoprecipitate was performed by RT-qPCR using primers specific for miR-330 ***(B)*** or for U6 ***(C)*** as a negative control. Quantifications are of three independent experiments (n=3), and error bars represent the SEM.

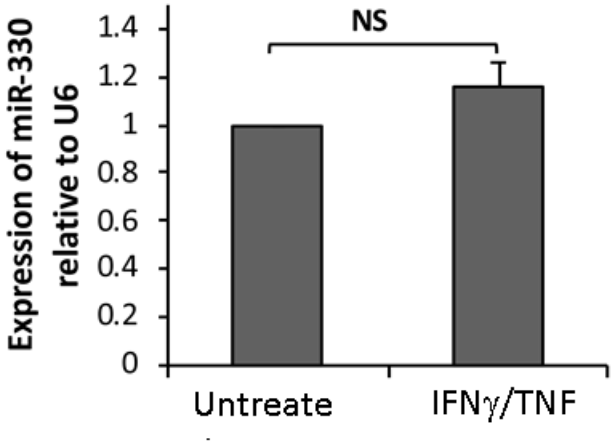


Figure 3.16. The expression of miR-330 does not change due to treatment of myotubes with IFNγ/TNFα.

miR-330 levels in C2C12 myotubes treated with or without IFNγ/TNFα for 24h was determined by RT-qPCR using primers specific to miR-330. Levels were standardized to U6 that was used as a negative control.

Next, we determined the impact of the predicted seed element of miR330, herein dubbed as miR-330 binding site (miR-330BS), on *STAT3*-3'UTR mediated translation as well as on its association with HuR. To achieve this, we generated Renilla-luciferase (R-*Luc*) constructs with the *STAT3* 3'UTR (R-*Luc*-3'STAT3) containing or not mutations in the miR-330BS (R-*Luc*-3'STAT3-*mut*-miR-330BS) (**Figure 3.17A**). These reporter constructs were then transfected into C2C12 cells and used in luciferase expression assays as well as in immunoprecipitation experiments with the anti-HuR antibody. Although deleting the miR-330BS did not affect the steady-state levels of R-*Luc* mRNA, this mutation increased luciferase activity by 3 folds when compared to the activity generated by intact R-*Luc*-3'STAT3 (**Figure 3.17B-C**). Moreover, there was a significant increase in the association of HuR with the R-*Luc*-3'STAT3-*mut*-miR-330BS mRNA when compared to the R-*Luc*-3'STAT3 control (**Figure 3.17D-E**). These results clearly indicate that preventing the recruitment of miR330 to its seed element enhances the binding of HuR to the *STAT3* 3'UTR.

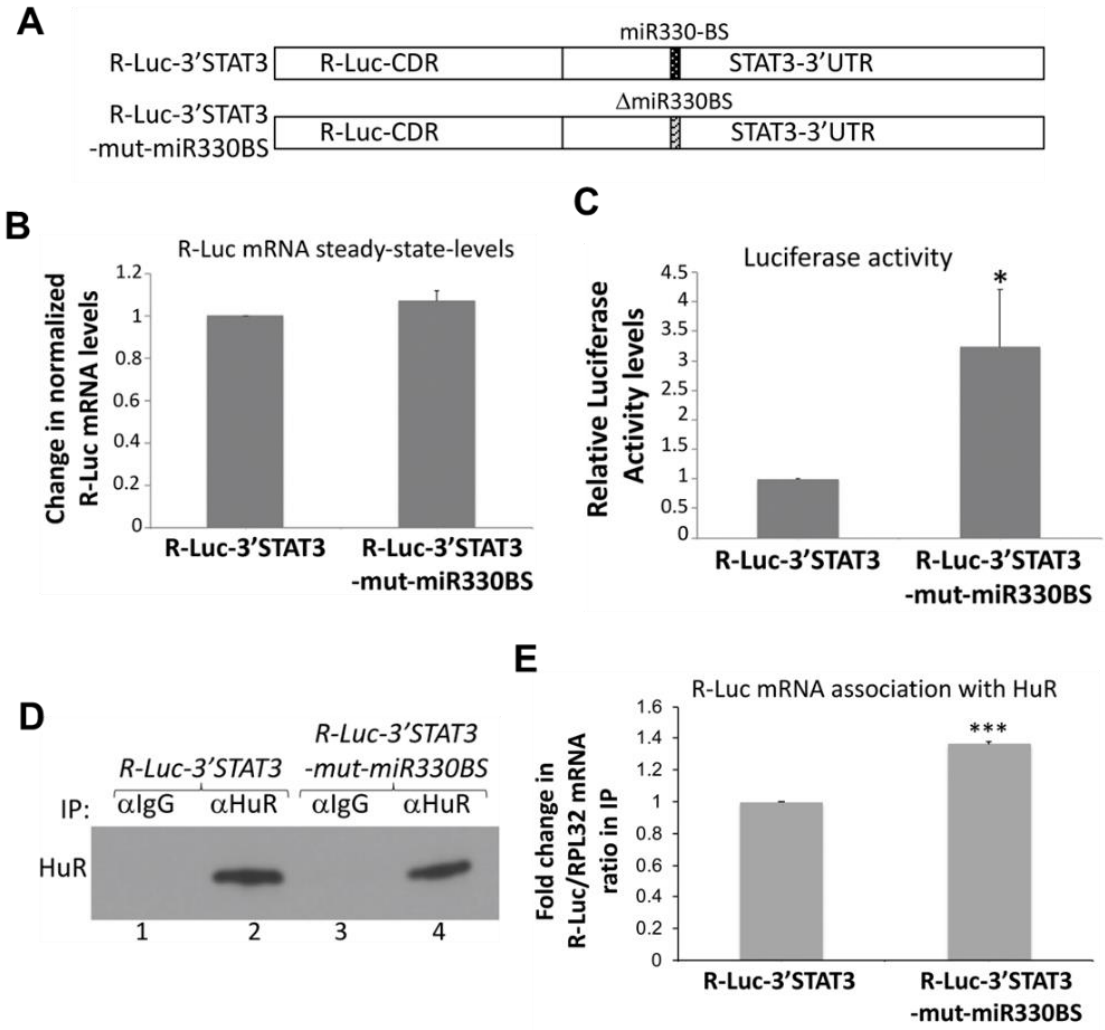


Fig. 3.17. The miR-330 seed element regulates the expression of STAT3.

(A) Schematic demonstrating the Renilla-luciferase (R-*Luc*) constructs with the *STAT3*-3'UTR (R-*Luc*-3'STAT3) or the *STAT3*-3'UTR mutant containing a mutation of the miR-330 seed element (R-*Luc*-3'STAT3-*mut*-miR330BS). ***(B-E)*** The reporter constructs described in *(A)* were transfected in C2C12 cells. R-Luc reporter mRNA steady state levels *(B)* as well as Luciferase activity *(C)* was determined for each construct. mRNA levels and luciferase activity for the R-*Luc*-3'STAT3-*mut*-miR330BS mRNA is shown relative to those obtained with the R-*Luc*-3'STAT3 construct. *(D, E)* Binding of HuR to the mRNA expressed from these constructs was determined by immunoprecipitating HuR *(D)* from lysates obtained from the cells described above and assessing by RT-qPCR *R-Luc* mRNA levels *(E)*. Levels were standardized to RPL32 mRNA levels. Quantifications for *(B, C and E)* are of three independent experiments (n=3), and error bars represent the SEM. Significance P-value in (C and E) was calculated using the unpaired T-test. *P<0.05, and ***P<0.001 from R-*Luc*-3'STAT3 samples.

The data outlined above raise the possibility that miR330 inhibits STAT3 translation by interfering with HuR binding to the *STAT3*-3'UTR. Indeed, overexpressing a miR-330 mimic in C2C12 cells significantly decreased STAT3 expression levels and, furthermore, significantly disrupted HuR association with the *STAT3* mRNA (**Figure 3.18A-B**). These data suggested that HuR promotes STAT3 expression by interfering with miR-330-mediated translation inhibition. If this is true, silencing miR-330 should rescue the expression of STAT3 in muscle cells depleted of endogenous HuR. Our data indicated that the expression of an anti-miR-330 (antagomir) in C2C12 muscle cells rescued (increased by at least two-fold) STAT3 protein levels in HuR knockdown cells when compared to cells treated with siHuR alone (**Figure 3.18C**). These results therefore indicate that HuR promotes STAT3 translation in muscle cells by binding to a *cis*-element 297nt apart from miR-330BS, partially alleviating the translation inhibition that is normally mediated by miR-330.

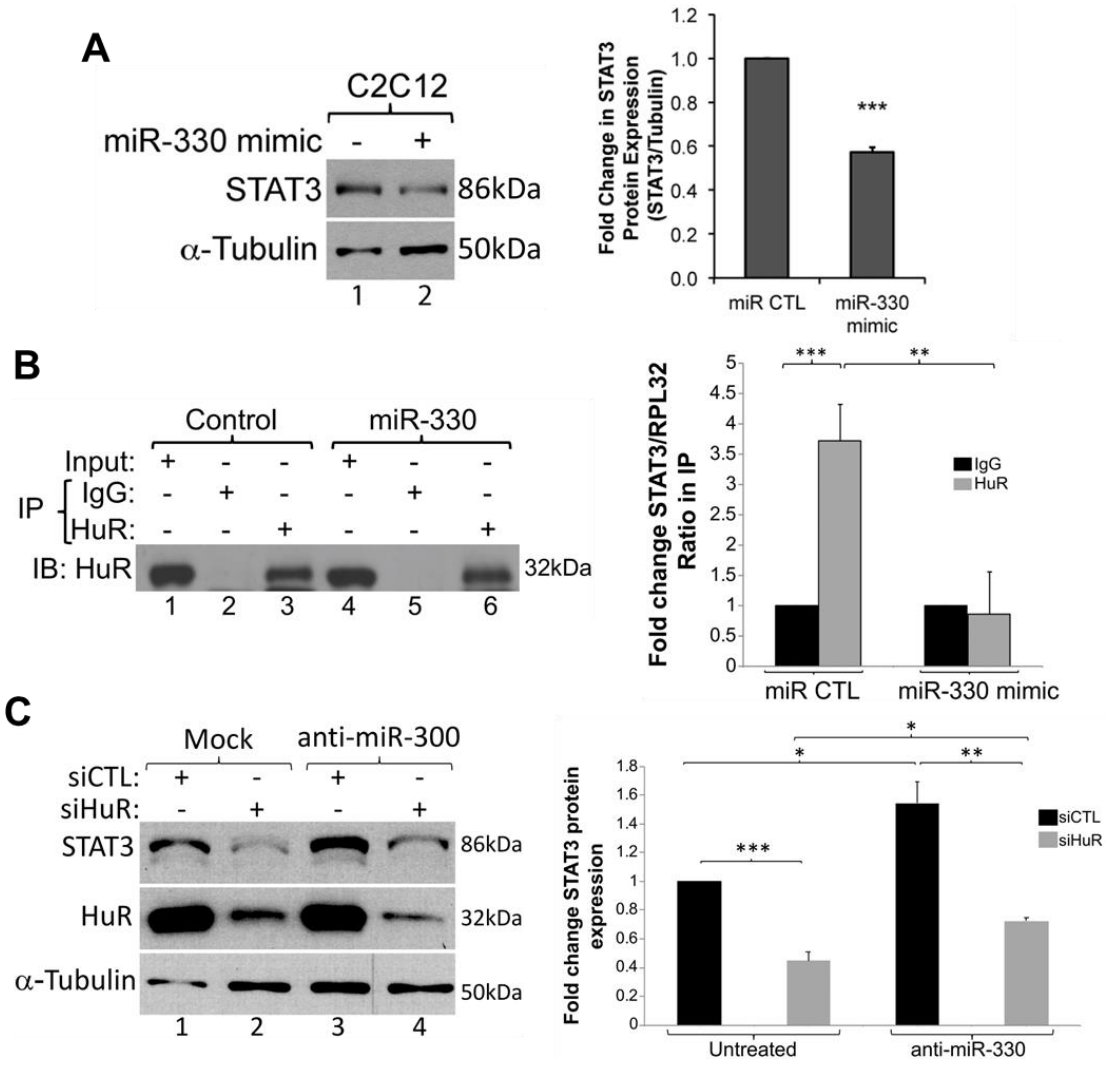

A
C2C12
miR-330 mimic - +
STAT3 86kDa
α-Tubulin 50kDa
1 2
Fold Change in STAT3 Protein Expression (STAT3/Tubulin)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
miR CTL
miR-330 mimic
B
Control
miR-330
Input: + - - + - -
IP
IgG: - + - - + -
HuR: - - + - - +
IB: HuR
32kDa
1 2 3 4 5 6
Fold change STAT3/RPL32 Ratio in IP
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
***
**
IgG
HuR
miR CTL
miR-330 mimic
C
Mock
anti-miR-300
siCTL: + - + -
siHuR: - + - +
STAT3 86kDa
HuR 32kDa
α-Tubulin 50kDa
1 2 3 4
Fold change STAT3 protein expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
**
***
siCTL
siHuR
Untreated
anti-miR-330
D
Myotubes treated with IFNγ and TNFα
1
HuR
STAT3 CDR
miR-330
3'UTR
STAT3 mRNA Translation
Muscle Wasting
2
HuR
STAT3 CDR
miR-330
3'UTR
STAT3 mRNA Translation
Muscle Wasting

Fig. 3.18. HuR negates the effect of miR-330 on the expression of STAT3.

(A) Total cell lysate from C2C12 cells transfected with a miR-330 mimic or a control miRNA were used for Western blot analysis **(left panel)** with antibodies against STAT3 and α-tubulin. **(right panel)** Densitometric quantification of STAT3 signal relative to α-tubulin signal. ***(B)*** Lysates obtained from C2C12 myotubes transfected with miR-330 mimic or miR control (negative control) were used for immunoprecipitation experiment using antibodies against HuR or IgG as a negative control. Western blot experiments showing immunoprecipitated HuR **(left panel)** and analysis by RT-qPCR of STAT3 mRNA associated to HuR **(right panel)** are shown. Levels were standardized to RPL32 mRNA levels. ***(C)*** C2C12 cells were transfected with siRNA targeting HuR or a non-specific control siRNA. The cells were then transfected with or without an antagomir against miR-330. Total cell lysate was used for Western blot analysis **(left panel)** using antibodies against STAT3, HuR, and α-tubulin. **(right panel)** Densitometric quantification of STAT3 levels were normalized to α-tubulin and is shown relative to the levels observed in untreated cells transfected with the control siRNA. All quantifications are of three independent experiments (n=3), and error bars represent the SEM. Significance P-values were calculated using the unpaired T-test. *P<0.05, **P<0.01, ***P<0.001 from equivalent miR CTL (A and B), or siCTL untreated (C) samples**. (D)** Model depicting the HuR-dependent posttranscriptional regulation of STAT3 expression during TNFα and IFNγ induced muscle wasting. In myotubes exposed to TNFα and IFNγ, HuR binds to a U-rich element in the 3'UTR of STAT3 mRNA promoting its translation. The association HuR with its binding element prevents binding of miR-330, thus inhibiting the affect of miR-330 on the translation of the STAT3 mRNA **(panel 1)**. However, under these conditions, the miR-330 mediated inhibition of STAT3 expression becomes active in the absence of HuR protein **(panel 2).**

3.6 Discussion

In this study we provide evidence supporting a key role of HuR in the progression of cytokine and cancer induced muscle atrophy both *in vitro* and *in vivo*. We identify *STAT3* mRNA as a novel target of HuR and we demonstrate that HuR is required for the expression of the STAT3 protein in wasting C2C12 myotubes and in skeletal muscle in mice. We also show that while HuR does not affect the stability nor the cellular movement of *STAT3* mRNA, HuR triggers muscle wasting by promoting the translation of the STAT3 protein. To achieve this, HuR binds specifically to a U-rich element in the *STAT3*-3'UTR to interfere, in part, with the miR-330-mediated inhibition of STAT3 translation. Together our data support a model whereby maintaining a high expression level of STAT3 protein in an HuR-dependent manner represents one of the key steps involved in the onset of muscle wasting (**Figure 3.18D**).

The RIP cDNA microarray analysis experiments clearly show that, consistent with its ability to bind high number of target mRNAs [527, 528], HuR associates with >2400 transcripts in untreated and fully formed myotubes (*Dataset 1*). As expected, many of these transcripts, such as *MyoD, Myogenin, HMGB1 and p21* mRNAs, are known targets of HuR and by regulating their expression, HuR plays a key role in myogenesis [471, 481, 483, 515, 529]. Surprisingly, however, in the presence of TNFα and IFNγ, HuR loses its ability to associate with the majority of its promyogenic mRNA partners and only binds to 74 messages, among which the mRNA encoding the procachectic factor *STAT3*. These observations raise the possibility that cytokines switch the function of HuR from a promoter of muscle fiber formation to an inducer of muscle wasting. In keeping with this observation, we have previously shown that TNFα and IFNγ change the function of HuR in muscle fibers *vis a vis* its promyogenic mRNA targets. Indeed, under these conditions the HuR-*MyoD* mRNA complex is disrupted, while at the same time HuR associates with and promotes the expression of the *iNOS* mRNA [506]. While these findings clearly provide a strong support for a functional switch of HuR in response to extracellular assaults such as cytokines, the molecular mechanisms behind this switch are still elusive.

The fact that the depletion of HuR prevents cytokine and cancer induced muscle wasting both *in vitro* and *in vivo* highlights the importance of HuR protein in the progression of this deadly syndrome. Indeed, our data establish that HuR participates in this process by promoting the translation of *STAT3* mRNA, which is one of the well-established drivers of muscle loss triggered as a result of inflammatory diseases such as cancer [130, 502, 503, 505]. HuR mediates this effect by directly binding to a U-rich element, P2B, in the *STAT3-3'UTR*, which consequently prevents miR-330-mediated inhibition of STAT3 translation. There are numerous examples of cross-talk between HuR and miRNA that lead to a collaborative or competitive regulation of the expression of numerous HuR mRNA targets in various systems. For example, HuR collaborates with the let-7 miRNA to inhibit the translation of *cMyc* mRNA in HeLa cells [525]. In contrast HuR, by competing with miR-195 for binding to the 3'UTR of the *stromal interaction molecule 1* (*stim1)* mRNA, modulates STIM1 expression and calcium release during wound healing [530]. Our laboratory has previously shown that HuR negates the effect of miR-1192 on HMGB1 expression despite the fact that they both simultaneously associate to the *HMGB1* mRNA in muscle cells [515]. In this study we demonstrate that the overexpression of miR-330 mimic decreases STAT3 protein levels while the use of an antagomir targeting this miRNA rescued, in part, the decreased expression of STAT3 in cells depleted of HuR. In addition, our data also show that overexpressing miR330 mimic interferes with the binding of HuR with *STAT3* mRNA, suggesting a competitive interplay between these two *trans*-acting factors. This observation raises the question as to how both HuR and miR-330 are found within the same complex despite their competition for binding to the *STAT3* mRNA? This could be explained by the existence of a dynamic equilibrium in the binding of miR-330 and HuR to the *STAT3* 3'UTR. In fact, a dynamic equilibrium between HuR and miR21 has been previously reported as a way to modulate the translation of *PDCD4* mRNA, where HuR directly binds to both the *PDCD4* 3'UTR as well as to the miR21 itself [531]. In our case therefore, it is possible that the strength of the binding of HuR or miR330 to its *cis*-element determines the outcome of STAT3 translation, promotion or inhibition. Our data, however, clearly show that the binding of one of these *trans*-acting factors to the *STAT3* 3'UTR reduces, although not completely, the binding of the other. Although this result explains in part

why these two *trans*-acting factors co-immunoprecipitate together in a *STAT3* 3'UTR-dependent manner, it also raises the possibility that the binding of either one of them affects the folding of the *STAT3* 3'UTR in a way that triggers the slow removal of the other factor. Testing experimentally this possibility will help determine the mechanisms behind this dynamic competitive interplay between HuR and miR330 in modulating STAT3 translation.

In this study we also show that depleting miR-330 activity did not fully restore STAT3 protein levels in HuR-knockdown muscle cells, raising the possibility of the involvement of other miRNAs. Indeed, it is well-known that the *STAT3* 3'UTR is targeted by numerous miRNAs [532, 533]. Therefore, it is possible that HuR also indirectly modulates the inhibition of STAT3 translation mediated by these miRNAs. While our data showing only a partial rescue of STAT3 expression with anti-miR330 antagomir in HuR depleted cells could be explained by this possibility, these results clearly links in part, HuR effect to its ability to interfere with miR-330 mediated translation inhibition. This observation also raises the possibility that other *trans*-acting factors are involved in regulating STAT3 expression. Indeed, it has been shown that the HuR-binding sites P2B and P16 that we describe in this study associate with other RBPs, such as Arid5a and CPEB1. Arid5a stabilizes *STAT3* mRNA by competing with the endoribonuclease Regnase-1 for binding to a stem-loop structure (1738-1765) that is found within the P16 element [534]. On the other hand, CPEB1 prevents the synthesis of STAT3 protein by binding to two putative U-rich-elements, one of which is located within the HuR-binding site P2B [535]. This later observation is consistent with our findings that P2B but not P16 element is mainly responsible for modulating the translation of *STAT3* mRNA. However, our data do not exclude the possibility of P16 involvement in regulating STAT3 expression posttranscriptionally at other levels such as mRNA stability [534]. Therefore, the possibility exists that competition or collaboration with these or other factors contribute to the HuR-mediated modulation of STAT3 expression in response to various stimuli.

Together, our data indicate that uncovering these mechanisms may lead to the identification of novel therapeutic options that can be exploited to interfere with STAT3-induced muscle wasting.

3.7 Materials and Methods

Plasmid construction

The pCMV-SPORT6 plasmid containing the full length STAT3 cDNA (accession number: BC003806) was purchased from Open Biosystems (catalogue number: MMM4769-99609717). The full-length 3′UTR of mouse STAT3 was subcloned into a pRL-SV40 vector (Promega) by PCR amplification of the pCMV-SPORT6 plasmid described above. The pRL-luc-3′STAT3-ΔP2B (containing the deleted ATTTTTTTTTTTAATTT sequence from the P2-B region), the pRL-luc-3′STAT3-ΔP16 (containing the deleted last 155nt from the STAT3-3'UTR as shown in *SI Appendix* Fig. S3) and the pRL-luc-3′STAT3-mut-miRBS (mutated miR-330 seed element) plasmids were generated by Norclone Biotech Laboratories, London, ON, Canada. The GFP and GFP-HuR plasmids were generated as described [493].

Cell culture and transfection

C2C12 cells were obtained from American Type Culture Collection (VA, USA) and grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing high glucose, L-glutamine, sodium pyruvate, and supplemented with 20% fetal bovine serum and 1% penicillin/streptomycin. Murine Lewis Lung carcinoma cells (LLC) were obtained from the ATCC and grown in DMEM with 10% FBS and 1% streptomycin–penicillin (Invitrogen). Transfections with siRNA specific for HuR or STAT3 or plasmids expressing GFP or GFP-HuR, as well as the pRL plasmids containing the STAT3 3'UTR with/without mutations of the P2 or P16 HuR binding sites or the miR-330 binding sites was performed using jetPrime reagent (PolyPlus Transfection) according to manufacturer's protocol. The siHuR (5'- CAUCAACACCGAGAUCAAAdTdT -3') was custom synthesized (Dharmacon) [483] while the siSTAT3 (5'-GCCUCAAGAUUGACCUAGAtt -3') was purchased from Invitrogen (Ambion). The mmu-miR-330-5p antagomir or mimic (Invitrogen, Ambion) were transfected at a final concentration of 200 nM using jetPrime reagent (PolyPlus Transfection) according to manufacturer's protocol.

miRNA Prediction

The miRNA predicted to associate with the STAT3 3'UTR was determined on the TargetScan (www.targetscan.org), miRanda (www.miRbase.org) and microrna.org websites.

Western blot analysis

Whole cell lysates were prepared by lysis in buffer containing 50 mm HEPES (pH 7.0), 150 mm NaCl, 10% glycerol, 1% Triton X-100, 10 mm sodium pyrophosphate, 100 mm NaF, 1 mm EGTA, 1.5 mm $MgCl_2$, 0.1 mM sodium ortho-vanadate, and complete EDTA-free protease inhibitors (Roche Applied Science). Primary antibodies used were total STAT3 (#9132 or 9139; Cell Signaling), □-tubulin (Developmental Studies Hybridoma Bank), HuR [482] and AGO2 [515].

Quantitative PCR (qPCR)

One microgram of total RNA was reverse transcribed with the M-MuLV Reverse Transcriptase (New England BioLabs) according to the manufacturer's protocol. Each sample was diluted 1/20 and used to detect the mRNA levels of *STAT3, R-Luc* and *RPL32* or *GAPDH* as a loading control using the SsoFast reagent (Bio-Rad Laboratories). The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method, where ΔΔCt is the difference in Ct values between the target and reference genes. Primers used for qPCR are as follows. STAT3 (F: 5' TAT CTT GGC CCT TTG GAA TG – 3', R: 5' - GCT GCT TGG TGT ATG GCT CT – 3'), GAPDH (F: 5' - AAG GTC ATC CCA GAG CTG AA - 3', R: 5' - AGG AGA CAA CCT GGT CCT CA – 3'), RPL32 (F: 5' - TTC TTC CTC GGC GCT GCC TAC GA – 3', R: 5' - AAC CTT CTC CGC ACC CTG TGG TCA – 3'), R-luc (F: 5' - TTG AAT CAT GGG ATG AAT GG - 3', R: 5' - TGT TGG ACG ACG AAC TTC AC - 3'). The miR-330 and U6 primer sets were purchased from Qiagen.

RNA-Immunoprecipitation (RIP)

Total cell extract (TCE) from C2C12 cells either untreated or treated with IFNγ/TNFα were prepared as previously described [482]. Immunoprecipitation was performed with

an antibody against HuR (3A2) or AGO2 [515] or IgG (Jackson ImmunoResearch Laboratories) as previously described [515]. The isolated mRNA or miRNA associating to HuR was determined by microarray analysis [506] or RNA-Sequencing respectively (Institute for Research in Immunology and Cancer (IRIC) Genomic Platform, University of Montreal) respectively. RT-qPCR experiments were then performed on the identified mRNA or miRNA to validate these associations. Analysis of miRNA was performed using the Universal cDNA synthesis kit (Exiqon) followed by qPCR analysis for miRNA using the SyBr Green Master Mix kit (Exiqon) as we have previously described [515].

RNA Electrophoretic Mobility Shift Assay (REMSA)

cDNA templates for the 16 cRNA probes spanning the STAT3 3'UTR were generated as previously described [515]. Briefly, cDNA for 14 of the 16 cDNAs spanning the STAT3 3'UTR, with the exception of P4 and P5, were amplified by PCR using the complete STAT3 3'UTR as a template and individual primer sets specific for each region (Supp. Table 2). PCR products for these regions were purified with an EZ-10 PCR product spin column purification kit (BioBasic). The P4 and P5 cDNA templates were obtained by annealing sense and anti-sense oligonucleotides spanning these regions. The radiolabelled probes were generated from these DNA templates by *in vitro* transcription reaction using radiolabelled □-^{32}P-UTP and T7 RNA polymerase (Promega). Recombinant GST or GST-HuR were incubated with 100 000 cpm of radiolabelled probe per reaction for 15 minutes and then with 100 □g of heparin for 15 min. After, samples were run for 2.5hrs at 180V on a native 5% acrylamide gel that had been pre-run for 1h at 80V. After fixation for 15 min, the gels were dried at 80°C for 1h and exposed at -80°C. For the K_d experiments (**Fig. 4*C***), 0, 10, 20 or 40 nM of GST-HuR were used. For REMSAs performed with RNase T1, recombinant GST or GST-HuR was first incubated with the radiolabelled mRNA for 15 min to allow complex formation, then heparin, followed by digestion with 300U RNase T1 (Thermo Fisher) for 10 min at room temperature.

Actinomycin D Pulse-Chase analysis

Actinomycin D pulse-chase experiments were performed as previously described [518]. C2C12 muscle cells were transfected with either a control siRNA or a siRNA specifically

targeting HuR using jetPrime reagent (PolyPlus Transfection) according to manufacturer's protocol. After 24h, cells were treated with IFNγ/TNFα for 24h before treatment with 5 μg mL^{-1} of ActD, a transcriptional inhibitor, for the indicated periods of time. *STAT3* and *G*APDH mRNA levels at each time point was determined by RT-qPCR using primers specific for both genes. *STAT3* mRNA levels were normalized to *GAPDH* levels and plotted as the percentage at the zero time point which is considered as 100%.

Polysome fractionation

Forty million C2C12 cells either untreated or treated with IFNγ/TNFα and either depleted of HuR or not were prepared for each sample. Cells were treated with the protein synthesis inhibitor cycloheximide (CHX; 100 mg mL^{-1}) for 15 min at 37°C immediately before harvesting. The cytoplasmic extracts of C2C12 cells were obtained by lysing pellets with a Dounce homogenizer and tight pestle. Isolated cytoplasmic fractions were centrifuged at 130,000g for 2h on a sucrose gradient (15-50%) and fractionated using a gradient fractionation system (Brandel) and absorbance was monitored at 254nm. RNA was extracted using phenol/chloroform from each fraction and the non-polysome (NP) fractions and polysome (P) fractions were combined. The levels of *STAT3* and *5.8S* mRNA were analysed using RT-qPCR. The *STAT3* mRNA levels were normalized to *5.8S* mRNA levels then the polysome / non-polysome (P/NP) ratio was determined and further normalized to the control siRNA, untreated condition.

Immunofluorescence

Cells were fixed in 3% para-formaldehyde for 20min, permeabilized with 0.1% Triton-X 100 in PBS for 20 minutes then incubated with antibodies against myoglobin (1:250; ab77232, Abcam) and myosin heavy chain (1:1000; clone MF-20, Developmental Studies Hybridoma Bank) [502]. Images were taken with a 40X magnification lens on an inverted Axiovert 200M microscope with an Axiocam MRm camera (Zeiss).

Fluorescent *in-situ* hybridization

in-situ hybridization were performed as previously described [515]. Briefly, the sense and anti-sense probes for *STAT3* mRNA were generated from PCR fragments with the

following sequence and fused to the indicated RNA polymerase promoter: anti-sense fused to T7 promoter (5' – TAA TAC GAC TCA CTA TAG GGT GCA CCA GCT GTA CAG CGA AC – 3'), sense fused to T3 promoter (5' AAT TAA CCC TCA CTA AAG GGG ATC CTG CAC TCG CTT CCG G – 3'). Probes were DIG-labeled according to manufacturer's instructions (Roche). C2C12 cells depleted or not of HuR were fixed and permeabilized as described above. Cells were incubated with DIG-labeled anti-sense or control sense probe to detect *STAT3* mRNA as well as the 3A2 (anti-HuR) antibody (1:1000). Cells were blocked with 1% goat serum for 10 min and incubated with anti-DIG and goat anti-mouse secondary antibody.

Luciferase expression/activity

R Luc mRNA steady state levels were determined by RT-qPCR using primers specific for R-Luc. Luciferase activity was furthermore measured using a Renilla luciferase assay system (Promega) following the manufacturer's instructions as previously described [515].

Muscle freezing and preparation of muscle/cell extracts

Quadriceps muscles were dissected from the mice, mounted on 7% tragacanth gum and then snap frozen in liquid-nitrogen-cooled isopentane for 10-20 sec. Muscle extracts were prepared by the homogenization of the frozen muscle tissue in extraction buffer (1x PBS,1% NP-40, 0.5% DOC, 0.1% SDS, 2mM SOV, 1X protease inhibitor (Roche)). Cell extracts were prepared by incubating C2C12 muscle cells with lysis buffer (50mM HEPES pH 7.0, 150mM NaCl, 10% glycerol, 1% Triton, 10mM pyrophosphate sodium, 100mM NaF, 1mM EGTA, 1,5mM MgCl2, 1X protease inhibitor (Roche) for 15min on ice, vortexing every 5 min. The lysed muscle/cells were then centrifuged at 12000 rpm for 15min at 4°C in order to collect the supernatant containing the proteins.

Animals

All experiments using animals were approved by the McGill University Faculty of Medicine, Animal Care Committee and comply with guidelines set by the Canadian Council of Animal Care. HuR muscle specific knockout mice (muHuR-KO) and their

control littermates, generated on a C57BL/6 background [521], were housed in a controlled environment and provided commercial laboratory food (Harlan #2018; 18% protein rodent diet; Madison, WI). They were grown in sterile cages with corn-cob bedding and they had free access to food and water.

Lewis Lung Carcinoma (LLC) xenograft

The LLC tumors were established in 8-9 weeks old male muHuR-KO or control littermates as described [521]. Tumours were formed due to the subcutaneous injections of 1×10^6 LLC cells in the right flank of the hindlimb of these mice. Subcutaneous injections of PBS in muHuR-KO mice or control littermates PBS were used as control. Quadriceps muscles were harvested from these mice 30 days post-injection of either PBS or LLC cells.

3.8 Acknowledgements

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Chapter 4 | General Discussion

4.1 Overview

The goal of this thesis was to tackle the dichotomic function of HuR in skeletal muscle, by deciphering mechanisms governing its pro-myogenic and pro-cachectic functions. To this end, we uncovered a new mechanism regulating the function of HuR during myogenesis and a new mechanism through which HuR promotes muscle wasting. In Chapter 2, we show that the posttranslational modification PARylation regulates the pro-myogenic function of HuR. We show that TNKS1 is required for the PARylation of HuR during myogenesis. TNKS1 binds HuR through the consensus TBM harbored in the hinge region of HuR, and promotes the localization of HuR to the cytoplasm as well as its caspase-mediated cleavage. Once in the cytoplasm, HuR regulates the expression of NPM and myogenin, resulting in the formation of muscle fibers. As such, we were able to reveal a novel regulatory mechanism for the well-known function of HuR in skeletal muscle fiber formation. Unlike its function in myogenesis, very little is known about the function of HuR in muscle wasting. Therefore, in Chapter 3, we identified a new molecular mechanism of cachexia which is mediated by the binding of HuR to the *STAT3* mRNA. In doing so, we showed that HuR induces the translation of the STAT3 message by inhibiting the actions of miR-330. In doing so, we further characterized the function of HuR in promoting muscle atrophy. Thus, we provided additional evidence demonstrating that HuR has opposing functions in skeletal muscle in either promoting muscle fiber formation or inducing muscle wasting.

Although these studies advance our knowledge about the function of HuR in skeletal muscle, much remains to be revealed. For example, our study in Chapter 2 shows that HuR PARylation promotes its function in regulating mRNA turnover. There are, however, several unanswered questions regarding the PARylation of HuR. For example, does this modification also regulate the ability of HuR to promote the translation of target mRNAs such as HMGB1? Does the PARylation of HuR affect its interaction with protein ligands, such as pp32 and KSRP, that mediate its cellular localization or RNA binding activity respectively? Moreover, thus far, only two pro-

cachectic targets of HuR have been identified, iNOS and STAT3. Since *iNOS* is a gene target of STAT3, are these mRNAs part of an RNA operon through which HuR induces muscle wasting [536]? If so, what are the other mRNAs that are part of this operon? Additionally, while the cleavage of HuR was identified as being the switch between its pro-survival and pro-apoptotic function in response to mild and severe stress, what mechanism(s) are in place in muscle to mediate the switch between HuR's pro-myogenic and pro-cachectic. Since PARP1 has been suggested to induce muscle wasting and was also shown to posttranslationally modify HuR in macrophages exposed to inflammatory stimuli, can PARP1-mediated PARylation of HuR (instead of TNKS1) impact its pro-cachectic function [421, 428, 429]? If this is the case, an investigation of the impact of PARP and PARG inhibitors in myopathies and cancer-cachexia would provide insights into their therapeutic potential.

4.2 Role of PARylation in the function of HuR

As previously mentioned, trans-acting factors cooperate with HuR to regulate the expression of various targets at different posttranscriptional levels in a spatial-temporal manner under various conditions. Indeed, during the early phase of muscle cell differentiation, HuR complexes with KSRP to promote the decay of the *NPM* mRNA [151]. Since KSRP and HuR associate in an RNA independent manner, their interaction is required for the recruitment of this complex to the *NPM* mRNA. Therefore, it is possible that the PARylation of HuR changes its structural conformation in a way that enables it to associate with its trans-acting factors such as KSRP. Indeed, we have recently shown that HuR regulates the stability of *Myogenin, MyoD* and the *p21* mRNA by interacting in an RNA independent manner with the RBP YB-1. Our data demonstrates that the HuR/YB-1 complex associates with a GRE in the 3'UTR of the mRNAs to promote their stability (unpublished data). One question that arises from this study is whether PARylation affects the formation of the HuR/YB-1 and, furthermore, the expression of these mRNAs.

The differential localization of HuR is known to mediate its function in cells. Indeed, the repertoire of trans-acting factors that HuR associates with under different cellular conditions most likely depends on its localization inside the cell. Our data clearly

demonstrate that PARylation promotes the cytoplasmic accumulation of HuR during myogenesis. This event might be a result of the acidic charged conferred by the pyrophosphates in pADPr. Indeed, one of the main impacts of PARylation on target proteins is a change in their localization, probably due to the acidity of the protein which occurs due to the modification. Several studies have drawn a relationship between the protein isoelectric point (pI) and subcellular localization [537-540]. It was shown that cytoplasmic, Golgi and vacuole targeted proteins are highly biased towards acidic pI, while nuclear, membrane and mitochondrial associated proteins have rather basic pI. Interestingly, acidic proteins seem to have higher interactions than basic proteins [541]. Therefore, the acidity conferred to HuR might be contributing to its cytoplasmic translocation, where it gets cleaved and accumulates in this compartment. As such, we show that PARylation is a key regulatory mechanism regulating the localization and cleavage of HuR during muscle cell differentiation likely by affecting its acidity resulting in its interaction with protein ligands.

Other posttranslational modifications of HuR, including phosphorylation and methylation, have been shown to modulate its RNA-binding activity and localization in various cell systems. We assessed the impact of phosphorylation and methylation of HuR during myogenesis and found that they do not affect its pro-myogenic activity. Therefore, PARylation, in addition to caspase-mediated cleavage, are likely the main posttranslational modification which affect the function of HuR in skeletal muscle. Nonetheless, it is possible that PARylation collaborates with other posttranslational modifications, such as its cleavage, by modifying HuR such that it renders it more accessible to enzymes which mediate these other modifications. Indeed, it was previously shown that PARylation of FoxO3 increased its phosphorylation in the cytosol [542]. Therefore, there might be an interplay between PARylation and other posttranslational modifications in regulating the function of HuR during myogenesis and potentially in other processes. Mass spectrometry analysis of the posttranslational modifications of GFP-HuR^{WT} compared to GFP-HuR^{G224D} might provide an initial insight on the potential interplay between PARylation and other posttranslational modifications of HuR in myogenesis.

4.3 Updated and potentially greater role of HuR in muscle wasting

Thus far, very little is known about the role of HuR in muscle wasting. Our lab has previously shown that HuR promotes the stability of *iNOS* mRNA, which is one of the main effectors of muscle wasting. In Chapter 3 of this thesis, we identified STAT3 as a new target of HuR that is regulated at the translation level. Although we decided to validate and focus our research on the HuR-mediated regulation of STAT3 (since it is a well-known promoter of muscle atrophy) other targets were also identified in the microarray results obtained by performing an RNP-immunoprecipitation experiments using extracts of myotubes treated with cytokines. Thus, there remains other unvalidated genes that are targeted by HuR in muscle wasting, which suggests that there is a larger set of genes that likely comprise an RNA operon that HuR may be affecting to promote muscle wasting.

In addition, HuR has been recently linked to Duchenne muscular dystrophy (DMD) [543]. This disease is caused by mutations and deletions in the dystrophin gene. Utrophin A is an autosomal homolog of dystrophin that can potentially be used as a treatment approach for DMD, since it has a high sequence identity and functional redundancy to dystrophin [544, 545]. Non-steroidal anti-inflammatory drugs (NSAIDs) and p38 MAPK activators have been previously shown to improve muscle function and elevate utrophin A expression [546-549]. In an attempt to test if celecoxib, an FDA approved cyclooxygenase-2 (COX2) inhibitor, that is a NSAID that also activates p38 MAPK signaling, could be beneficial in ameliorating DMD, HuR protein level was found to be increased *in vivo,* in response to celecoxib treatment [543, 550]. In addition, HuR was found to bind to *utrophin A* mRNA, suggesting that utrophin A expression is regulated by HuR.

HuR was shown to regulate pathways, in non muscle cells, that were shown to be involved in skeletal muscle wasting. Indeed, in smooth muscle cells, HuR was shown to be involved modulating autophagy through the regulation of AMPKα1 and AMPKα2 expression [551]. HuR binds to AREs in the 3'UTR of these mRNAs thereby increasing their stability and translation. AMPK is a key factor in cellular energy metabolism and a key upstream activator of autophagy [552]. The depletion of HuR reduced p-AMPK and

the activation of the downstream autophagy-related protein LC3II, underlining the significant role of HuR in autophagy. As mentioned in the introduction, autophagy is involved in cancer-cachexia-induced muscle wasting. Therefore, there is potentially a bigger role for HuR in muscle wasting, through the activation of different pathways, besides the STAT3 and iNOS pathways. Although these mRNAs were not identified in our microarray analysis (Chapter 3), perhaps repeating the analysis by performing a PAR-CLIP (photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation) analysis would give a better characterization of the repertoire of mRNAs targeted by HuR during muscle wasting.

Lately, an increasing number of studies show that miRNAs, including miR-206, miR-21, and miR-378 are associated with cachexia [553-556]. Previous studies show that miRNAs modulate the function of HuR, and additionally, our miRNA sequencing analysis identified 15 miRNAs that interact with HuR in muscle fibers (Chapter 3) [152, 169, 272]. Therefore, it would not be surprising that HuR may be found to interact with different, pro-cachectic miRNAs if a miRNA sequencing in wasting muscle fiber is performed.

4.4 Potential role of PARylation in regulating the pro-cachectic function of HuR

Mechanisms regulating the function of HuR in skeletal muscle have not been fully discovered. In this thesis, we show that PARylation by TNKS1 promotes the pro-myogenic function of HuR. Nonetheless, another enzyme from the same family as TNKS1, PARP1, which is the best-characterized PARP, also modifies HuR with the same modification, albeit to promote the pro-inflammatory function of HuR in stimulated macrophage cells [345]. HuR has been previously shown to compete with miR181 to promote the stabilization of *TNFα* mRNA [175]. Similarly, HuR competes with miR51 to alleviate the decay of the *Cxcl2* mRNA. Importantly, this event was shown to be modulated by the oligomerization of HuR, which is induced by PARP1-mediated PARylation [350]. This PARylation of HuR was shown to promote the stabilization of pro-inflammatory messages such as *Cxcl2* [345]. Therefore, it is possible that TNKS-1-mediated PARylation promotes the pro-myogenic function of HuR, while PARP1-mediated PARylation promotes the pro-cachectic function of HuR, since it involves the

stabilization of the pro-inflammatory factor. This hypothesis can be supported by the phenotype of PARP1 KO mice which have an attenuated muscle wasting phenotype compared to their controls [428]. In addition, PARP inhibitors reduce muscle wasting, making this a plausible hypothesis [422, 426, 429, 557]. Moreover, it was previously shown that PARP1 levels decrease in myotubes compared to myoblasts [468]. While in models of muscle wasting resulting from various sources, such as ageing [423], burn injuries [424], myopathies [467], PARP1 levels and activity increased. Therefore, it is possible that during myogenesis, TNKS1 is activated and PARylates HuR, while during muscle wasting, PARP1 levels increase, and PARP1 PARylates HuR to promote wasting. The switch in PARPs, from TNKS1 to PARP1, in mediating the PARylation of HuR under inflammatory conditions thus could provide an explanation as to how HuR switches from being a pro-myogenic to a pro-cachectic factor in skeletal muscle.

Variations in the levels of PARPs might explain the seemingly higher impact of PARP1 in muscle wasting. However, it is well established that PARP1 and TNKS1 do not catalyze the same pADPr structure, where PARP1 forms branched pADPr and TNKS1 forms linear polymers. Although the functional difference between both structures of the modification is not clear, recent studies suggest that the length and structure of pADPr polymers affect cellular outcome [558]. In fact, longer, branched pADPr polymers have a longer half-life than linear or shorter branched polymers. Additionally, longer branched polymers seem to be responsible for parthanatos, and form stronger non-covalent binding bonds with proteins containing pADPr-binding domains. This may further support the hypothesis that TNKS1-mediated PARylation of HuR, which involved linear polymers, affects its function in a different manner than the PARP1-mediated PARylation, which involves branched polymers. However, clear distinction between both types of pADPr modifications of a single protein remains unknown.

4.5 Therapeutic impact of PARP and PARG inhibitors in muscle wasting

The benefits of using PARP inhibitors in muscle wasting have been previously explored. The significance of the role of PARP1 in muscle wasting is underlined by the ameliorated cachectic phenotype of PARP1 KO mice [428]. In fact, acetylation of FoxO3

did not increase in these mice compared to their controls. It was shown that using the PARP1 inhibitor, BGP-15, protected against cachexia and improved cardiac and skeletal myopathies [429]. Although the exact mechanisms have not been fully deciphered, PARP inhibitors were shown to reduce the expression of inflammatory factors (including TNFα, IFNγ, and IL-6) in skeletal muscles of patients with third degree burn [426]. This is supported by the well-known function of PARP1 in inflammation, especially its involvement in the STAT3 and NFκB pathways, which are key in mediating muscle wasting.

Additionally, based on the results of Chapter 2, the use of PARG inhibitors might prove beneficial to improve muscle differentiation to enhance the activity of TNKS1-mediated PARylation. Since PARP1 inhibitors ameliorate muscle wasting one could potentially consider combining these inhibitors with PARG inhibitors to treat muscle related pathologies. Although this might seem controversial, it is currently under investigation as possible combinatory therapies for cancer. Indeed, PARG inhibitors seem to complement and increase the impact of PARP inhibitors as a potential combinatory therapy to treat cancers marked by genomic instabilities [559]. So far, PARG inhibitors use have proven to be challenging due to undesirable characteristics for therapeutic use, such as low cell permeability, low activity in cells, off-target effects, and low bioavailability. Recent studies are investigating new PARG inhibitors that may provide potential therapeutics for cancer [560]. If proven efficient, their use in combination with PARP1 inhibitors might be beneficial for muscle wasting by inhibiting the activity of PARP1 and enhancing the pro-myogenic activity of TNKS1.

It is noteworthy that HuR has been shown to bind the mRNA of PARG and upregulates its expression through stabilization [561]. Silencing HuR was shown to increase the impact of PARP inhibitors in pancreatic ductal adenocarcinomas. HuR-KO-mediated downregulation of PARG enhanced the trapping of PARPs on DNA and thus increase cytotoxicity of PARP inhibitors, which is an impact of PARG inhibitors that is being described in various models. Therefore, the interplay between HuR and PARylation might be more significant than being a simple modification, where there

might be a feedback loop involving HuR and PARylation, which can be taken advantage of in therapy.

4.6 Potential role of PARPs in Muscle Wasting.

Although mechanisms underlying the role of PARPs in muscle wasting are not fully understood, it seems that several PARPs have been shown to be involved in autophagy. For instance, PARP1 seems to have a prominent role in positively regulating autophagy in cardiac and smooth muscle [542, 562, 563]. PARP1 activation was shown to induce excessive autophagy in these cells through activation of the AMPK/mTOR pathway [563]. This event was important for the development of hyperplasia and hypertrophy of smooth muscle cells, which lead to airway remodelling in asthmatic patients. Similarly, PARP1-mediated activation of the AMPK/mTOR pathway induced autophagy and apoptosis of cardiomyocytes in myocardial infarction condition [562]. In both case, PARP-1 mediated apoptosis and excessive autophagy negatively impacts the condition. PARP1 was also shown to promote autophagy in cardiomyocytes by modulating FoxO3a transcriptional activity [542]. PARP1 and its catalytic activity displace histone 1 from the FoxO3a promoter of autophagy-related genes, recruits FoxO3a to the nucleus, and thereby promotes its transcriptional activity. Since several studies have shown that FoxO3 is targeted by PARP1-mediated PARylation [564] it would not surprising that PARP1 might be involved in muscle wasting by regulating the autophagy related activity of FoxO3.

Unlike PARP1, PARP2 is believed to inhibit autophagy [374]. In fact, PARP2 deletion resulted in the accumulation of autophagosomes in murine skeletal C2C12 muscle cells and embryonic fibroblasts (MEFs). PARP2 and its enzymatic activity induced the breakdown of autophagosomes through the activation of AMPK and mTORC2, but not mTORC1. Although AMPK is known to induce autophagy, this group observed that there seems to be a causative role of the inhibition of AMPK in the induction of autophagy.

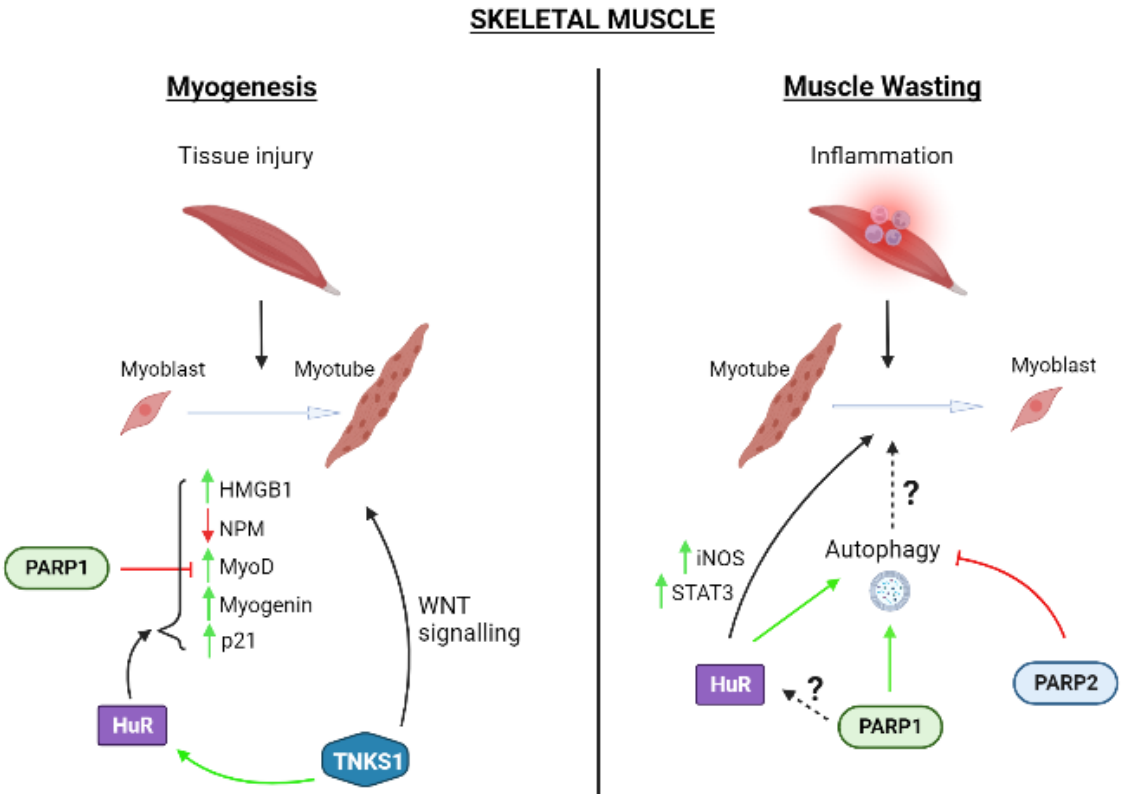

Figure 4.1 Potential role of PARylation in myogenesis and muscle wasting.

(Left panel) Myogenesis. HuR promotes myogenesis by promoting the translation of HMGB1 [152], the decay of NPM [151], and the stability of MyoD, myogenin, and p21 mRNAs [183, 325]. We showed in chapter 2 that TNKS1 promotes the pro-myogenic function of HuR. Others have shown that Wnt signalling, which is stimulated by the activity of TNKS1, regulates embryogenic and postnatal myogenesis [384, 401, 402, 404-406, 565]. PARP1 negatively impacts myogenesis by preventing the transcriptional activity of MyoD [400]. (Right panel) Muscle wasting. HuR promotes muscle wasting by stabilizing iNOS mRNA and promoting the translation of STAT3 (Chapter 3) [107]. In smooth muscle, HuR promotes autophagy by stabilizing AMPKα and AMPKβ [551]. PARP1 causes excessive autophagy in smooth and cardiac muscle through the activation of AMPK/mTOR and FoxO3a pathway [542, 562, 563]. PARP2 is involved in autophagosomes clearance in murine C2C12 skeletal muscle cells and in MEFs [374]. While the involvement of HuR, PARP1, and PARP2 in autophagy have been demonstrated, their importance in muscle wasting linked autophagy has not been shown.

TNKS is another PARP that was shown to be involved in a specific type of autophagy which targets peroxisomes (pexophagy) [399]. TNKS1 and TNKS2 were shown to interact with the peroxisomal protein PEX14 and with the autophagosomal protein ATG9A. TNKS1 and TNKS2 function as chaperones by promoting the colocalization of these two proteins and promoting pexophagy. Although pexophagy has not been shown to be involved in muscle wasting, it seems that PARPs have an increasingly growing role in modulating autophagy.

Additionally, one of the main functions of TNKS1 is the promotion of telomere elongation through the targeting of the TRF1, which is a telomere-specific DNA-binding protein that negatively regulates telomere length maintenance. In fact, TNKS1 was discovered through the investigation of TRF1-binding proteins [462]. Telomeres are DNA-protein complexes located at the ends of chromosomes, promoting chromosomal stability and gene expression. With every cycle of cell division, telomeric length decreases. Once it reaches a minimum length, cells enter an irreversible growth arrest state, termed replicative senescence. Telomerase is an enzyme that elongates telomeres, and, in senescent cells, the activity of this enzyme is decreased [566, 567]. In various models of muscle atrophy, it is observed that satellite cells become increasingly senescent, making them unable to maintain muscle tissue renewal. For example, decreased telomere length in satellite cells was associated with the impaired muscle regenerative capacity in individuals with COPD suffering from muscle wasting (approximately 30-40% of COPD patients suffer from muscle atrophy) [568]. Additionally, in muscle dystrophy patients suffering from DMD or LGMD2C, satellite cells seem to prematurely senesce due to their repeated cellular division to repair the continuous muscle damage, as evidenced by their shortened minimal telomeric length [569, 570]. Thus, it is possible that increasing the activity of TNKS1 might ameliorate or delay muscle wasting in these patients.

4.7 Conclusion and Future Directions

This work deciphers two different mechanisms involving opposite functions of HuR in skeletal muscle. It provides a regulatory mechanism for the pro-myogenic function of HuR, and further characterizes the pro-cachectic function. These studies

provide initial findings that should be further investigated *in vivo* and potentially be used in clinical trials for muscle-related diseases. This can be done by using PARG inhibitors to ameliorate skeletal muscle formation, or using the new technology being developed to deliver miRNAs, such as miR-330, to specific tissues, such as cachectic skeletal muscle. Indeed, miRNA drugs are in clinical studies for cancer, hepatitis C, heart failure, wound healing, and other conditions [571, 572]. Delivery methods for miRNAs involve micelles, liposomes, nanoparticles, and intranasal delivery methods. The findings in Chapter 2 also widen our understanding of the potential impact of PARP inhibitors, specifically TNKS inhibitors since it proved to be highly important in muscle fiber formation. Finding regulatory mechanisms for the function of HuR would be beneficial since HuR inhibitors are toxic [184]. One of the challenges in using them is that HuR functions in many processes. Therefore, targeting HuR in a specific process through targeting a posttranslational modification required to drive its function might be a viable option for therapy, especially that PARP inhibitors are already FDA-approved, and many are in clinical trials.

In Chapter 3, although we focused on STAT3, our microarray analysis identified a variety of potential targets of HuR during muscle wasting. Similarly, various miRNAs interacting with HuR were identified by the miRNA-sequencing analysis. These data provide a basis for future analysis for the function of HuR in cytokine-induced muscle wasting. Additionally, in contrast to the function of HuR in myogenesis, no protein ligands have been identified to regulate in trans the pro-cachectic function of HuR. While trans-acting factors have been identified in the pro-myogenic function of HuR, the impact of PARylation on these factors needs to be further studied.

Appendix

i. Extended List of Publications

Journal Publications

1. **Souad Mubaid**, Jennifer F. Ma, Amr Omer, Xian J. Lian, Brenda J. Sanchez, Samantha Robinson, Anne Cammas, Virginie Dormoy-Raclet, Sergio Di Marco, Sridar Chittur, Scott A. Tenenbaum, Imed-Eddine Gallouzi. *HuR counteracts miR-330 to promote STAT3 translation during inflammation-induced muscle wasting. – Published August 2019 in PNAS* 10.1073/pnas.1905172116

2. Derek T. Hall, Takla Griss, Jennifer F. Ma, Brenda J. Sanchez, Jason Sadek, Anne Marie K. Tremblay, **Souad Mubaid**, Amr Omer, Rebecca J. Ford, Nathalie Bedard, Arnim Pause, Simon S. Wing, Sergio Di Marco, Gregory R. Steinberg, Russell G. Jones, and Imed-Eddine Gallouzi. *The AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), but not metformin, prevents inflammation-associated cachectic muscle wasting*. - *Published May 2018 in EMBO Molecular Medecine* 10(7):e8307.

3. Brenda J. Sánchez, Anne-Marie K. Tremblay, Derek T. Hall, Erzsebet Kovacs, Jennifer F. Ma, **Souad Mubaid**, Patricia L. Hallauer, Brittany L. Phillips, Katherine E. Vest, Anita H. Corbett, Dimitris L. Kontoyiannis, Kenneth E. M. Hastings, Sergio Di Marco, and Imed-Eddine Gallouzi. *Depletion of HuR in murine skeletal muscle enhances exercise endurance and prevents cancer-induced muscle atrophy. - Published Sept. 2019 in Nature Comm.* 10.1038/s41467-019-12186-6.

4. Brenda J. Sánchez, **Souad Mubaid**, Sandrine Busque, Yossef De Los Santos, Khouloud Ashour, Jason Sadek, Xian Jin Lian, Sergio Di Marco, Shahryar Khattak, Imed-Eddine Gallouzi. *The formation of HuR/YB1 complex is required for the stabilization of target mRNA to promote myogenesis.* – Accepted for publication in *Nucleic Acid Research – November 2022.*

Papers in Preparation or under Revision

5. **Souad Mubaid**, Pauline Adjibade, Derek T. Hall, Xian Jin Lian, Sandrine Brusque, Khouloud Ashour, Graeme Carlile, Jean-Philippe Gagné, Sergio Di Marco, David Y. Thomas, Guy G. Poirier, Imed-Eddine Gallouzi. *Tankyrase-1 regulates RBP-mediated mRNA turnover to promote muscle fiber formation.* 2022 – In review.

6. Anne-Marie K Tremblay, Brenda J. Sanchez, Bianca Colalillo, Jason Sadek, **Souad Mubaid**, et al. HuR promotes muscle cell fate determination by inhibiting adipogenic fate. 2022 - *Manuscript in preparation.*

7. Anne-Marie K Tremblay, Brenda J. Sanchez, Bianca Colalillo, Jason Sadek, **Souad Mubaid**, et al. Sex-related dimorphic function of HuR in skeletal muscle and brown adipocyte metabolic dynamic. 2022 - *Manuscript in preparation.*

ii. References

All schematics, including Figures 1.1 to 1.3, 1.5 to 1.8, 2.14, and 4.1, were created with **Biorender.com**, and the publication licensing rights were obtained through subscription to the Student plan.

Agreement numbers for the figures are: 1.1 (ZB24DNQUKE), 1.2 (TH24DNS1J4), 1.3 (XQ24DNQHWS), 1.5 (YL24DNR8Y6), 1.6 (CO24DNRN3S), 1.7 (WN24DNRV4L), 1.8 (WA24DNP7JE), 2.14 (XL24DNQP09), 4.1 (RU24DNR2JE)

1. Charge, S.B. and M.A. Rudnicki, *Cellular and molecular regulation of muscle regeneration.* Physiol Rev, 2004. **84**(1): p. 209-38.
2. Kaeser, M.D. and B.M. Emerson, *Remodeling plans for cellular specialization: unique styles for every room.* Curr Opin Genet Dev, 2006. **16**(5): p. 508-12.
3. Yin, H., F. Price, and M.A. Rudnicki, *Satellite cells and the muscle stem cell niche.* Physiol Rev, 2013. **93**(1): p. 23-67.
4. Feige, P., et al., *Orienting Muscle Stem Cells for Regeneration in Homeostasis, Aging, and Disease.* Cell Stem Cell, 2018. **23**(5): p. 653-664.
5. Tajbakhsh, S., *Skeletal muscle stem cells in developmental versus regenerative myogenesis.* J Intern Med, 2009. **266**(4): p. 372-89.
6. von Roretz, C., et al., *HuR and myogenesis: being in the right place at the right time.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2011. **1813**(9): p. 1663-1667.
7. Rudnicki, M.A., et al., *MyoD or Myf-5 is required for the formation of skeletal muscle.* Cell, 1993. **75**(7): p. 1351-9.
8. Qaisar, R., S. Bhaskaran, and H. Van Remmen, *Muscle fiber type diversification during exercise and regeneration.* Free Radic Biol Med, 2016. **98**: p. 56-67.
9. Asfour, H.A., M.Z. Allouh, and R.S. Said, *Myogenic regulatory factors: The orchestrators of myogenesis after 30 years of discovery.* Exp Biol Med (Maywood), 2018. **243**(2): p. 118-128.
10. Hasty, P., et al., *Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene.* Nature, 1993. **364**(6437): p. 501-6.
11. Nabeshima, Y., et al., *Myogenin gene disruption results in perinatal lethality because of severe muscle defect.* Nature, 1993. **364**(6437): p. 532-5.
12. Bentzinger, C.F., Y.X. Wang, and M.A. Rudnicki, *Building muscle: molecular regulation of myogenesis.* Cold Spring Harb Perspect Biol, 2012. **4**(2).
13. Singh, K. and F.J. Dilworth, *Differential modulation of cell cycle progression distinguishes members of the myogenic regulatory factor family of transcription factors.* Febs j, 2013. **280**(17): p. 3991-4003.
14. Davis, R.L. and H. Weintraub, *Acquisition of myogenic specificity by replacement of three amino acid residues from MyoD into E12.* Science, 1992. **256**(5059): p. 1027-30.
15. Zammit, P.S., *Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis.* Semin Cell Dev Biol, 2017. **72**: p. 19-32.
16. Ciciliot, S., et al., *Muscle type and fiber type specificity in muscle wasting.* Int J Biochem Cell Biol, 2013. **45**(10): p. 2191-9.
17. Schiaffino, S. and C. Mammucari, *Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models.* Skelet Muscle, 2011. **1**(1): p. 4.

18. Bassel-Duby, R. and E.N. Olson, *Signaling pathways in skeletal muscle remodeling.* Annu Rev Biochem, 2006. **75**: p. 19-37.
19. Zammit, P.S., et al., *Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers.* Exp Cell Res, 2002. **281**(1): p. 39-49.
20. Davis, R.L., H. Weintraub, and A.B. Lassar, *Expression of a single transfected cDNA converts fibroblasts to myoblasts.* Cell, 1987. **51**(6): p. 987-1000.
21. Weintraub, H., et al., *Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD.* Proc Natl Acad Sci U S A, 1989. **86**(14): p. 5434-8.
22. Zhang, J.M., et al., *Coupling of the cell cycle and myogenesis through the cyclin D1-dependent interaction of MyoD with cdk4.* Embo j, 1999. **18**(4): p. 926-33.
23. Deato, M.D., et al., *MyoD targets TAF3/TRF3 to activate myogenin transcription.* Mol Cell, 2008. **32**(1): p. 96-105.
24. Cao, Y., et al., *Genome-wide MyoD binding in skeletal muscle cells: a potential for broad cellular reprogramming.* Dev Cell, 2010. **18**(4): p. 662-74.
25. Blais, A., et al., *An initial blueprint for myogenic differentiation.* Genes Dev, 2005. **19**(5): p. 553-69.
26. Karimian, A., Y. Ahmadi, and B. Yousefi, *Multiple functions of p21 in cell cycle, apoptosis and transcriptional regulation after DNA damage.* DNA Repair (Amst), 2016. **42**: p. 63-71.
27. Wang, Y., et al., *Intrinsic disorder mediates the diverse regulatory functions of the Cdk inhibitor p21.* Nat Chem Biol, 2011. **7**(4): p. 214-21.
28. Bertoli, C., J.M. Skotheim, and R.A. de Bruin, *Control of cell cycle transcription during G1 and S phases.* Nat Rev Mol Cell Biol, 2013. **14**(8): p. 518-28.
29. Chinzei, N., et al., *P21 deficiency delays regeneration of skeletal muscular tissue.* PLoS One, 2015. **10**(5): p. e0125765.
30. Liu, Q.C., et al., *Comparative expression profiling identifies differential roles for Myogenin and p38α MAPK signaling in myogenesis.* J Mol Cell Biol, 2012. **4**(6): p. 386-97.
31. Cao, Y., et al., *Global and gene-specific analyses show distinct roles for Myod and Myog at a common set of promoters.* Embo j, 2006. **25**(3): p. 502-11.
32. Adhikari, A., W. Kim, and J. Davie, *Myogenin is required for assembly of the transcription machinery on muscle genes during skeletal muscle differentiation.* PLoS One, 2021. **16**(1): p. e0245618.
33. O'Donnell, K.A., et al., *c-Myc-regulated microRNAs modulate E2F1 expression.* Nature, 2005. **435**(7043): p. 839-43.
34. Sylvestre, Y., et al., *An E2F/miR-20a autoregulatory feedback loop.* J Biol Chem, 2007. **282**(4): p. 2135-43.
35. Nagel, S., et al., *Activation of miR-17-92 by NK-like homeodomain proteins suppresses apoptosis via reduction of E2F1 in T-cell acute lymphoblastic leukemia.* Leuk Lymphoma, 2009. **50**(1): p. 101-8.
36. Zhang, W., R.R. Behringer, and E.N. Olson, *Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies.* Genes Dev, 1995. **9**(11): p. 1388-99.
37. Hinterberger, T.J., et al., *Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development.* Dev Biol, 1991. **147**(1): p. 144-56.
38. Moretti, I., et al., *MRF4 negatively regulates adult skeletal muscle growth by repressing MEF2 activity.* Nat Commun, 2016. **7**: p. 12397.
39. Acharyya, S., et al., *Cancer cachexia is regulated by selective targeting of skeletal muscle gene products.* J Clin Invest, 2004. **114**(3): p. 370-8.
40. Baracos, V.E., et al., *Cancer-associated cachexia.* Nat Rev Dis Primers, 2018. **4**: p. 17105.

41. Remels, A.H., et al., *The mechanisms of cachexia underlying muscle dysfunction in COPD.* J Appl Physiol (1985), 2013. **114**(9): p. 1253-62.
42. Song, J., et al., *Skeletal muscle wasting after a severe burn is a consequence of cachexia and sarcopenia.* JPEN J Parenter Enteral Nutr, 2021. **45**(8): p. 1627-1633.
43. Von Roenn, J.H., E.L. Roth, and R. Craig, *HIV-related cachexia: potential mechanisms and treatment.* Oncology, 1992. **49 Suppl 2**: p. 50-4.
44. Argilés, J.M., et al., *The role of cytokines in cancer cachexia.* Curr Opin Support Palliat Care, 2009. **3**(4): p. 263-8.
45. Tisdale, M.J., *Mechanisms of cancer cachexia.* Physiol Rev, 2009. **89**(2): p. 381-410.
46. Webster, J.M., et al., *Inflammation and Skeletal Muscle Wasting During Cachexia.* Front Physiol, 2020. **11**: p. 597675.
47. Fearon, K., et al., *Definition and classification of cancer cachexia: an international consensus.* Lancet Oncol, 2011. **12**(5): p. 489-95.
48. Ryan, A.M., et al., *Effects of weight loss and sarcopenia on response to chemotherapy, quality of life, and survival.* Nutrition, 2019. **67-68**: p. 110539.
49. Deboer, M.D., *Animal models of anorexia and cachexia.* Expert Opin Drug Discov, 2009. **4**(11): p. 1145-1155.
50. Tisdale, M.J., *Cancer cachexia.* Curr Opin Gastroenterol, 2010. **26**(2): p. 146-51.
51. Tsoli, M. and G. Robertson, *Cancer cachexia: malignant inflammation, tumorkines, and metabolic mayhem.* Trends Endocrinol Metab, 2013. **24**(4): p. 174-83.
52. Moses, A.G., et al., *Pro-inflammatory cytokine release by peripheral blood mononuclear cells from patients with advanced pancreatic cancer: relationship to acute phase response and survival.* Oncol Rep, 2009. **21**(4): p. 1091-5.
53. Sandri, M., *Protein breakdown in cancer cachexia.* Semin Cell Dev Biol, 2016. **54**: p. 11-9.
54. Morita, M., et al., *mTOR coordinates protein synthesis, mitochondrial activity and proliferation.* Cell Cycle, 2015. **14**(4): p. 473-80.
55. Bhat, M., et al., *Targeting the translation machinery in cancer.* Nat Rev Drug Discov, 2015. **14**(4): p. 261-78.
56. Yoon, M.S., *mTOR as a Key Regulator in Maintaining Skeletal Muscle Mass.* Front Physiol, 2017. **8**: p. 788.
57. Schmitt, T.L., et al., *Activity of the Akt-dependent anabolic and catabolic pathways in muscle and liver samples in cancer-related cachexia.* J Mol Med (Berl), 2007. **85**(6): p. 647-54.
58. Asp, M.L., et al., *Evidence for the contribution of insulin resistance to the development of cachexia in tumor-bearing mice.* Int J Cancer, 2010. **126**(3): p. 756-63.
59. Di Marco, S., et al., *The translation inhibitor pateamine A prevents cachexia-induced muscle wasting in mice.* Nat Commun, 2012. **3**: p. 896.
60. Eley, H.L., et al., *Increased expression of phosphorylated forms of RNA-dependent protein kinase and eukaryotic initiation factor 2alpha may signal skeletal muscle atrophy in weight-losing cancer patients.* Br J Cancer, 2008. **98**(2): p. 443-9.
61. Lecker, S.H., A.L. Goldberg, and W.E. Mitch, *Protein degradation by the ubiquitin-proteasome pathway in normal and disease states.* J Am Soc Nephrol, 2006. **17**(7): p. 1807-19.
62. Cohen, S., et al., *During muscle atrophy, thick, but not thin, filament components are degraded by MuRF1-dependent ubiquitylation.* J Cell Biol, 2009. **185**(6): p. 1083-95.
63. Kedar, V., et al., *Muscle-specific RING finger 1 is a bona fide ubiquitin ligase that degrades cardiac troponin I.* Proc Natl Acad Sci U S A, 2004. **101**(52): p. 18135-40.
64. Polge, C., et al., *Muscle actin is polyubiquitinylated in vitro and in vivo and targeted for breakdown by the E3 ligase MuRF1.* Faseb j, 2011. **25**(11): p. 3790-802.

65. Lagirand-Cantaloube, J., et al., *The initiation factor eIF3-f is a major target for atrogin1/MAFbx function in skeletal muscle atrophy.* Embo j, 2008. **27**(8): p. 1266-76.
66. Bodine, S.C. and L.M. Baehr, *Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and MAFbx/atrogin-1.* Am J Physiol Endocrinol Metab, 2014. **307**(6): p. E469-84.
67. Sandri, M., *Protein breakdown in muscle wasting: role of autophagy-lysosome and ubiquitin-proteasome.* Int J Biochem Cell Biol, 2013. **45**(10): p. 2121-9.
68. Sanchez, A.M., et al., *AMPK promotes skeletal muscle autophagy through activation of forkhead FoxO3a and interaction with Ulk1.* J Cell Biochem, 2012. **113**(2): p. 695-710.
69. Kim, J., et al., *AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1.* Nat Cell Biol, 2011. **13**(2): p. 132-41.
70. Penna, F., et al., *Autophagic degradation contributes to muscle wasting in cancer cachexia.* Am J Pathol, 2013. **182**(4): p. 1367-78.
71. Yang, Z.J., et al., *The role of autophagy in cancer: therapeutic implications.* Mol Cancer Ther, 2011. **10**(9): p. 1533-41.
72. Pettersen, K., et al., *Cancer cachexia associates with a systemic autophagy-inducing activity mimicked by cancer cell-derived IL-6 trans-signaling.* Sci Rep, 2017. **7**(1): p. 2046.
73. Aversa, Z., et al., *Autophagy is induced in the skeletal muscle of cachectic cancer patients.* Sci Rep, 2016. **6**: p. 30340.
74. Grumati, P., et al., *Physical exercise stimulates autophagy in normal skeletal muscles but is detrimental for collagen VI-deficient muscles.* Autophagy, 2011. **7**(12): p. 1415-23.
75. Pigna, E., et al., *Aerobic Exercise and Pharmacological Treatments Counteract Cachexia by Modulating Autophagy in Colon Cancer.* Sci Rep, 2016. **6**: p. 26991.
76. Puppa, M.J., et al., *The effect of exercise on IL-6-induced cachexia in the Apc (Min/+) mouse.* J Cachexia Sarcopenia Muscle, 2012. **3**(2): p. 117-37.
77. Chang, N.C., F.P. Chevalier, and M.A. Rudnicki, *Satellite Cells in Muscular Dystrophy - Lost in Polarity.* Trends Mol Med, 2016. **22**(6): p. 479-496.
78. He, W.A., et al., *NF-κB-mediated Pax7 dysregulation in the muscle microenvironment promotes cancer cachexia.* J Clin Invest, 2013. **123**(11): p. 4821-35.
79. Marchildon, F., et al., *Expression of CCAAT/Enhancer Binding Protein Beta in Muscle Satellite Cells Inhibits Myogenesis in Cancer Cachexia.* PLoS One, 2015. **10**(12): p. e0145583.
80. Marchildon, F., et al., *CCAAT/enhancer binding protein beta is expressed in satellite cells and controls myogenesis.* Stem Cells, 2012. **30**(12): p. 2619-30.
81. Inaba, S., et al., *Muscle regeneration is disrupted by cancer cachexia without loss of muscle stem cell potential.* PLoS One, 2018. **13**(10): p. e0205467.
82. Riccardi, D., et al., *Plasma Lipid Profile and Systemic Inflammation in Patients With Cancer Cachexia.* Front Nutr, 2020. **7**: p. 4.
83. Op den Kamp, C.M., et al., *Nuclear transcription factor κ B activation and protein turnover adaptations in skeletal muscle of patients with progressive stages of lung cancer cachexia.* Am J Clin Nutr, 2013. **98**(3): p. 738-48.
84. Chiappalupi, S., et al., *Targeting RAGE prevents muscle wasting and prolongs survival in cancer cachexia.* J Cachexia Sarcopenia Muscle, 2020. **11**(4): p. 929-946.
85. Iwase, S., et al., *Steep elevation of blood interleukin-6 (IL-6) associated only with late stages of cachexia in cancer patients.* Eur Cytokine Netw, 2004. **15**(4): p. 312-6.
86. Songür, N., et al., *Serum interleukin-6 levels correlate with malnutrition and survival in patients with advanced non-small cell lung cancer.* Tumori, 2004. **90**(2): p. 196-200.
87. Baltgalvis, K.A., et al., *Muscle wasting and interleukin-6-induced atrogin-I expression in the cachectic Apc (Min/+) mouse.* Pflugers Arch, 2009. **457**(5): p. 989-1001.

88. Fujita, J., et al., *Anti-interleukin-6 receptor antibody prevents muscle atrophy in colon-26 adenocarcinoma-bearing mice with modulation of lysosomal and ATP-ubiquitin-dependent proteolytic pathways.* Int J Cancer, 1996. **68**(5): p. 637-43.
89. Tsujinaka, T., et al., *Interleukin 6 receptor antibody inhibits muscle atrophy and modulates proteolytic systems in interleukin 6 transgenic mice.* J Clin Invest, 1996. **97**(1): p. 244-9.
90. Baltgalvis, K.A., et al., *Interleukin-6 and cachexia in ApcMin/+ mice.* Am J Physiol Regul Integr Comp Physiol, 2008. **294**(2): p. R393-401.
91. Bonetto, A., et al., *JAK/STAT3 pathway inhibition blocks skeletal muscle wasting downstream of IL-6 and in experimental cancer cachexia.* Am J Physiol Endocrinol Metab, 2012. **303**(3): p. E410-21.
92. Beutler, B.A., I.W. Milsark, and A. Cerami, *Cachectin/tumor necrosis factor: production, distribution, and metabolic fate in vivo.* J Immunol, 1985. **135**(6): p. 3972-7.
93. Tracey, K.J., et al., *Cachectin/tumor necrosis factor induces cachexia, anemia, and inflammation.* J Exp Med, 1988. **167**(3): p. 1211-27.
94. Llovera, M., F.J. López-Soriano, and J.M. Argilés, *Effects of tumor necrosis factor-alpha on muscle-protein turnover in female Wistar rats.* J Natl Cancer Inst, 1993. **85**(16): p. 1334-9.
95. Oliff, A., et al., *Tumors secreting human TNF/cachectin induce cachexia in mice.* Cell, 1987. **50**(4): p. 555-63.
96. Llovera, M., et al., *Protein turnover in skeletal muscle of tumour-bearing transgenic mice overexpressing the soluble TNF receptor-1.* Cancer Lett, 1998. **130**(1-2): p. 19-27.
97. Ramamoorthy, S., M. Donohue, and M. Buck, *Decreased Jun-D and myogenin expression in muscle wasting of human cachexia.* Am J Physiol Endocrinol Metab, 2009. **297**(2): p. E392-401.
98. Ghosh, S. and M. Karin, *Missing pieces in the NF-kappaB puzzle.* Cell, 2002. **109 Suppl**: p. S81-96.
99. Li, Y.P. and M.B. Reid, *NF-kappaB mediates the protein loss induced by TNF-alpha in differentiated skeletal muscle myotubes.* Am J Physiol Regul Integr Comp Physiol, 2000. **279**(4): p. R1165-70.
100. Tidball, J.G. and S.A. Villalta, *Regulatory interactions between muscle and the immune system during muscle regeneration.* Am J Physiol Regul Integr Comp Physiol, 2010. **298**(5): p. R1173-87.
101. Goldberg, R.M., et al., *Pentoxifylline for treatment of cancer anorexia and cachexia? A randomized, double-blind, placebo-controlled trial.* J Clin Oncol, 1995. **13**(11): p. 2856-9.
102. Jatoi, A., et al., *A placebo-controlled, double-blind trial of infliximab for cancer-associated weight loss in elderly and/or poor performance non-small cell lung cancer patients (N01C9).* Lung Cancer, 2010. **68**(2): p. 234-9.
103. Wiedenmann, B., et al., *A multicenter, phase II study of infliximab plus gemcitabine in pancreatic cancer cachexia.* J Support Oncol, 2008. **6**(1): p. 18-25.
104. Di Marco, S., et al., *NF-kappa B-mediated MyoD decay during muscle wasting requires nitric oxide synthase mRNA stabilization, HuR protein, and nitric oxide release.* Mol Cell Biol, 2005. **25**(15): p. 6533-45.
105. Hall, D.T., et al., *The AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), but not metformin, prevents inflammation-associated cachectic muscle wasting.* EMBO Mol Med, 2018. **10**(7).
106. Ma, J.F., et al., *STAT3 promotes IFNgamma/TNFalpha-induced muscle wasting in an NF-kappaB-dependent and IL-6-independent manner.* EMBO Mol Med, 2017. **9**(5): p. 622-637.
107. Mubaid, S., et al., *HuR counteracts miR-330 to promote STAT3 translation during inflammation-induced muscle wasting.* Proc Natl Acad Sci U S A, 2019. **116**(35): p. 17261-17270.
108. Guttridge, D.C., et al., *NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia.* Science, 2000. **289**(5488): p. 2363-6.

109. Matthys, P., et al., *Severe cachexia in mice inoculated with interferon-gamma-producing tumor cells.* Int J Cancer, 1991. **49**(1): p. 77-82.
110. Langstein, H.N., et al., *The roles of gamma-interferon and tumor necrosis factor alpha in an experimental rat model of cancer cachexia.* Cancer Res, 1991. **51**(9): p. 2302-6.
111. Strijdom, H., N. Chamane, and A. Lochner, *Nitric oxide in the cardiovascular system: a simple molecule with complex actions.* Cardiovasc J Afr, 2009. **20**(5): p. 303-10.
112. Radi, R., *Peroxynitrite, a stealthy biological oxidant.* J Biol Chem, 2013. **288**(37): p. 26464-72.
113. Szabó, C., H. Ischiropoulos, and R. Radi, *Peroxynitrite: biochemistry, pathophysiology and development of therapeutics.* Nat Rev Drug Discov, 2007. **6**(8): p. 662-80.
114. Wiseman, D.A. and D.C. Thurmond, *The good and bad effects of cysteine S-nitrosylation and tyrosine nitration upon insulin exocytosis: a balancing act.* Curr Diabetes Rev, 2012. **8**(4): p. 303-15.
115. Barreiro, E., et al., *Both oxidative and nitrosative stress are associated with muscle wasting in tumour-bearing rats.* FEBS Lett, 2005. **579**(7): p. 1646-52.
116. Buck, M. and M. Chojkier, *Muscle wasting and dedifferentiation induced by oxidative stress in a murine model of cachexia is prevented by inhibitors of nitric oxide synthesis and antioxidants.* Embo j, 1996. **15**(8): p. 1753-65.
117. Sadek, J., et al., *Pharmacological or genetic inhibition of iNOS prevents cachexia-mediated muscle wasting and its associated metabolism defects.* EMBO Mol Med, 2021. **13**(7): p. e13591.
118. Kamei, Y., et al., *Skeletal muscle FOXO1 (FKHR) transgenic mice have less skeletal muscle mass, down-regulated Type I (slow twitch/red muscle) fiber genes, and impaired glycemic control.* J Biol Chem, 2004. **279**(39): p. 41114-23.
119. Reed, S.A., et al., *Inhibition of IkappaB kinase alpha (IKKα) or IKKbeta (IKKβ) plus forkhead box O (Foxo) abolishes skeletal muscle atrophy.* Biochem Biophys Res Commun, 2011. **405**(3): p. 491-6.
120. Sandri, M., et al., *Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy.* Cell, 2004. **117**(3): p. 399-412.
121. Zhuang, P., et al., *Reversal of muscle atrophy by Zhimu and Huangbai herb pair via activation of IGF-1/Akt and autophagy signal in cancer cachexia.* Support Care Cancer, 2016. **24**(3): p. 1189-98.
122. Cai, D., et al., *IKKbeta/NF-kappaB activation causes severe muscle wasting in mice.* Cell, 2004. **119**(2): p. 285-98.
123. Miao, C., et al., *Pyrrolidine Dithiocarbamate (PDTC) Attenuates Cancer Cachexia by Affecting Muscle Atrophy and Fat Lipolysis.* Front Pharmacol, 2017. **8**: p. 915.
124. Hall, D.T., et al., *Inducible nitric oxide synthase (iNOS) in muscle wasting syndrome, sarcopenia, and cachexia.* Aging (Albany NY), 2011. **3**(8): p. 702-15.
125. Shinozaki, S., et al., *Inflammatory stimuli induce inhibitory S-nitrosylation of the deacetylase SIRT1 to increase acetylation and activation of p53 and p65.* Sci Signal, 2014. **7**(351): p. ra106.
126. Der-Torossian, H., et al., *Metabolic derangements in the gastrocnemius and the effect of Compound A therapy in a murine model of cancer cachexia.* J Cachexia Sarcopenia Muscle, 2013. **4**(2): p. 145-55.
127. Reich, N.C., *STATs get their move on.* Jakstat, 2013. **2**(4): p. e27080.
128. Takeda, K., et al., *Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality.* Proc Natl Acad Sci U S A, 1997. **94**(8): p. 3801-4.
129. Delgoffe, G.M. and D.A. Vignali, *STAT heterodimers in immunity: A mixed message or a unique signal?* Jakstat, 2013. **2**(1): p. e23060.
130. Bonetto, A., et al., *STAT3 activation in skeletal muscle links muscle wasting and the acute phase response in cancer cachexia.* PLoS One, 2011. **6**(7): p. e22538.

131. Silva, K.A., et al., *Inhibition of Stat3 activation suppresses caspase-3 and the ubiquitin-proteasome system, leading to preservation of muscle mass in cancer cachexia.* J Biol Chem, 2015. **290**(17): p. 11177-87.
132. Zhang, L., et al., *Stat3 activation links a C/EBPδ to myostatin pathway to stimulate loss of muscle mass.* Cell Metab, 2013. **18**(3): p. 368-79.
133. Kim, S.J. and S. Yoon, *Activated Rac1 regulates the degradation of IκBα and the nuclear translocation of STAT3-NFκB complexes in starved cancer cells.* Exp Mol Med, 2016. **48**(5): p. e231.
134. Yamada, E., et al., *Mouse skeletal muscle fiber-type-specific macroautophagy and muscle wasting are regulated by a Fyn/STAT3/Vps34 signaling pathway.* Cell Rep, 2012. **1**(5): p. 557-69.
135. Qin, B., et al., *IL-6 Inhibits Starvation-induced Autophagy via the STAT3/Bcl-2 Signaling Pathway.* Sci Rep, 2015. **5**: p. 15701.
136. You, L., et al., *The role of STAT3 in autophagy.* Autophagy, 2015. **11**(5): p. 729-39.
137. Fan, M., et al., *The critical role of STAT3 in biogenesis of tumor-derived exosomes with potency of inducing cancer cachexia in vitro and in vivo.* Oncogene, 2022. **41**(7): p. 1050-1062.
138. Chitti, S.V., P. Fonseka, and S. Mathivanan, *Emerging role of extracellular vesicles in mediating cancer cachexia.* Biochem Soc Trans, 2018. **46**(5): p. 1129-1136.
139. Zhang, G., et al., *Tumor induces muscle wasting in mice through releasing extracellular Hsp70 and Hsp90.* Nat Commun, 2017. **8**(1): p. 589.
140. Rudnicki, M.A., et al., *The molecular regulation of muscle stem cell function.* Cold Spring Harb Symp Quant Biol, 2008. **73**: p. 323-31.
141. Figliola, R., et al., *Regulation of p57(KIP2) during muscle differentiation: role of Egr1, Sp1 and DNA hypomethylation.* J Mol Biol, 2008. **380**(2): p. 265-77.
142. Berkes, C.A. and S.J. Tapscott, *MyoD and the transcriptional control of myogenesis.* Semin Cell Dev Biol, 2005. **16**(4-5): p. 585-95.
143. Skapek, S.X., et al., *Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase.* Science, 1995. **267**(5200): p. 1022-4.
144. Freire, P.P., et al., *The Pathway to Cancer Cachexia: MicroRNA-Regulated Networks in Muscle Wasting Based on Integrative Meta-Analysis.* Int J Mol Sci, 2019. **20**(8).
145. Shen, L., et al., *Cachexia-related long noncoding RNA, CAAlnc1, suppresses adipogenesis by blocking the binding of HuR to adipogenic transcription factor mRNAs.* Int J Cancer, 2019. **145**(7): p. 1809-1821.
146. Van der Giessen, K., et al., *RNAi-mediated HuR depletion leads to the inhibition of muscle cell differentiation.* Journal of Biological Chemistry, 2003. **278**(47): p. 47119-47128.
147. Beauchamp, P., et al., *The cleavage of HuR interferes with its transportin-2-mediated nuclear import and promotes muscle fiber formation.* 2010. **17**(10): p. 1588-1599.
148. Briata, P., et al., *p38-dependent phosphorylation of the mRNA decay-promoting factor KSRP controls the stability of select myogenic transcripts.* 2005. **20**(6): p. 891-903.
149. Janice Sánchez, B., et al., *Depletion of HuR in murine skeletal muscle enhances exercise endurance and prevents cancer-induced muscle atrophy.* Nat Commun, 2019. **10**(1): p. 4171.
150. Phillips, B.L., et al., *Post-transcriptional regulation of Pabpn1 by the RNA binding protein HuR.* Nucleic Acids Research, 2018. **46**(15): p. 7643-7661.
151. Cammas, A., et al., *Destabilization of nucleophosmin mRNA by the HuR/KSRP complex is required for muscle fibre formation.* 2014. **5**.
152. Dormoy-Raclet, V., et al., *HuR and miR-1192 regulate myogenesis by modulating the translation of HMGB1 mRNA.* Nat Commun, 2013. **4**: p. 2388.
153. Beutler, B. and A. Cerami, *Cachectin/tumor necrosis factor: an endogenous mediator of shock and inflammation.* Immunol Res, 1986. **5**(4): p. 281-93.

154. Beutler, B. and A. Cerami, *Cachectin and tumour necrosis factor as two sides of the same biological coin.* Nature, 1986. **320**(6063): p. 584-8.
155. Guttridge, D.C., et al., *NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1.* Mol Cell Biol, 1999. **19**(8): p. 5785-99.
156. Zerbini, L.F., et al., *Constitutive activation of nuclear factor kappaB p50/p65 and Fra-1 and JunD is essential for deregulated interleukin 6 expression in prostate cancer.* Cancer Res, 2003. **63**(9): p. 2206-15.
157. Jacobson, A. and S.W. Peltz, *Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells.* Annu Rev Biochem, 1996. **65**: p. 693-739.
158. Wang, W., et al., *HuR regulates cyclin A and cyclin B1 mRNA stability during cell proliferation.* Embo j, 2000. **19**(10): p. 2340-50.
159. Lin, S., et al., *Down-regulation of cyclin D1 expression by prostaglandin A(2) is mediated by enhanced cyclin D1 mRNA turnover.* Mol Cell Biol, 2000. **20**(21): p. 7903-13.
160. Rodriguez-Pascual, F., et al., *Complex contribution of the 3'-untranslated region to the expressional regulation of the human inducible nitric-oxide synthase gene. Involvement of the RNA-binding protein HuR.* J Biol Chem, 2000. **275**(34): p. 26040-9.
161. Nabors, L.B., et al., *HuR, a RNA stability factor, is expressed in malignant brain tumors and binds to adenine- and uridine-rich elements within the 3' untranslated regions of cytokine and angiogenic factor mRNAs.* Cancer Res, 2001. **61**(5): p. 2154-61.
162. Brennan, C. and J. Steitz, *HuR and mRNA stability.* Cellular and Molecular Life Sciences CMLS, 2001. **58**(2): p. 266-277.
163. Gallouzi, I.E. and J.A. Steitz, *Delineation of mRNA export pathways by the use of cell-permeable peptides.* Science, 2001. **294**(5548): p. 1895-901.
164. Kullmann, M., et al., *ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5'UTR.* Genes Dev, 2002. **16**(23): p. 3087-99.
165. Wang, W., et al., *Loss of HuR is linked to reduced expression of proliferative genes during replicative senescence.* Mol Cell Biol, 2001. **21**(17): p. 5889-98.
166. Meijlink, F., et al., *Removal of a 67-base-pair sequence in the noncoding region of protooncogene fos converts it to a transforming gene.* Proc Natl Acad Sci U S A, 1985. **82**(15): p. 4987-91.
167. Veyrune, J.L., J. Hesketh, and J.M. Blanchard, *3' untranslated regions of c-myc and c-fos mRNAs: multifunctional elements regulating mRNA translation, degradation and subcellular localization.* Prog Mol Subcell Biol, 1997. **18**: p. 35-63.
168. Neininger, A., et al., *MK2 targets AU-rich elements and regulates biosynthesis of tumor necrosis factor and interleukin-6 independently at different post-transcriptional levels.* J Biol Chem, 2002. **277**(5): p. 3065-8.
169. Abdelmohsen, K., et al., *miR-519 reduces cell proliferation by lowering RNA-binding protein HuR levels.* Proc Natl Acad Sci U S A, 2008. **105**(51): p. 20297-302.
170. Barker, A., et al., *Sequence requirements for RNA binding by HuR and AUF1.* J Biochem, 2012. **151**(4): p. 423-37.
171. Brennan, C.M., I.E. Gallouzi, and J.A. Steitz, *Protein ligands to HuR modulate its interaction with target mRNAs in vivo.* J Cell Biol, 2000. **151**(1): p. 1-14.
172. Cammas, A., et al., *Destabilization of nucleophosmin mRNA by the HuR/KSRP complex is required for muscle fibre formation.* Nature communications, 2014. **5**.
173. Chang, N., et al., *HuR uses AUF1 as a cofactor to promote p16INK4 mRNA decay.* Mol Cell Biol, 2010. **30**(15): p. 3875-86.
174. Cho, S.J., J. Zhang, and X. Chen, *RNPC1 modulates the RNA-binding activity of, and cooperates with, HuR to regulate p21 mRNA stability.* Nucleic Acids Res, 2010. **38**(7): p. 2256-67.

175. Dan, C., et al., *Modulation of TNF-α mRNA stability by human antigen R and miR181s in sepsis-induced immunoparalysis.* EMBO Mol Med, 2015. **7**(2): p. 140-57.
176. Kawai, T., et al., *Translational control of cytochrome c by RNA-binding proteins TIA-1 and HuR.* Mol Cell Biol, 2006. **26**(8): p. 3295-307.
177. Kim, H.H., et al., *HuR recruits let-7/RISC to repress c-Myc expression.* Genes Dev, 2009. **23**(15): p. 1743-8.
178. Abdelmohsen, K., et al., *Posttranscriptional orchestration of an anti-apoptotic program by HuR.* Cell Cycle, 2007. **6**(11): p. 1288-92.
179. Lal, A., et al., *Antiapoptotic function of RNA-binding protein HuR effected through prothymosin alpha.* Embo j, 2005. **24**(10): p. 1852-62.
180. Durie, D., et al., *RNA-binding protein HuR mediates cytoprotection through stimulation of XIAP translation.* Oncogene, 2011. **30**(12): p. 1460-9.
181. Mazroui, R., et al., *Caspase-mediated cleavage of HuR in the cytoplasm contributes to pp32/PHAP-I regulation of apoptosis.* The Journal of cell biology, 2008. **180**(1): p. 113-127.
182. Von Roretz, C., et al., *Apoptotic-induced cleavage shifts HuR from being a promoter of survival to an activator of caspase-mediated apoptosis.* Cell Death & Differentiation, 2013. **20**(1): p. 154-168.
183. Figueroa, A., et al., *Role of HuR in skeletal myogenesis through coordinate regulation of muscle differentiation genes.* Mol Cell Biol, 2003. **23**(14): p. 4991-5004.
184. Wu, M., et al., *The RNA Binding Protein HuR: A Promising Drug Target for Anticancer Therapy.* Curr Cancer Drug Targets, 2019. **19**(5): p. 382-399.
185. Lang, M., et al., *HuR Small-Molecule Inhibitor Elicits Differential Effects in Adenomatosis Polyposis and Colorectal Carcinogenesis.* Cancer Res, 2017. **77**(9): p. 2424-2438.
186. Ma, W.-J., et al., *Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein.* Journal of Biological Chemistry, 1996. **271**(14): p. 8144-8151.
187. Peng, S.S.Y., et al., *RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein.* The EMBO journal, 1998. **17**(12): p. 3461-3470.
188. Campos, A.R. and K. White, *Mutant alleles at the locus elav in Drosophila melanogaster lead to nervous system defects. A developmental-genetic analysis.* Journal of neurogenetics, 1985. **2**(3): p. 197-218.
189. Robinow, S., et al., *The elav gene product of Drosophila, required in neurons, has three RNP consensus motifs.* Science, 1988. **242**(4885): p. 1570-1572.
190. Khabar, K.S., *Hallmarks of cancer and AU-rich elements.* Wiley Interdiscip Rev RNA, 2017. **8**(1).
191. von Roretz, C., A.M. Macri, and I.-E. Gallouzi, *Transportin 2 regulates apoptosis through the RNA-binding protein HuR.* Journal of Biological Chemistry, 2011. **286**(29): p. 25983-25991.
192. Abdelmohsen, K., et al., *Posttranscriptional gene regulation by RNA-binding proteins during oxidative stress: implications for cellular senescence.* Biol Chem, 2008. **389**(3): p. 243-55.
193. Fan, X.C. and J.A. Steitz, *Overexpression of HuR, a nuclear–cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs.* The EMBO journal, 1998. **17**(12): p. 3448-3460.
194. Chung, S., et al., *Purification and properties of HuD, a neuronal RNA-binding protein.* J Biol Chem, 1996. **271**(19): p. 11518-24.
195. Fialcowitz-White, E.J., et al., *Specific protein domains mediate cooperative assembly of HuR oligomers on AU-rich mRNA-destabilizing sequences.* J Biol Chem, 2007. **282**(29): p. 20948-59.
196. Yeap, B.B., et al., *Novel binding of HuR and poly(C)-binding protein to a conserved UC-rich motif within the 3'-untranslated region of the androgen receptor messenger RNA.* J Biol Chem, 2002. **277**(30): p. 27183-92.

197. Wang, H., et al., *The structure of the ARE-binding domains of Hu antigen R (HuR) undergoes conformational changes during RNA binding.* Acta Crystallogr D Biol Crystallogr, 2013. **69**(Pt 3): p. 373-80.
198. Doller, A., et al., *Tandem phosphorylation of serines 221 and 318 by protein kinase Cdelta coordinates mRNA binding and nucleocytoplasmic shuttling of HuR.* Mol Cell Biol, 2010. **30**(6): p. 1397-410.
199. Millard, S.S., et al., *A U-rich element in the 5' untranslated region is necessary for the translation of p27 mRNA.* Mol Cell Biol, 2000. **20**(16): p. 5947-59.
200. Meng, Z., et al., *The ELAV RNA-stability factor HuR binds the 5'-untranslated region of the human IGF-IR transcript and differentially represses cap-dependent and IRES-mediated translation.* Nucleic Acids Res, 2005. **33**(9): p. 2962-79.
201. Galbán, S., et al., *RNA-binding proteins HuR and PTB promote the translation of hypoxia-inducible factor 1alpha.* Mol Cell Biol, 2008. **28**(1): p. 93-107.
202. Chen, C.Y. and A.B. Shyu, *AU-rich elements: characterization and importance in mRNA degradation.* Trends Biochem Sci, 1995. **20**(11): p. 465-70.
203. von Roretz, C. and I.E. Gallouzi, *Decoding ARE-mediated decay: is microRNA part of the equation?* J Cell Biol, 2008. **181**(2): p. 189-94.
204. Brennan, C., J.J.C. Steitz, and M.L.S. CMLS, *HuR and mRNA stability.* 2001. **58**(2): p. 266-277.
205. Chang, S.H., et al., *Antagonistic function of the RNA-binding protein HuR and miR-200b in post-transcriptional regulation of vascular endothelial growth factor-A expression and angiogenesis.* J Biol Chem, 2013. **288**(7): p. 4908-21.
206. Izquierdo, J.M., *Hu antigen R (HuR) functions as an alternative pre-mRNA splicing regulator of Fas apoptosis-promoting receptor on exon definition.* J Biol Chem, 2008. **283**(27): p. 19077-84.
207. Zhu, H., et al., *Hu proteins regulate polyadenylation by blocking sites containing U-rich sequences.* J Biol Chem, 2007. **282**(4): p. 2203-10.
208. Hinman, M.N. and H. Lou, *Diverse molecular functions of Hu proteins.* Cell Mol Life Sci, 2008. **65**(20): p. 3168-81.
209. Dickson, A.M., et al., *Dephosphorylation of HuR protein during alphavirus infection is associated with HuR relocalization to the cytoplasm.* Journal of Biological Chemistry, 2012. **287**(43): p. 36229-36238.
210. Jiang, X., et al., *Distinctive roles of PHAP proteins and prothymosin-alpha in a death regulatory pathway.* Science, 2003. **299**(5604): p. 223-6.
211. Mitchell, P. and D. Tollervey, *mRNA stability in eukaryotes.* Curr Opin Genet Dev, 2000. **10**(2): p. 193-8.
212. Kelemen, O., et al., *Function of alternative splicing.* Gene, 2013. **514**(1): p. 1-30.
213. Lee, Y. and D.C. Rio, *Mechanisms and Regulation of Alternative Pre-mRNA Splicing.* Annu Rev Biochem, 2015. **84**: p. 291-323.
214. Chang, S.H., et al., *ELAVL1 regulates alternative splicing of eIF4E transporter to promote postnatal angiogenesis.* Proc Natl Acad Sci U S A, 2014. **111**(51): p. 18309-14.
215. Poganik, J.R., et al., *Post-transcriptional regulation of Nrf2-mRNA by the mRNA-binding proteins HuR and AUF1.* Faseb j, 2019. **33**(12): p. 14636-14652.
216. Cheng, J., et al., *Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule.* Science, 1994. **263**(5154): p. 1759-62.
217. Berget, S.M., *Exon recognition in vertebrate splicing.* J Biol Chem, 1995. **270**(6): p. 2411-4.
218. Ferraiuolo, M.A., et al., *A role for the eIF4E-binding protein 4E-T in P-body formation and mRNA decay.* J Cell Biol, 2005. **170**(6): p. 913-24.
219. Kamenska, A., et al., *Human 4E-T represses translation of bound mRNAs and enhances microRNA-mediated silencing.* Nucleic Acids Res, 2014. **42**(5): p. 3298-313.

220. Ma, Q., *Role of nrf2 in oxidative stress and toxicity.* Annu Rev Pharmacol Toxicol, 2013. **53**: p. 401-26.
221. Shahbabian, K. and P. Chartrand, *Control of cytoplasmic mRNA localization.* Cell Mol Life Sci, 2012. **69**(4): p. 535-52.
222. Gallouzi, I.E., et al., *HuR binding to cytoplasmic mRNA is perturbed by heat shock.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3073-8.
223. Gallouzi, I.E., C.M. Brennan, and J.A. Steitz, *Protein ligands mediate the CRM1-dependent export of HuR in response to heat shock.* Rna, 2003. **9**(11): p. 1410.
224. Güttinger, S., et al., *Transportin2 functions as importin and mediates nuclear import of HuR.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 2918-23.
225. Rebane, A., A. Aab, and J.A. Steitz, *Transportins 1 and 2 are redundant nuclear import factors for hnRNP A1 and HuR.* Rna, 2004. **10**(4): p. 590-9.
226. Wang, W., et al., *AMP-activated protein kinase-regulated phosphorylation and acetylation of importin alpha1: involvement in the nuclear import of RNA-binding protein HuR.* J Biol Chem, 2004. **279**(46): p. 48376-88.
227. Zou, T., et al., *Polyamines modulate the subcellular localization of RNA-binding protein HuR through AMP-activated protein kinase-regulated phosphorylation and acetylation of importin alpha1.* Biochem J, 2008. **409**(2): p. 389-98.
228. Abdelmohsen, K., et al., *Phosphorylation of HuR by CHK2 regulates SIRT1 expression.* Mol Cell, 2007. **25**(4): p. 543-57.
229. Doller, A., et al., *Posttranslational modification of the AU-rich element binding protein HuR by protein kinase Cdelta elicits angiotensin II-induced stabilization and nuclear export of cyclooxygenase 2 mRNA.* Mol Cell Biol, 2008. **28**(8): p. 2608-25.
230. Kim, H.H., et al., *Nuclear HuR accumulation through phosphorylation by Cdk1.* Genes Dev, 2008. **22**(13): p. 1804-15.
231. Kim, H.H. and M. Gorospe, *Phosphorylated HuR shuttles in cycles.* Cell Cycle, 2008. **7**(20): p. 3124-6.
232. Tiedje, C., et al., *The p38/MK2-driven exchange between tristetraprolin and HuR regulates AU-rich element-dependent translation.* PLoS Genet, 2012. **8**(9): p. e1002977.
233. Fan, X.C. and J.A. Steitz, *Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs.* Embo j, 1998. **17**(12): p. 3448-60.
234. Doller, A., et al., *RNA-dependent association with myosin IIA promotes F-actin-guided trafficking of the ELAV-like protein HuR to polysomes.* Nucleic Acids Res, 2013. **41**(19): p. 9152-67.
235. Cheng, Y.C., et al., *MPT0B098, a novel microtubule inhibitor that destabilizes the hypoxia-inducible factor-1α mRNA through decreasing nuclear-cytoplasmic translocation of RNA-binding protein HuR.* Mol Cancer Ther, 2013. **12**(7): p. 1202-12.
236. Eberhardt, W., et al., *Cytoskeleton-Dependent Transport as a Potential Target for Interfering with Post-transcriptional HuR mRNA Regulons.* Front Pharmacol, 2016. **7**: p. 251.
237. Antic, D. and J.D. Keene, *Messenger ribonucleoprotein complexes containing human ELAV proteins: interactions with cytoskeleton and translational apparatus.* J Cell Sci, 1998. **111 (Pt 2)**: p. 183-97.
238. Wu, X. and G. Brewer, *The regulation of mRNA stability in mammalian cells: 2.0.* Gene, 2012. **500**(1): p. 10-21.
239. Körner, C.G. and E. Wahle, *Poly(A) tail shortening by a mammalian poly(A)-specific 3'-exoribonuclease.* J Biol Chem, 1997. **272**(16): p. 10448-56.
240. Körner, C.G., et al., *The deadenylating nuclease (DAN) is involved in poly(A) tail removal during the meiotic maturation of Xenopus oocytes.* Embo j, 1998. **17**(18): p. 5427-37.

241. Bakheet, T., B.R. Williams, and K.S. Khabar, *ARED 2.0: an update of AU-rich element mRNA database.* Nucleic Acids Res, 2003. **31**(1): p. 421-3.
242. Barreau, C., L. Paillard, and H.B. Osborne, *AU-rich elements and associated factors: are there unifying principles?* Nucleic Acids Res, 2005. **33**(22): p. 7138-50.
243. Chen, C.Y., et al., *AU binding proteins recruit the exosome to degrade ARE-containing mRNAs.* Cell, 2001. **107**(4): p. 451-64.
244. Linker, K., et al., *Involvement of KSRP in the post-transcriptional regulation of human iNOS expression-complex interplay of KSRP with TTP and HuR.* Nucleic Acids Res, 2005. **33**(15): p. 4813-27.
245. Pabis, M., et al., *HuR biological function involves RRM3-mediated dimerization and RNA binding by all three RRMs.* Nucleic Acids Res, 2018.
246. Dean, J.L., et al., *The 3' untranslated region of tumor necrosis factor alpha mRNA is a target of the mRNA-stabilizing factor HuR.* Mol Cell Biol, 2001. **21**(3): p. 721-30.
247. Katsanou, V., et al., *HuR as a negative posttranscriptional modulator in inflammation.* Mol Cell, 2005. **19**(6): p. 777-89.
248. Kozak, M., *Regulation of translation via mRNA structure in prokaryotes and eukaryotes.* Gene, 2005. **361**: p. 13-37.
249. Furuichi, Y. and A.J. Shatkin, *Viral and cellular mRNA capping: past and prospects.* Adv Virus Res, 2000. **55**: p. 135-84.
250. Gingras, A.C., B. Raught, and N. Sonenberg, *eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation.* Annu Rev Biochem, 1999. **68**: p. 913-63.
251. von der Haar, T., et al., *The mRNA cap-binding protein eIF4E in post-transcriptional gene expression.* Nat Struct Mol Biol, 2004. **11**(6): p. 503-11.
252. Kozak, M., *Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes.* Cell, 1986. **44**(2): p. 283-92.
253. Kozak, M., *Translation of insulin-related polypeptides from messenger RNAs with tandemly reiterated copies of the ribosome binding site.* Cell, 1983. **34**(3): p. 971-8.
254. Kozak, M., *The scanning model for translation: an update.* J Cell Biol, 1989. **108**(2): p. 229-41.
255. Wang, L. and S.R. Wessler, *Role of mRNA secondary structure in translational repression of the maize transcriptional activator Lc(1,2).* Plant Physiol, 2001. **125**(3): p. 1380-7.
256. Goossen, B. and M.W. Hentze, *Position is the critical determinant for function of iron-responsive elements as translational regulators.* Mol Cell Biol, 1992. **12**(5): p. 1959-66.
257. Mazan-Mamczarz, K., et al., *RNA-binding protein HuR enhances p53 translation in response to ultraviolet light irradiation.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8354-9.
258. Winkler, C., et al., *Attenuation of the ELAV1-like protein HuR sensitizes adenocarcinoma cells to the intrinsic apoptotic pathway by increasing the translation of caspase-2L.* Cell death & disease, 2014. **5**(7): p. e1321.
259. Srikantan, S., K. Tominaga, and M. Gorospe, *Functional interplay between RNA-binding protein HuR and microRNAs.* Curr Protein Pept Sci, 2012. **13**(4): p. 372-9.
260. Ke, X.S., et al., *MicroRNAs: key participants in gene regulatory networks.* Curr Opin Chem Biol, 2003. **7**(4): p. 516-23.
261. Li, X., et al., *AGO2 and its partners: a silencing complex, a chromatin modulator, and new features.* Crit Rev Biochem Mol Biol, 2020. **55**(1): p. 33-53.
262. Horak, M., J. Novak, and J. Bienertova-Vasku, *Muscle-specific microRNAs in skeletal muscle development.* Dev Biol, 2016. **410**(1): p. 1-13.
263. Fabian, M.R., N. Sonenberg, and W. Filipowicz, *Regulation of mRNA translation and stability by microRNAs.* Annu Rev Biochem, 2010. **79**: p. 351-79.

264. Vasudevan, S., Y. Tong, and J.A. Steitz, *Switching from repression to activation: microRNAs can up-regulate translation.* Science, 2007. **318**(5858): p. 1931-4.
265. Kedde, M. and R. Agami, *Interplay between microRNAs and RNA-binding proteins determines developmental processes.* Cell Cycle, 2008. **7**(7): p. 899-903.
266. Höck, J., et al., *Proteomic and functional analysis of Argonaute-containing mRNA-protein complexes in human cells.* EMBO Rep, 2007. **8**(11): p. 1052-60.
267. Bhattacharyya, S.N., et al., *Relief of microRNA-mediated translational repression in human cells subjected to stress.* Cell, 2006. **125**(6): p. 1111-24.
268. Young, L.E., et al., *The mRNA stability factor HuR inhibits microRNA-16 targeting of COX-2.* Mol Cancer Res, 2012. **10**(1): p. 167-80.
269. Srikantan, S., et al., *Translational control of TOP2A influences doxorubicin efficacy.* Mol Cell Biol, 2011. **31**(18): p. 3790-801.
270. Tominaga, K., et al., *Competitive regulation of nucleolin expression by HuR and miR-494.* Mol Cell Biol, 2011. **31**(20): p. 4219-31.
271. Epis, M.R., et al., *The RNA-binding protein HuR opposes the repression of ERBB-2 gene expression by microRNA miR-331-3p in prostate cancer cells.* J Biol Chem, 2011. **286**(48): p. 41442-41454.
272. Chen, J., et al., *Interaction of RNA-binding protein HuR and miR-466i regulates GM-CSF expression.* Sci Rep, 2017. **7**(1): p. 17233.
273. Mukherjee, N., et al., *Integrative regulatory mapping indicates that the RNA-binding protein HuR couples pre-mRNA processing and mRNA stability.* Mol Cell, 2011. **43**(3): p. 327-39.
274. Kundu, P., et al., *HuR protein attenuates miRNA-mediated repression by promoting miRISC dissociation from the target RNA.* Nucleic Acids Res, 2012. **40**(11): p. 5088-100.
275. Glorian, V., et al., *HuR-dependent loading of miRNA RISC to the mRNA encoding the Ras-related small GTPase RhoB controls its translation during UV-induced apoptosis.* Cell Death Differ, 2011. **18**(11): p. 1692-701.
276. Wang, W., et al., *HuR regulates p21 mRNA stabilization by UV light.* Mol Cell Biol, 2000. **20**(3): p. 760-9.
277. Cherry, J., et al., *HuR, an RNA-binding protein, involved in the control of cellular differentiation.* In Vivo, 2006. **20**(1): p. 17-23.
278. Jain, R.G., et al., *Ectopic expression of Hel-N1, an RNA-binding protein, increases glucose transporter (GLUT1) expression in 3T3-L1 adipocytes.* Mol Cell Biol, 1997. **17**(2): p. 954-62.
279. Grammatikakis, I., K. Abdelmohsen, and M. Gorospe, *Posttranslational control of HuR function.* Wiley Interdisciplinary Reviews: RNA, 2017. **8**(1).
280. Talwar, S., et al., *Caspase-mediated cleavage of RNA-binding protein HuR regulates c-Myc protein expression after hypoxic stress.* Journal of Biological Chemistry, 2011. **286**(37): p. 32333-32343.
281. Srikantan, S. and M. Gorospe, *HuR function in disease.* Front Biosci (Landmark Ed), 2012. **17**: p. 189-205.
282. Katsanou, V., et al., *The RNA-binding protein Elavl1/HuR is essential for placental branching morphogenesis and embryonic development.* Mol Cell Biol, 2009. **29**(10): p. 2762-76.
283. Liu, L., et al., *HuR Enhances Early Restitution of the Intestinal Epithelium by Increasing Cdc42 Translation.* Mol Cell Biol, 2017. **37**(7).
284. Chi, M.N., et al., *The RNA-binding protein ELAVL1/HuR is essential for mouse spermatogenesis, acting both at meiotic and postmeiotic stages.* Mol Biol Cell, 2011. **22**(16): p. 2875-85.
285. Sun, K., et al., *Correction: Neuron-Specific HuR-Deficient Mice Spontaneously Develop Motor Neuron Disease.* J Immunol, 2018.

286. Zhu, Z., et al., *Cytoplasmic HuR expression correlates with P-gp, HER-2 positivity, and poor outcome in breast cancer.* Tumour Biol, 2013. **34**(4): p. 2299-308.
287. Prislei, S., et al., *MiR-200c and HuR in ovarian cancer.* BMC Cancer, 2013. **13**: p. 72.
288. Vázquez-Chantada, M., et al., *HuR/methyl-HuR and AUF1 regulate the MAT expressed during liver proliferation, differentiation, and carcinogenesis.* Gastroenterology, 2010. **138**(5): p. 1943-53.
289. Kang, M.J., et al., *NF-kappaB activates transcription of the RNA-binding factor HuR, via PI3K-AKT signaling, to promote gastric tumorigenesis.* Gastroenterology, 2008. **135**(6): p. 2030-42, 2042.e1-3.
290. Zhang, C., et al., *Cytoplasmic expression of the ELAV-like protein HuR as a potential prognostic marker in esophageal squamous cell carcinoma.* Tumour Biol, 2014. **35**(1): p. 73-80.
291. Peng, W., et al., *Elevated HuR in Pancreas Promotes a Pancreatitis-Like Inflammatory Microenvironment That Facilitates Tumor Development.* Mol Cell Biol, 2018. **38**(3).
292. Ziegeler, G., et al., *Embryonic lethal abnormal vision-like HuR-dependent mRNA stability regulates post-transcriptional expression of cyclin-dependent kinase inhibitor p27Kip1.* J Biol Chem, 2010. **285**(20): p. 15408-19.
293. Janakiraman, H., et al., *Repression of caspase-3 and RNA-binding protein HuR cleavage by cyclooxygenase-2 promotes drug resistance in oral squamous cell carcinoma.* 2016.
294. Talwar, S., et al., *Inhibition of caspases protects mice from radiation-induced oral mucositis and abolishes the cleavage of RNA-binding protein HuR.* 2014. **289**(6): p. 3487-3500.
295. Ma, W.-J., et al., *Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein.* 1996. **271**(14): p. 8144-8151.
296. Robinow, S., et al., *The elav gene product of Drosophila, required in neurons, has three RNP consensus motifs.* 1988. **242**(4885): p. 1570-1572.
297. Dickson, A.M., et al., *Dephosphorylation of HuR protein during alphavirus infection is associated with HuR relocalization to the cytoplasm.* 2012. **287**(43): p. 36229-36238.
298. Mazroui, R., et al., *Caspase-mediated cleavage of HuR in the cytoplasm contributes to pp32/PHAP-I regulation of apoptosis.* 2008. **180**(1): p. 113-127.
299. Chen, J., et al., *Posttranscriptional gene regulation of IL-17 by the RNA-binding protein HuR is required for initiation of experimental autoimmune encephalomyelitis.* J Immunol, 2013. **191**(11): p. 5441-50.
300. Chen, J., et al., *The RNA-binding protein HuR contributes to neuroinflammation by promoting C-C chemokine receptor 6 (CCR6) expression on Th17 cells.* J Biol Chem, 2017. **292**(35): p. 14532-14543.
301. Sung, S.C., et al., *7-Ketocholesterol upregulates interleukin-6 via mechanisms that are distinct from those of tumor necrosis factor-alpha, in vascular smooth muscle cells.* J Vasc Res, 2009. **46**(1): p. 36-44.
302. Zhou, H., et al., *HIV protease inhibitors increase TNF-alpha and IL-6 expression in macrophages: involvement of the RNA-binding protein HuR.* Atherosclerosis, 2007. **195**(1): p. e134-43.
303. Wang, J.G., et al., *LFA-1-dependent HuR nuclear export and cytokine mRNA stabilization in T cell activation.* J Immunol, 2006. **176**(4): p. 2105-13.
304. Costantino, C.L., et al., *The role of HuR in gemcitabine efficacy in pancreatic cancer: HuR Up-regulates the expression of the gemcitabine metabolizing enzyme deoxycytidine kinase.* 2009. **69**(11): p. 4567-4572.
305. Subramaniam, D., et al., *Gastrin-mediated interleukin-8 and cyclooxygenase-2 gene expression: differential transcriptional and posttranscriptional mechanisms.* Gastroenterology, 2008. **134**(4): p. 1070-82.

306. Winzen, R., et al., *Distinct domains of AU-rich elements exert different functions in mRNA destabilization and stabilization by p38 mitogen-activated protein kinase or HuR.* Mol Cell Biol, 2004. **24**(11): p. 4835-47.
307. Dixon, D.A., et al., *Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells.* J Clin Invest, 2001. **108**(11): p. 1657-65.
308. Sengupta, S., et al., *The RNA-binding protein HuR regulates the expression of cyclooxygenase-2.* J Biol Chem, 2003. **278**(27): p. 25227-33.
309. Cok, S.J., S.J. Acton, and A.R. Morrison, *The proximal region of the 3'-untranslated region of cyclooxygenase-2 is recognized by a multimeric protein complex containing HuR, TIA-1, TIAR, and the heterogeneous nuclear ribonucleoprotein U.* J Biol Chem, 2003. **278**(38): p. 36157-62.
310. Matsui, K., et al., *Natural antisense transcript stabilizes inducible nitric oxide synthase messenger RNA in rat hepatocytes.* Hepatology, 2008. **47**(2): p. 686-97.
311. Krishnamurthy, P., et al., *IL-10 inhibits inflammation and attenuates left ventricular remodeling after myocardial infarction via activation of STAT3 and suppression of HuR.* Circ Res, 2009. **104**(2): p. e9-18.
312. Rajasingh, J., et al., *IL-10-induced TNF-alpha mRNA destabilization is mediated via IL-10 suppression of p38 MAP kinase activation and inhibition of HuR expression.* Faseb j, 2006. **20**(12): p. 2112-4.
313. Cuneo, A.A., D. Herrick, and M.V. Autieri, *Il-19 reduces VSMC activation by regulation of mRNA regulatory factor HuR and reduction of mRNA stability.* J Mol Cell Cardiol, 2010. **49**(4): p. 647-54.
314. Sugihara, M., et al., *Effects of infliximab therapy on gene expression levels of tumor necrosis factor alpha, tristetraprolin, T cell intracellular antigen 1, and Hu antigen R in patients with rheumatoid arthritis.* Arthritis Rheum, 2007. **56**(7): p. 2160-9.
315. Suzuki, E., et al., *Expression of TNF-alpha, tristetraprolin, T-cell intracellular antigen-1 and Hu antigen R genes in synovium of patients with rheumatoid arthritis.* Int J Mol Med, 2006. **18**(2): p. 273-8.
316. Nieminen, R., et al., *Aurothiomalate inhibits COX-2 expression in chondrocytes and in human cartilage possibly through its effects on COX-2 mRNA stability.* Eur J Pharmacol, 2008. **587**(1-3): p. 309-16.
317. Di Mari, J.F., et al., *HETEs enhance IL-1-mediated COX-2 expression via augmentation of message stability in human colonic myofibroblasts.* Am J Physiol Gastrointest Liver Physiol, 2007. **293**(4): p. G719-28.
318. Esnault, S. and J.S. Malter, *Hyaluronic acid or TNF-alpha plus fibronectin triggers granulocyte macrophage-colony-stimulating factor mRNA stabilization in eosinophils yet engages differential intracellular pathways and mRNA binding proteins.* J Immunol, 2003. **171**(12): p. 6780-7.
319. van der Giessen, K. and I.E. Gallouzi, *Involvement of transportin 2-mediated HuR import in muscle cell differentiation.* Mol Biol Cell, 2007. **18**(7): p. 2619-29.
320. Legnini, I., et al., *A feedforward regulatory loop between HuR and the long noncoding RNA linc-MD1 controls early phases of myogenesis.* Mol Cell, 2014. **53**(3): p. 506-14.
321. Apponi, L.H., A.H. Corbett, and G.K. Pavlath, *RNA-binding proteins and gene regulation in myogenesis.* Trends in pharmacological sciences, 2011. **32**(11): p. 652-658.
322. Lai, R.Y., et al., *Effect of chronic contractile activity on mRNA stability in skeletal muscle.* Am J Physiol Cell Physiol, 2010. **299**(1): p. C155-63.
323. Li, H., et al., *Lipopolysaccharide-induced methylation of HuR, an mRNA-stabilizing protein, by CARM1. Coactivator-associated arginine methyltransferase.* J Biol Chem, 2002. **277**(47): p. 44623-30.

324. Beauchamp, P., et al., *The cleavage of HuR interferes with its transportin-2-mediated nuclear import and promotes muscle fiber formation.* Cell Death & Differentiation, 2010. **17**(10): p. 1588-1599.
325. Van der Giessen, K., et al., *RNAi-mediated HuR depletion leads to the inhibition of muscle cell differentiation.* 2003. **278**(47): p. 47119-47128.
326. Apponi, L.H., A.H. Corbett, and G.K. Pavlath, *RNA-binding proteins and gene regulation in myogenesis.* Trends Pharmacol Sci, 2011. **32**(11): p. 652-8.
327. Lv, W., et al., *lncMGPF is a novel positive regulator of muscle growth and regeneration.* J Cachexia Sarcopenia Muscle, 2020. **11**(6): p. 1723-1746.
328. Gherzi, R., et al., *Akt2-mediated phosphorylation of Pitx2 controls Ccnd1 mRNA decay during muscle cell differentiation.* Cell Death Differ, 2010. **17**(6): p. 975-83.
329. Martínez-Fernandez, S., et al., *Pitx2c overexpression promotes cell proliferation and arrests differentiation in myoblasts.* Dev Dyn, 2006. **235**(11): p. 2930-9.
330. Briata, P., et al., *The Wnt/beta-catenin-->Pitx2 pathway controls the turnover of Pitx2 and other unstable mRNAs.* Mol Cell, 2003. **12**(5): p. 1201-11.
331. Deschênes-Furry, J., et al., *The RNA-binding protein HuR binds to acetylcholinesterase transcripts and regulates their expression in differentiating skeletal muscle cells.* J Biol Chem, 2005. **280**(27): p. 25361-8.
332. Engel, A.G., K. Ohno, and S.M. Sine, *Congenital myasthenic syndromes: recent advances.* Arch Neurol, 1999. **56**(2): p. 163-7.
333. Ohno, K., et al., *Human endplate acetylcholinesterase deficiency caused by mutations in the collagen-like tail subunit (ColQ) of the asymmetric enzyme.* Proc Natl Acad Sci U S A, 1998. **95**(16): p. 9654-9.
334. Prives, J.M. and B.M. Paterson, *Differentiation of cell membranes in cultures of embryonic chick breast muscle.* Proc Natl Acad Sci U S A, 1974. **71**(8): p. 3208-11.
335. Fuentes, M.E. and P. Taylor, *Control of acetylcholinesterase gene expression during myogenesis.* Neuron, 1993. **10**(4): p. 679-87.
336. Vallette, F.M., M. Fauquet, and M.A. Teillet, *Difference in the expression of asymmetric acetylcholinesterase molecular forms during myogenesis in early avian dermomyotomes and limb buds in ovo and in vitro.* Dev Biol, 1987. **120**(1): p. 77-84.
337. Moro, C., S. Bajpeyi, and S.R. Smith, *Determinants of intramyocellular triglyceride turnover: implications for insulin sensitivity.* Am J Physiol Endocrinol Metab, 2008. **294**(2): p. E203-13.
338. Mynatt, R.L., et al., *The RNA binding protein HuR influences skeletal muscle metabolic flexibility in rodents and humans.* Metabolism, 2019. **97**: p. 40-49.
339. Stone, A.C., et al., *Female Mice Are Protected from Metabolic Decline Associated with Lack of Skeletal Muscle HuR.* Biology (Basel), 2021. **10**(6).
340. Pilon, G., P. Dallaire, and A. Marette, *Inhibition of inducible nitric-oxide synthase by activators of AMP-activated protein kinase: a new mechanism of action of insulin-sensitizing drugs.* J Biol Chem, 2004. **279**(20): p. 20767-74.
341. Ravel-Chapuis, A., et al., *A novel CARM1-HuR axis involved in muscle differentiation and plasticity misregulated in spinal muscular atrophy.* Hum Mol Genet, 2022. **31**(9): p. 1453-1470.
342. Fan, X., et al., *HnRNP A1 and A/B interaction with PABPN1 in oculopharyngeal muscular dystrophy.* Can J Neurol Sci, 2003. **30**(3): p. 244-51.
343. Gantt, K., et al., *An early event in adipogenesis, the nuclear selection of the CCAAT enhancer-binding protein {beta} (C/EBP{beta}) mRNA by HuR and its translocation to the cytosol.* J Biol Chem, 2005. **280**(26): p. 24768-74.
344. Abdelmohsen, K., et al., *Ubiquitin-mediated proteolysis of HuR by heat shock.* Embo j, 2009. **28**(9): p. 1271-82.

345. Ke, Y., et al., *PARP1 promotes gene expression at the post-transcriptional level by modulating the RNA-binding protein HuR.* 2017. **8**: p. ncomms14632.
346. Masuda, K., et al., *Global dissociation of HuR-mRNA complexes promotes cell survival after ionizing radiation.* Embo j, 2011. **30**(6): p. 1040-53.
347. Pang, L., et al., *Loss of CARM1 is linked to reduced HuR function in replicative senescence.* BMC Mol Biol, 2013. **14**: p. 15.
348. Vigouroux, C., et al., *Methyl(R217)HuR and MCM6 are inversely correlated and are prognostic markers in non small cell lung carcinoma.* Lung Cancer, 2015. **89**(2): p. 189-96.
349. Ke, Y., et al., *PARP1 promotes gene expression at the post-transcriptional level by modulating the RNA-binding protein HuR.* Nature communications, 2017. **8**: p. ncomms14632.
350. Ke, Y., et al., *Poly(ADP-ribosyl)ation enhances HuR oligomerization and contributes to pro-inflammatory gene mRNA stabilization.* Cell Mol Life Sci, 2021. **78**(4): p. 1817-1835.
351. Chambon, P., J.D. Weill, and P. Mandel, *Nicotinamide mononucleotide activation of new DNA-dependent polyadenylic acid synthesizing nuclear enzyme.* Biochem Biophys Res Commun, 1963. **11**: p. 39-43.
352. Daniels, C.M., S.E. Ong, and A.K. Leung, *Phosphoproteomic approach to characterize protein mono- and poly(ADP-ribosyl)ation sites from cells.* J Proteome Res, 2014. **13**(8): p. 3510-22.
353. Daniels, C.M., S.E. Ong, and A.K. Leung, *The Promise of Proteomics for the Study of ADP-Ribosylation.* Mol Cell, 2015. **58**(6): p. 911-24.
354. Alemasova, E.E. and O.I. Lavrik, *Poly(ADP-ribosyl)ation by PARP1: reaction mechanism and regulatory proteins.* Nucleic Acids Res, 2019. **47**(8): p. 3811-3827.
355. DaRosa, P.A., et al., *Allosteric activation of the RNF146 ubiquitin ligase by a poly(ADP-ribosyl)ation signal.* Nature, 2015. **517**(7533): p. 223-6.
356. Das, R., et al., *Allosteric regulation of E2:E3 interactions promote a processive ubiquitination machine.* Embo j, 2013. **32**(18): p. 2504-16.
357. Simbulan-Rosenthal, C.M., et al., *Involvement of PARP and poly(ADP-ribosyl)ation in the early stages of apoptosis and DNA replication.* Mol Cell Biochem, 1999. **193**(1-2): p. 137-48.
358. Li, N., et al., *Poly-ADP ribosylation of PTEN by tankyrases promotes PTEN degradation and tumor growth.* Genes Dev, 2015. **29**(2): p. 157-70.
359. Alemasova, E.E., et al., *Poly(ADP-ribosyl)ation as a new posttranslational modification of YB-1.* Biochimie, 2015. **119**: p. 36-44.
360. Asher, G., et al., *Poly (ADP-ribose) polymerase 1 participates in the phase entrainment of circadian clocks to feeding.* 2010. **142**(6): p. 943-953.
361. Meder, V.S., et al., *PARP-1 and PARP-2 interact with nucleophosmin/B23 and accumulate in transcriptionally active nucleoli.* J Cell Sci, 2005. **118**(Pt 1): p. 211-22.
362. Grimaldi, G., et al., *PARPs and PAR as novel pharmacological targets for the treatment of stress granule-associated disorders.* Biochem Pharmacol, 2019. **167**: p. 64-75.
363. McGurk, L., et al., *Poly(ADP-Ribose) Prevents Pathological Phase Separation of TDP-43 by Promoting Liquid Demixing and Stress Granule Localization.* Mol Cell, 2018. **71**(5): p. 703-717.e9.
364. Bock, F.J., T.T. Todorova, and P.J.M.c. Chang, *RNA regulation by poly (ADP-ribose) polymerases.* 2015. **58**(6): p. 959-969.
365. Ji, Y. and A.V. Tulin, *Poly(ADP-ribosyl)ation of heterogeneous nuclear ribonucleoproteins modulates splicing.* Nucleic Acids Res, 2009. **37**(11): p. 3501-13.
366. Di Giammartino, D.C., Y. Shi, and J.L.J.M.c. Manley, *PARP1 represses PAP and inhibits polyadenylation during heat shock.* 2013. **49**(1): p. 7-17.
367. Isabelle, M., et al., *Quantitative proteomics and dynamic imaging reveal that G3BP-mediated stress granule assembly is poly(ADP-ribose)-dependent following exposure to MNNG-induced DNA alkylation.* J Cell Sci, 2012. **125**(Pt 19): p. 4555-66.

368. Gibson, B.A. and W.L. Kraus, *New insights into the molecular and cellular functions of poly (ADP-ribose) and PARPs.* Nature reviews Molecular cell biology, 2012. **13**(7): p. 411-424.
369. Soldani, C. and A.I. Scovassi, *Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update.* Apoptosis, 2002. **7**(4): p. 321-8.
370. Jubin, T., et al., *Poly ADP-ribose polymerase-1: Beyond transcription and towards differentiation.* Semin Cell Dev Biol, 2017. **63**: p. 167-179.
371. Erener, S., et al., *Inflammasome-activated caspase 7 cleaves PARP1 to enhance the expression of a subset of NF-kappaB target genes.* Mol Cell, 2012. **46**(2): p. 200-11.
372. Hu, B., et al., *Regulation of myofibroblast differentiation by poly(ADP-ribose) polymerase 1.* Am J Pathol, 2013. **182**(1): p. 71-83.
373. Marton, J., et al., *Poly(ADP-ribose) polymerase-2 is a lipid-modulated modulator of muscular lipid homeostasis.* Biochim Biophys Acta Mol Cell Biol Lipids, 2018. **1863**(11): p. 1399-1412.
374. Jankó, L., et al., *Silencing of PARP2 Blocks Autophagic Degradation.* Cells, 2020. **9**(2).
375. Smith, S., et al., *Tankyrase, a poly (ADP-ribose) polymerase at human telomeres.* Science, 1998. **282**(5393): p. 1484-1487.
376. Smith, S. and T. de Lange, *Cell cycle dependent localization of the telomeric PARP, tankyrase, to nuclear pore complexes and centrosomes.* J Cell Sci, 1999. **112 (Pt 21)**: p. 3649-56.
377. Mizutani, A. and H. Seimiya, *Tankyrase promotes primary precursor miRNA processing to precursor miRNA.* Biochem Biophys Res Commun, 2020. **522**(4): p. 945-951.
378. Lehtiö, L., N.W. Chi, and S. Krauss, *Tankyrases as drug targets.* Febs j, 2013. **280**(15): p. 3576-93.
379. Chiang, Y.J., et al., *Tankyrase 1 and tankyrase 2 are essential but redundant for mouse embryonic development.* PLoS One, 2008. **3**(7): p. e2639.
380. Rouleau, M., et al., *PARP inhibition: PARP1 and beyond.* Nature Reviews Cancer, 2010. **10**(4): p. 293-301.
381. Muramatsu, Y., et al., *Cross-species difference in telomeric function of tankyrase 1.* Cancer Sci, 2007. **98**(6): p. 850-7.
382. Yeh, T.Y., J.I. Sbodio, and N.W. Chi, *Mitotic phosphorylation of tankyrase, a PARP that promotes spindle assembly, by GSK3.* Biochem Biophys Res Commun, 2006. **350**(3): p. 574-9.
383. Chang, W., J.N. Dynek, and S. Smith, *NuMA is a major acceptor of poly(ADP-ribosyl)ation by tankyrase 1 in mitosis.* Biochem J, 2005. **391**(Pt 2): p. 177-84.
384. Huang, S.M., et al., *Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling.* Nature, 2009. **461**(7264): p. 614-20.
385. Chi, N.W. and H.F. Lodish, *Tankyrase is a golgi-associated mitogen-activated protein kinase substrate that interacts with IRAP in GLUT4 vesicles.* J Biol Chem, 2000. **275**(49): p. 38437-44.
386. Su, Z., et al., *Tankyrase modulates insulin sensitivity in skeletal muscle cells by regulating the stability of GLUT4 vesicle proteins.* J Biol Chem, 2018. **293**(22): p. 8578-8587.
387. Ye, P., et al., *Tankyrases maintain homeostasis of intestinal epithelium by preventing cell death.* PLoS Genet, 2018. **14**(9): p. e1007697.
388. Zou, L.H., et al., *TNKS1BP1 functions in DNA double-strand break repair though facilitating DNA-PKcs autophosphorylation dependent on PARP-1.* Oncotarget, 2015. **6**(9): p. 7011-22.
389. Mariotti, L., et al., *Tankyrase Requires SAM Domain-Dependent Polymerization to Support Wnt-beta-Catenin Signaling.* Mol Cell, 2016. **63**(3): p. 498-513.
390. Koirala, S., et al., *Tissue-Specific Regulation of the Wnt/β-Catenin Pathway by PAGE4 Inhibition of Tankyrase.* Cell Rep, 2020. **32**(3): p. 107922.
391. Xu, D., et al., *USP25 regulates Wnt signaling by controlling the stability of tankyrases.* Genes Dev, 2017. **31**(10): p. 1024-1035.
392. Langelier, M.F., et al., *PARP family enzymes: regulation and catalysis of the poly(ADP-ribose) posttranslational modification.* Curr Opin Struct Biol, 2018. **53**: p. 187-198.

393. DaRosa, P.A., R.E. Klevit, and W. Xu, *Structural basis for tankyrase-RNF146 interaction reveals noncanonical tankyrase-binding motifs.* Protein Sci, 2018. **27**(6): p. 1057-1067.
394. Eisemann, T., M.F. Langelier, and J.M. Pascal, *Structural and functional analysis of parameters governing tankyrase-1 interaction with telomeric repeat-binding factor 1 and GDP-mannose 4,6-dehydratase.* J Biol Chem, 2019. **294**(40): p. 14574-14590.
395. Yeh, T.Y., et al., *Insulin-stimulated exocytosis of GLUT4 is enhanced by IRAP and its partner tankyrase.* Biochem J, 2007. **402**(2): p. 279-90.
396. Li, Z., et al., *Herpes simplex virus requires poly(ADP-ribose) polymerase activity for efficient replication and induces extracellular signal-related kinase-dependent phosphorylation and ICP0-dependent nuclear localization of tankyrase 1.* J Virol, 2012. **86**(1): p. 492-503.
397. Kim, M.K., C. Dudognon, and S. Smith, *Tankyrase 1 regulates centrosome function by controlling CPAP stability.* EMBO Rep, 2012. **13**(8): p. 724-32.
398. Zamudio-Martinez, E., et al., *Tankyrases as modulators of pro-tumoral functions: molecular insights and therapeutic opportunities.* 2021. **40**(1): p. 1-15.
399. Li, X., et al., *Proteomic Analysis of the Human Tankyrase Protein Interaction Network Reveals Its Role in Pexophagy.* Cell Rep, 2017. **20**(3): p. 737-749.
400. Matteini, F., et al., *Poly(ADP-ribose) Polymerase 1 (PARP1) restrains MyoD-dependent gene expression during muscle differentiation.* Sci Rep, 2020. **10**(1): p. 15086.
401. Liu, D., et al., *Podocan affects C2C12 myogenic differentiation by enhancing Wnt/beta-catenin signaling.* J Cell Physiol, 2019. **234**(7): p. 11130-11139.
402. Abraham, S.T., *A role for the Wnt3a/beta-catenin signaling pathway in the myogenic program of C2C12 cells.* In Vitro Cell Dev Biol Anim, 2016. **52**(9): p. 935-941.
403. Thorvaldsen, T.E., et al., *Differential Roles of AXIN1 and AXIN2 in Tankyrase Inhibitor-Induced Formation of Degradasomes and β-Catenin Degradation.* PLoS One, 2017. **12**(1): p. e0170508.
404. Li, J., et al., *Fibronectin type III domain containing four promotes differentiation of C2C12 through the Wnt/β-catenin signaling pathway.* Faseb j, 2020.
405. Cisternas, P., et al., *Wnt signaling in skeletal muscle dynamics: myogenesis, neuromuscular synapse and fibrosis.* Mol Neurobiol, 2014. **49**(1): p. 574-89.
406. von Maltzahn, J., et al., *Wnt signaling in myogenesis.* Trends Cell Biol, 2012. **22**(11): p. 602-9.
407. Otto, A., et al., *Canonical Wnt signalling induces satellite-cell proliferation during adult skeletal muscle regeneration.* J Cell Sci, 2008. **121**(Pt 17): p. 2939-50.
408. van Amerongen, R. and A. Berns, *Knockout mouse models to study Wnt signal transduction.* Trends Genet, 2006. **22**(12): p. 678-89.
409. Tajbakhsh, S., et al., *Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5.* Development, 1998. **125**(21): p. 4155-4162.
410. Brack, A.S., et al., *A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis.* Cell stem cell, 2008. **2**(1): p. 50-59.
411. Aon, M.A., N. Bhatt, and S.C. Cortassa, *Mitochondrial and cellular mechanisms for managing lipid excess.* Front Physiol, 2014. **5**: p. 282.
412. Kelley, D.E. and J.A. Simoneau, *Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus.* J Clin Invest, 1994. **94**(6): p. 2349-56.
413. Puigserver, P., et al., *Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction.* Nature, 2003. **423**(6939): p. 550-5.
414. Yoon, J.C., et al., *Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1.* Nature, 2001. **413**(6852): p. 131-8.
415. Finck, B.N. and D.P. Kelly, *PGC-1 coactivators: inducible regulators of energy metabolism in health and disease.* J Clin Invest, 2006. **116**(3): p. 615-22.

416. Mensink, M., et al., *Improved skeletal muscle oxidative enzyme activity and restoration of PGC-1 alpha and PPAR beta/delta gene expression upon rosiglitazone treatment in obese patients with type 2 diabetes mellitus.* Int J Obes (Lond), 2007. **31**(8): p. 1302-10.
417. Semple, R.K., et al., *Expression of the thermogenic nuclear hormone receptor coactivator PGC-1alpha is reduced in the adipose tissue of morbidly obese subjects.* Int J Obes Relat Metab Disord, 2004. **28**(1): p. 176-9.
418. Wang, H., et al., *Tankyrase inhibition ameliorates lipid disorder via suppression of PGC-1alpha PARylation in db/db mice.* Int J Obes (Lond), 2020.
419. Bai, P., et al., *PARP-2 regulates SIRT1 expression and whole-body energy expenditure.* Cell Metab, 2011. **13**(4): p. 450-460.
420. Mohamed, J.S., et al., *MicroRNA-149 inhibits PARP-2 and promotes mitochondrial biogenesis via SIRT-1/PGC-1α network in skeletal muscle.* Diabetes, 2014. **63**(5): p. 1546-59.
421. Bai, P., et al., *PARP-1 inhibition increases mitochondrial metabolism through SIRT1 activation.* Cell Metab, 2011. **13**(4): p. 461-468.
422. Chacon-Cabrera, A., et al., *MicroRNA expression and protein acetylation pattern in respiratory and limb muscles of Parp-1(-/-) and Parp-2(-/-) mice with lung cancer cachexia.* Biochim Biophys Acta, 2015. **1850**(12): p. 2530-43.
423. Mohamed, J.S., et al., *Dysregulation of SIRT-1 in aging mice increases skeletal muscle fatigue by a PARP-1-dependent mechanism.* Aging (Albany NY), 2014. **6**(10): p. 820-34.
424. Ahmad, A., et al., *The clinically used PARP inhibitor olaparib improves organ function, suppresses inflammatory responses and accelerates wound healing in a murine model of third-degree burn injury.* Br J Pharmacol, 2018. **175**(2): p. 232-245.
425. Sadler, J.B.A., et al., *The deubiquitinating enzyme USP25 binds tankyrase and regulates trafficking of the facilitative glucose transporter GLUT4 in adipocytes.* Sci Rep, 2019. **9**(1): p. 4710.
426. Olah, G., et al., *Increased poly(ADP-ribosyl)ation in skeletal muscle tissue of pediatric patients with severe burn injury: prevention by propranolol treatment.* Shock, 2011. **36**(1): p. 18-23.
427. Cantó, C., K.J. Menzies, and J. Auwerx, *NAD(+) Metabolism and the Control of Energy Homeostasis: A Balancing Act between Mitochondria and the Nucleus.* Cell Metab, 2015. **22**(1): p. 31-53.
428. Chacon-Cabrera, A., et al., *Role of PARP activity in lung cancer-induced cachexia: Effects on muscle oxidative stress, proteolysis, anabolic markers, and phenotype.* J Cell Physiol, 2017. **232**(12): p. 3744-3761.
429. Campelj, D.G., et al., *The Paradoxical Effect of PARP Inhibitor BGP-15 on Irinotecan-Induced Cachexia and Skeletal Muscle Dysfunction.* Cancers (Basel), 2020. **12**(12).
430. Ubersax, J.A. and J.E. Ferrell, *Mechanisms of specificity in protein phosphorylation.* Nat Rev Mol Cell Biol, 2007. **8**(7): p. 530-41.
431. Choudhary, C., et al., *The growing landscape of lysine acetylation links metabolism and cell signalling.* Nat Rev Mol Cell Biol, 2014. **15**(8): p. 536-50.
432. Reily, C., et al., *Glycosylation in health and disease.* Nat Rev Nephrol, 2019. **15**(6): p. 346-366.
433. Rape, M., *Ubiquitylation at the crossroads of development and disease.* Nat Rev Mol Cell Biol, 2018. **19**(1): p. 59-70.
434. Seeler, J.S. and A. Dejean, *SUMO and the robustness of cancer.* Nat Rev Cancer, 2017. **17**(3): p. 184-197.
435. Hess, D.T., et al., *Protein S-nitrosylation: purview and parameters.* Nat Rev Mol Cell Biol, 2005. **6**(2): p. 150-66.
436. Mattei, A.L., N. Bailly, and A. Meissner, *DNA methylation: a historical perspective.* Trends Genet, 2022. **38**(7): p. 676-707.

437. Jiang, H., et al., *Protein Lipidation: Occurrence, Mechanisms, Biological Functions, and Enabling Technologies.* Chem Rev, 2018. **118**(3): p. 919-988.
438. Basu, B., et al., *Targeting the DNA damage response in oncology: past, present and future perspectives.* Curr Opin Oncol, 2012. **24**(3): p. 316-24.
439. Curtin, N.J. and C. Szabo, *Poly(ADP-ribose) polymerase inhibition: past, present and future.* Nat Rev Drug Discov, 2020. **19**(10): p. 711-736.
440. Berger, N.A., et al., *Opportunities for the repurposing of PARP inhibitors for the therapy of non-oncological diseases.* Br J Pharmacol, 2018. **175**(2): p. 192-222.
441. Berger, F., M.H. Ramirez-Hernandez, and M. Ziegler, *The new life of a centenarian: signalling functions of NAD(P).* Trends Biochem Sci, 2004. **29**(3): p. 111-8.
442. Yamada, M., M. Miwa, and T. Sugimura, *Studies on poly (adenosine diphosphate-ribose). X. Properties of a partially purified poly (adenosine diphosphate-ribose) polymerase.* Arch Biochem Biophys, 1971. **146**(2): p. 579-86.
443. Lüscher, B., et al., *ADP-ribosyltransferases, an update on function and nomenclature.* Febs j, 2021.
444. Vyas, S., et al., *Family-wide analysis of poly(ADP-ribose) polymerase activity.* Nat Commun, 2014. **5**: p. 4426.
445. Kleine, H., et al., *Substrate-assisted catalysis by PARP10 limits its activity to mono-ADP-ribosylation.* Mol Cell, 2008. **32**(1): p. 57-69.
446. Rippmann, J.F., K. Damm, and A. Schnapp, *Functional characterization of the poly(ADP-ribose) polymerase activity of tankyrase 1, a potential regulator of telomere length.* J Mol Biol, 2002. **323**(2): p. 217-24.
447. Tan, E.S., K.A. Krukenberg, and T.J. Mitchison, *Large-scale preparation and characterization of poly(ADP-ribose) and defined length polymers.* Anal Biochem, 2012. **428**(2): p. 126-36.
448. Alvarez-Gonzalez, R. and M.K. Jacobson, *Characterization of polymers of adenosine diphosphate ribose generated in vitro and in vivo.* Biochemistry, 1987. **26**(11): p. 3218-24.
449. Min, A. and S.A. Im, *PARP Inhibitors as Therapeutics: Beyond Modulation of PARylation.* Cancers (Basel), 2020. **12**(2).
450. Eisemann, T. and J.M. Pascal, *Poly(ADP-ribose) polymerase enzymes and the maintenance of genome integrity.* Cell Mol Life Sci, 2020. **77**(1): p. 19-33.
451. Bilokapic, S., et al., *Bridging of DNA breaks activates PARP2-HPF1 to modify chromatin.* Nature, 2020. **585**(7826): p. 609-613.
452. Li, N., et al., *Tankyrase disrupts metabolic homeostasis and promotes tumorigenesis by inhibiting LKB1-AMPK signalling.* Nat Commun, 2019. **10**(1): p. 4363.
453. Boehler, C., et al., *Phenotypic characterization of Parp-1 and Parp-2 deficient mice and cells.* Methods Mol Biol, 2011. **780**: p. 313-36.
454. Ali, A.A., et al., *The zinc-finger domains of PARP1 cooperate to recognize DNA strand breaks.* 2012. **19**(7): p. 685-692.
455. Ding, L., et al., *PARP1 Suppresses the Transcription of PD-L1 by Poly(ADP-Ribosyl)ating STAT3.* Cancer Immunol Res, 2019. **7**(1): p. 136-149.
456. Hassa, P.O. and M.O. Hottiger, *The diverse biological roles of mammalian PARPS, a small but powerful family of poly-ADP-ribose polymerases.* Front Biosci, 2008. **13**: p. 3046-82.
457. Mashimo, M. and J. Moss, *Functional Role of ADP-Ribosyl-Acceptor Hydrolase 3 in poly(ADP-Ribose) Polymerase-1 Response to Oxidative Stress.* Curr Protein Pept Sci, 2016. **17**(7): p. 633-640.
458. Slattery, E., et al., *Purification and analysis of a factor which suppresses nick-induced transcription by RNA polymerase II and its identity with poly(ADP-ribose) polymerase.* J Biol Chem, 1983. **258**(9): p. 5955-9.

459. Meisterernst, M., G. Stelzer, and R.G. Roeder, *Poly(ADP-ribose) polymerase enhances activator-dependent transcription in vitro.* Proc Natl Acad Sci U S A, 1997. **94**(6): p. 2261-5.
460. Obaji, E., T. Haikarainen, and L. Lehtio, *Structural basis for DNA break recognition by ARTD2/PARP2.* Nucleic Acids Res, 2018. **46**(22): p. 12154-12165.
461. Dregalla, R.C., et al., *Regulatory roles of tankyrase 1 at telomeres and in DNA repair: suppression of T-SCE and stabilization of DNA-PKcs.* Aging (Albany NY), 2010. **2**(10): p. 691-708.
462. Smith, S., et al., *Tankyrase, a poly (ADP-ribose) polymerase at human telomeres.* 1998. **282**(5393): p. 1484-1487.
463. Melikishvili, M., et al., *Transcriptome-wide identification of the RNA-binding landscape of the chromatin-associated protein PARP1 reveals functions in RNA biogenesis.* Cell Discov, 2017. **3**: p. 17043.
464. Jungmichel, S., et al., *Proteome-wide identification of poly(ADP-Ribosyl)ation targets in different genotoxic stress responses.* Mol Cell, 2013. **52**(2): p. 272-85.
465. Gagné, J.-P., et al., *Proteome-wide identification of poly (ADP-ribose) binding proteins and poly (ADP-ribose)-associated protein complexes.* Nucleic acids research, 2008. **36**(22): p. 6959-6976.
466. Ryu, D., et al., *NAD+ repletion improves muscle function in muscular dystrophy and counters global PARylation.* Sci Transl Med, 2016. **8**(361): p. 361ra139.
467. Aguennouz, M., et al., *Telomere shortening is associated to TRF1 and PARP1 overexpression in Duchenne muscular dystrophy.* Neurobiol Aging, 2011. **32**(12): p. 2190-7.
468. Olah, G., et al., *Differentiation-Associated Downregulation of Poly(ADP-Ribose) Polymerase-1 Expression in Myoblasts Serves to Increase Their Resistance to Oxidative Stress.* PLoS One, 2015. **10**(7): p. e0134227.
469. White, J.P., et al., *IL-6 regulation on skeletal muscle mitochondrial remodeling during cancer cachexia in the ApcMin/+ mouse.* Skelet Muscle, 2012. **2**: p. 14.
470. Caplan, A.I. and M.J. Rosenberg, *Interrelationship between poly (ADP-Rib) synthesis, intracellular NAD levels, and muscle or cartilage differentiation from mesodermal cells of embryonic chick limb.* Proc Natl Acad Sci U S A, 1975. **72**(5): p. 1852-7.
471. von Roretz, C., et al., *HuR and myogenesis: being in the right place at the right time.* Biochimica et biophysica acta, 2011. **1813**(9): p. 1663-7.
472. Yaffe, D. and O. Saxel, *Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle.* Nature, 1977. **270**(5639): p. 725-7.
473. Kirby, C.A., et al., *Structure of human tankyrase 1 in complex with small-molecule inhibitors PJ34 and XAV939.* Acta Crystallogr Sect F Struct Biol Cryst Commun, 2012. **68**(Pt 2): p. 115-8.
474. Karlberg, T., et al., *Structural basis for the interaction between tankyrase-2 and a potent Wnt-signaling inhibitor.* J Med Chem, 2010. **53**(14): p. 5352-5.
475. Le Bihan, M.C., et al., *Cellular Proteome Dynamics during Differentiation of Human Primary Myoblasts.* J Proteome Res, 2015. **14**(8): p. 3348-61.
476. Yan, J., et al., *circRNAome profiling reveals circFgfr2 regulates myogenesis and muscle regeneration via a feedback loop.* J Cachexia Sarcopenia Muscle, 2022. **13**(1): p. 696-712.
477. Liu, T.Y., et al., *Muscle developmental defects in heterogeneous nuclear Ribonucleoprotein A1 knockout mice.* Open Biol, 2017. **7**(1).
478. Senderek, J., et al., *Autosomal-dominant distal myopathy associated with a recurrent missense mutation in the gene encoding the nuclear matrix protein, matrin 3.* Am J Hum Genet, 2009. **84**(4): p. 511-8.
479. Espinoza-Lewis, R.A., et al., *Poly(C)-binding protein 1 (Pcbp1) regulates skeletal muscle differentiation by modulating microRNA processing in myoblasts.* J Biol Chem, 2017. **292**(23): p. 9540-9550.

480. Song, Y.J. and H. Lee, *YB1/p32, a nuclear Y-box binding protein 1, is a novel regulator of myoblast differentiation that interacts with Msx1 homeoprotein.* Exp Cell Res, 2010. **316**(4): p. 517-29.
481. Beauchamp, P., et al., *The cleavage of HuR interferes with its transportin-2-mediated nuclear import and promotes muscle fiber formation.* Cell Death Differ, 2010. **17**(10): p. 1588-99.
482. Cammas, A., et al., *Destabilization of nucleophosmin mRNA by the HuR/KSRP complex is required for muscle fibre formation.* Nat Commun, 2014. **5**: p. 4190.
483. van der Giessen, K., et al., *RNAi-mediated HuR depletion leads to the inhibition of muscle cell differentiation.* The Journal of biological chemistry, 2003. **278**(47): p. 47119-28.
484. Daniels, C.M., *Characterization of the ADP-ribosylated proteome by mass spectrometry*. 2015, Johns Hopkins University.
485. Loseva, O., et al., *PARP-3 is a mono-ADP-ribosylase that activates PARP-1 in the absence of DNA.* J Biol Chem, 2010. **285**(11): p. 8054-60.
486. Apponi, L.H., A.H. Corbett, and G.K. Pavlath, *RNA-binding proteins and gene regulation in myogenesis.* Trends in pharmacological sciences, 2011. **32**(11): p. 652-8.
487. Sbodio, J.I. and N.-W. Chi, *Identification of a Tankyrase-binding Motif Shared by IRAP, TAB182, and Human TRF1 but Not Mouse TRF1 NuMA CONTAINS THIS RXXPDG MOTIF AND IS A NOVEL TANKYRASE PARTNER.* Journal of Biological Chemistry, 2002. **277**(35): p. 31887-31892.
488. Guettler, S., et al., *Structural basis and sequence rules for substrate recognition by Tankyrase explain the basis for cherubism disease.* Cell, 2011. **147**(6): p. 1340-54.
489. Pollock, K., et al., *Identifying and Validating Tankyrase Binders and Substrates: A Candidate Approach.* Methods Mol Biol, 2017. **1608**: p. 445-473.
490. Li, B., et al., *Crystal structure of a tankyrase 1-telomere repeat factor 1 complex.* Acta Crystallogr F Struct Biol Commun, 2016. **72**(Pt 4): p. 320-7.
491. Tajbakhsh, S., et al., *Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5.* Development, 1998. **125**(21): p. 4155-62.
492. Münsterberg, A., et al., *Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite.* Genes & development, 1995. **9**(23): p. 2911-2922.
493. Mazroui, R., et al., *Caspase-mediated cleavage of HuR in the cytoplasm contributes to pp32/PHAP-I regulation of apoptosis.* The Journal of cell biology, 2008. **180**(1): p. 113-27.
494. Briata, P., et al., *KSRP, many functions for a single protein.* Front Biosci (Landmark Ed), 2011. **16**: p. 1787-96.
495. Xu, Y., et al., *Post-translational modification control of RNA-binding protein hnRNPK function.* Open Biol, 2019. **9**(3): p. 180239.
496. Gherzi, R., et al., *Akt2-mediated phosphorylation of Pitx2 controls Ccnd1 mRNA decay during muscle cell differentiation.* Cell death and differentiation, 2010. **17**(6): p. 975-83.
497. Clement, S.L., et al., *Phosphorylation of tristetraprolin by MK2 impairs AU-rich element mRNA decay by preventing deadenylase recruitment.* Mol Cell Biol, 2011. **31**(2): p. 256-66.
498. Velázquez-Cruz, A., et al., *Post-translational Control of RNA-Binding Proteins and Disease-Related Dysregulation.* Front Mol Biosci, 2021. **8**: p. 658852.
499. Frontera, W.R. and J. Ochala, *Skeletal muscle: a brief review of structure and function.* Calcif Tissue Int, 2015. **96**(3): p. 183-95.
500. Robert, F., et al., *Targeting protein synthesis in a Myc/mTOR-driven model of anorexia-cachexia syndrome delays its onset and prolongs survival.* Cancer research, 2012. **72**(3): p. 747-56.
501. Fearon, K.H., *Selective androgen receptor modulators in cancer cachexia?* Lancet Oncol, 2013. **14**(4): p. 271-2.

502. Ma, J.F., et al., *STAT3 promotes IFNgamma/TNFalpha-induced muscle wasting in an NF-kappaB-dependent and IL-6-independent manner.* EMBO Mol Med, 2017.
503. Zimmers, T.A., M.L. Fishel, and A. Bonetto, *STAT3 in the systemic inflammation of cancer cachexia.* Semin Cell Dev Biol, 2016. **54**: p. 28-41.
504. Hall, D.T., et al., *Inducible nitric oxide synthase (iNOS) in muscle wasting syndrome, sarcopenia, and cachexia.* Aging, 2011. **3**(8): p. 702-15.
505. Bonetto, A., et al., *JAK/STAT3 pathway inhibition blocks skeletal muscle wasting downstream of IL-6 and in experimental cancer cachexia.* American journal of physiology. Endocrinology and metabolism, 2012. **303**(3): p. E410-21.
506. Di Marco, S., et al., *NF-(kappa)B-mediated MyoD decay during muscle wasting requires nitric oxide synthase mRNA stabilization, HuR protein, and nitric oxide release.* Molecular and cellular biology, 2005. **25**(15): p. 6533-45.
507. Zhang, L., et al., *Stat3 activation links a C/EBPdelta to myostatin pathway to stimulate loss of muscle mass.* Cell Metab, 2013. **18**(3): p. 368-79.
508. Wu, P., et al., *Prognostic role of STAT3 in solid tumors: a systematic review and meta-analysis.* Oncotarget, 2016. **7**(15): p. 19863-83.
509. Xu, Y.H. and S. Lu, *A meta-analysis of STAT3 and phospho-STAT3 expression and survival of patients with non-small-cell lung cancer.* Eur J Surg Oncol, 2014. **40**(3): p. 311-7.
510. Anderson, D.M., et al., *Severe muscle wasting and denervation in mice lacking the RNA-binding protein ZFP106.* Proc Natl Acad Sci U S A, 2016. **113**(31): p. E4494-503.
511. Li, J., et al., *miR-29b contributes to multiple types of muscle atrophy.* Nat Commun, 2017. **8**: p. 15201.
512. Farooq, F., et al., *p38 Mitogen-activated protein kinase stabilizes SMN mRNA through RNA binding protein HuR.* Hum Mol Genet, 2009. **18**(21): p. 4035-45.
513. Fallini, C., G.J. Bassell, and W. Rossoll, *The ALS disease protein TDP-43 is actively transported in motor neuron axons and regulates axon outgrowth.* Hum Mol Genet, 2012. **21**(16): p. 3703-18.
514. Milani, P., et al., *Posttranscriptional regulation of SOD1 gene expression under oxidative stress: Potential role of ELAV proteins in sporadic ALS.* Neurobiol Dis, 2013. **60**: p. 51-60.
515. Dormoy-Raclet, V., et al., *HuR and miR-1192 regulate myogenesis by modulating the translation of HMGB1 mRNA.* Nature communications, 2013. **4**: p. 2388.
516. Figueroa, A., et al., *Role of HuR in skeletal myogenesis through coordinate regulation of muscle differentiation genes.* Molecular and cellular biology, 2003. **23**(14): p. 4991-5004.
517. Tenenbaum, S.A., et al., *Identifying mRNA subsets in messenger ribonucleoprotein complexes by using cDNA arrays.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(26): p. 14085-90.
518. Di Marco, S., et al., *The translation inhibitor pateamine A prevents cachexia-induced muscle wasting in mice.* Nature communications, 2012. **3**: p. 896.
519. Hall, D.T., et al., *The AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), but not metformin, prevents inflammation-associated cachectic muscle wasting.* EMBO Mol Med, 2018.
520. Mi, H., et al., *PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements.* Nucleic Acids Res, 2017. **45**(D1): p. D183-d189.
521. Sánchez, J.B., et al., *Depletion of HuR in murine skeletal muscle enhances exercise endurance and prevents cancer-induced muscle atrophy.* In Press, 2019.
522. Drury, G.L., et al., *FasL expression in activated T lymphocytes involves HuR-mediated stabilization.* The Journal of biological chemistry, 2010. **285**(41): p. 31130-8.

523. Di Marco, S., et al., *Polymorphism in the 3'-untranslated region of TNFalpha mRNA impairs binding of the post-transcriptional regulatory protein HuR to TNFalpha mRNA.* Nucleic acids research, 2001. **29**(4): p. 863-71.
524. Zhang, L., et al., *The RNA-binding protein CUGBP1 regulates stability of tumor necrosis factor mRNA in muscle cells: implications for myotonic dystrophy.* J Biol Chem, 2008. **283**(33): p. 22457-63.
525. Kim, H.H., et al., *HuR recruits let-7/RISC to repress c-Myc expression.* Genes & development, 2009.
526. Kundu, P., et al., *HuR protein attenuates miRNA-mediated repression by promoting miRISC dissociation from the target RNA.* Nucleic acids research, 2012. **40**(11): p. 5088-100.
527. Nicholson, C.O., M. Friedersdorf, and J.D. Keene, *Quantifying RNA binding sites transcriptome-wide using DO-RIP-seq.* RNA, 2017. **23**(1): p. 32-46.
528. Simone, L.E. and J.D. Keene, *Mechanisms coordinating ELAV/Hu mRNA regulons.* Current opinion in genetics & development, 2013. **23**(1): p. 35-43.
529. van der Giessen, K. and I.E. Gallouzi, *Involvement of transportin 2-mediated HuR import in muscle cell differentiation.* Molecular biology of the cell, 2007. **18**(7): p. 2619-29.
530. Zhuang, R., et al., *miR-195 competes with HuR to modulate stim1 mRNA stability and regulate cell migration.* Nucleic Acids Res, 2013. **41**(16): p. 7905-19.
531. Poria, D.K., et al., *RNA-binding protein HuR sequesters microRNA-21 to prevent translation repression of proinflammatory tumor suppressor gene programmed cell death 4.* Oncogene, 2016. **35**(13): p. 1703-15.
532. Haghikia, A., et al., *STAT3 regulation of and by microRNAs in development and disease.* JAKSTAT, 2012. **1**(3): p. 143-50.
533. Kurdi, M., C. Zgheib, and G.W. Booz, *Recent Developments on the Crosstalk Between STAT3 and Inflammation in Heart Function and Disease.* Front Immunol, 2018. **9**: p. 3029.
534. Masuda, K., et al., *Arid5a regulates naive CD4+ T cell fate through selective stabilization of Stat3 mRNA.* J Exp Med, 2016. **213**(4): p. 605-19.
535. Alexandrov, I.M., et al., *Cytoplasmic polyadenylation element binding protein deficiency stimulates PTEN and Stat3 mRNA translation and induces hepatic insulin resistance.* PLoS Genet, 2012. **8**(1): p. e1002457.
536. Keene, J.D. and S.A. Tenenbaum, *Eukaryotic mRNPs may represent posttranscriptional operons.* Mol Cell, 2002. **9**(6): p. 1161-7.
537. Ho, E., A. Hayen, and M.R. Wilkins, *Characterisation of organellar proteomes: a guide to subcellular proteomic fractionation and analysis.* Proteomics, 2006. **6**(21): p. 5746-57.
538. Kiraga, J., et al., *The relationships between the isoelectric point and: length of proteins, taxonomy and ecology of organisms.* BMC Genomics, 2007. **8**: p. 163.
539. Kurotani, A., et al., *Localization-specific distributions of protein pI in human proteome are governed by local pH and membrane charge.* BMC Mol Cell Biol, 2019. **20**(1): p. 36.
540. Tokmakov, A.A., A. Kurotani, and K.I. Sato, *Protein pI and Intracellular Localization.* Front Mol Biosci, 2021. **8**: p. 775736.
541. Chasapis, C.T. and G. Konstantinoudis, *Protein isoelectric point distribution in the interactomes across the domains of life.* Biophys Chem, 2020. **256**: p. 106269.
542. Wang, C., et al., *PARP1 promote autophagy in cardiomyocytes via modulating FoxO3a transcription.* Cell Death Dis, 2018. **9**(11): p. 1047.
543. Péladeau, C., N.J. Adam, and B.J. Jasmin, *Celecoxib treatment improves muscle function in mdx mice and increases utrophin A expression.* Faseb j, 2018. **32**(9): p. 5090-5103.
544. Love, D.R., et al., *An autosomal transcript in skeletal muscle with homology to dystrophin.* Nature, 1989. **339**(6219): p. 55-8.

545. Tinsley, J.M. and K.E. Davies, *Utrophin: a potential replacement for dystrophin?* Neuromuscul Disord, 1993. **3**(5-6): p. 537-9.
546. Serra, F., et al., *Inflammation in muscular dystrophy and the beneficial effects of non-steroidal anti-inflammatory drugs.* Muscle Nerve, 2012. **46**(5): p. 773-84.
547. Miyatake, S., et al., *Anti-inflammatory drugs for Duchenne muscular dystrophy: focus on skeletal muscle-releasing factors.* Drug Des Devel Ther, 2016. **10**: p. 2745-58.
548. Amirouche, A., et al., *Activation of p38 signaling increases utrophin A expression in skeletal muscle via the RNA-binding protein KSRP and inhibition of AU-rich element-mediated mRNA decay: implications for novel DMD therapeutics.* Hum Mol Genet, 2013. **22**(15): p. 3093-111.
549. Péladeau, C., et al., *Combinatorial therapeutic activation with heparin and AICAR stimulates additive effects on utrophin A expression in dystrophic muscles.* Hum Mol Genet, 2016. **25**(1): p. 24-43.
550. Farooq, F., et al., *Celecoxib increases SMN and survival in a severe spinal muscular atrophy mouse model via p38 pathway activation.* Hum Mol Genet, 2013. **22**(17): p. 3415-24.
551. Liu, S., et al., *Smooth muscle-specific HuR knockout induces defective autophagy and atherosclerosis.* Cell Death Dis, 2021. **12**(4): p. 385.
552. Saikia, R. and J. Joseph, *AMPK: a key regulator of energy stress and calcium-induced autophagy.* J Mol Med (Berl), 2021. **99**(11): p. 1539-1551.
553. Camargo, R.G., et al., *Cancer Cachexia and MicroRNAs.* Mediators Inflamm, 2015. **2015**: p. 367561.
554. Soares, R.J., et al., *Involvement of microRNAs in the regulation of muscle wasting during catabolic conditions.* J Biol Chem, 2014. **289**(32): p. 21909-25.
555. Kulyté, A., et al., *MicroRNA profiling links miR-378 to enhanced adipocyte lipolysis in human cancer cachexia.* Am J Physiol Endocrinol Metab, 2014. **306**(3): p. E267-74.
556. Miao, C., et al., *Cancer-derived exosome miRNAs induce skeletal muscle wasting by Bcl-2-mediated apoptosis in colon cancer cachexia.* Mol Ther Nucleic Acids, 2021. **24**: p. 923-938.
557. Kennedy, T.L., et al., *BGP-15 Improves Aspects of the Dystrophic Pathology in mdx and dko Mice with Differing Efficacies in Heart and Skeletal Muscle.* Am J Pathol, 2016. **186**(12): p. 3246-3260.
558. Reber, J.M. and A. Mangerich, *Why structure and chain length matter: on the biological significance underlying the structural heterogeneity of poly(ADP-ribose).* Nucleic Acids Res, 2021. **49**(15): p. 8432-8448.
559. Slade, D., *PARP and PARG inhibitors in cancer treatment.* Genes Dev, 2020. **34**(5-6): p. 360-394.
560. Houl, J.H., et al., *Selective small molecule PARG inhibitor causes replication fork stalling and cancer cell death.* Nat Commun, 2019. **10**(1): p. 5654.
561. Chand, S.N., et al., *Post-transcriptional regulation of PARG mRNA by HuR facilitates DNA repair and resistance to PARP inhibitors.* Cancer Res, 2017.
562. Liu, X., et al., *MicroRNA-223 protects neonatal rat cardiomyocytes and H9c2 cells from hypoxia-induced apoptosis and excessive autophagy via the Akt/mTOR pathway by targeting PARP-1.* J Mol Cell Cardiol, 2018. **118**: p. 133-146.
563. Sun, P., et al., *MiR-21 modulates proliferation and apoptosis of human airway smooth muscle cells by regulating autophagy via PARP-1/AMPK/mTOR signalling pathway.* Respir Physiol Neurobiol, 2022. **301**: p. 103891.
564. Lu, J., et al., *The poly (ADP-ribosyl) ation of FoxO3 mediated by PARP1 participates in isoproterenol-induced cardiac hypertrophy.* 2016. **1863**(12): p. 3027-3039.
565. Brack, A.S., et al., *A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis.* 2008. **2**(1): p. 50-59.
566. Harley, C.B., A.B. Futcher, and C.W. Greider, *Telomeres shorten during ageing of human fibroblasts.* Nature, 1990. **345**(6274): p. 458-60.

567. Greider, C.W. and E.H. Blackburn, *Identification of a specific telomere terminal transferase activity in Tetrahymena extracts.* Cell, 1985. **43**(2 Pt 1): p. 405-13.
568. Thériault, M.E., et al., *Satellite cells senescence in limb muscle of severe patients with COPD.* PLoS One, 2012. **7**(6): p. e39124.
569. Decary, S., et al., *Shorter telomeres in dystrophic muscle consistent with extensive regeneration in young children.* Neuromuscul Disord, 2000. **10**(2): p. 113-20.
570. Sacco, A., et al., *Short telomeres and stem cell exhaustion model Duchenne muscular dystrophy in mdx/mTR mice.* Cell, 2010. **143**(7): p. 1059-71.
571. Wang, P., Y. Zhou, and A.M. Richards, *Effective tools for RNA-derived therapeutics: siRNA interference or miRNA mimicry.* Theranostics, 2021. **11**(18): p. 8771-8796.
572. Diener, C., A. Keller, and E. Meese, *Emerging concepts of miRNA therapeutics: from cells to clinic.* Trends Genet, 2022. **38**(6): p. 613-626.