Characterizing the Role of HuR in Skeletal Muscle of Mice with Spinal Muscular Atrophy

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Thesis submitted to the Faculty of
Graduate and Postdoctoral Studies in partial fulfillment
of the requirements for the degree of Master of Science
in Cellular and Molecular Medicine

September 25, 2017

Cellular and Molecular Medicine
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ABSTRACT

Spinal muscular atrophy (SMA) is a debilitating neuromuscular disorder characterized by insufficient SMN protein, resulting in motoneuron death. Initially, it was thought that motoneuronal death is followed by muscle atrophy; however, recent research is beginning to reveal possible muscle intrinsic defects, independent of motoneuron defects, in SMA. Previous studies have elucidated the cooperative involvement of CARM1, HuD and SMN in motoneurons, revealing HuD as a possible key player in the SMA phenotype. In this study, we focus on HuR, a ubiquitous family member of HuD, and the possibility that it plays a similar key role with CARM1 and SMN in skeletal muscle. Through the use of an shCARM1 stable line of C2C12s, we show that CARM1 is necessary for HuR functionality during differentiation. We further show that the methylation of HuR is necessary for its capability to translocate cytoplasmically during differentiation. We confirm an interaction between HuR and SMN, suggestive of a similar mechanism as was shown previously with HuD. In light of these findings, we next progressed to determine whether HuR is misregulated in an SMA mouse model. We report increased CARM1 levels in skeletal muscles of these mice. We further discovered that a deficiency in SMN protein impairs HuR upregulation and cytoplasmic translocation in response to HuR activation through sciatic nerve denervation. These findings were correlated with aberrant mRNA expression of HuR targets upon denervation. Taken together, these results show that HuR methylation is essential for proper myogenesis, and that the mechanism by which it acts likely requires sufficient SMN protein levels. In a deficiency of SMN, HuR shows signs of misregulation that may play a role in the inability to maintain or repair muscle in SMA.

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LIST OF ABBREVIATIONS

AChE – Acetylcholinesterase

AChR – Acetylcholine receptor

ARE – AU-rich element

MHC – Myosin heavy chain

DMEM – Dulbecco's modified Eagle's medium

cDNA – Complementary Deoxyribonucleic acid

CARM1 – Coactivator-associated arginine methyltransferase 1

PRMT – Protein arginine methyltransferase

SAM – S-adenosylmethionine

aDMA – asymmetrical dimethylated arginine

sDMA – symmetrical dimethylated arginine

MMA – Mono-methylated arginine

FBS – Fetal bovine serum

R217K – HuR with arginine 217 substituted for a lysine

R217W – HuR with arginine 217 substituted for a tryptophan

HRP – Horseradish peroxidase

PBS – Phosphate buffered saline

PBST – Phosphate buffered saline with tween

PCR – Polymerase chain reaction

p21 – Cyclin-dependent kinase inhibitor 1

GRIP1 – Glutamate receptor interacting protein 1

NMJ – Neuromuscular junction

mRNA – Messenger RNA

snRNP – Small nuclear ribonucleoprotein

snRNA – Small nuclear ribonucleic acid

qPCR – Quantitative polymerase chain reaction

RNA – Ribonucleic acid

RRM – RNA recognition motif

HNS – HuR nucleocytoplasmic shuttling sequence

RT-PCR – Reverse transcriptase polymerase chain reaction

SDS – Sodium dodecyl sulphate

SMA – Spinal muscular atrophy

UV - Ultraviolet

shRNA – Short hairpin RNA

MuRF1 – Muscle RING-finger protein 1

MAFbx – Muscle specific F-box protein

SMN – Survival motor neuron

SMNΔ7 – SMN protein lacking exon 7

UTR – Untranslated region

SMN1 – Survival motor neuron 1 gene

SMN2 – Survival motor neuron 2 gene

hnRNP – Heterogeneous nuclear ribonucleoprotein

Myf5 – Myogenic factor 5

HSA – Human skeletal actin

KSRP – KH-type splicing regulatory protein

OE - Overexpression

CTRL - Control

PKC – Protein kinase C

CP1 – Cleavage product 1

IP injection – Intraperitoneal injection

WT – Wild type

MEF2 – Myoctye enhancer factor 2

GFP – Green fluorescent protein

CRISPR – Clustered regularly interspaced short palindromic repeats

ACKNOWLEDGEMENTS

First and foremost, I would like to thank both my supervisors, Dr. Jocelyn Côté and Dr. Bernard Jasmin, for their support, guidance and mentoring throughout my degree. It truly has been an invaluable learning experience that I cannot have achieved without their help and encouragement. I would further like to thank my advisory committee members, Dr. Rashmi Kothary and Dr. Nadine Wiper-Bergeron, for their guidance and direction. I would also like to express my gratitude to Dr. Rashmi Kothary for providing the mice for the *in vivo* studies.

Special thanks to Dr. Aymeric Ravel-Chapuis for his mentorship and support. I would like to thank John Lunde, Dr. Guy Belanger, Dr. Tara Crawford Parks and Geneviève Paris for their technical expertise and assistance. Thank you to all members of the Jasmin and Côté labs for their friendship and advice.

Finally, thank you to all my friends and family that have supported me and lifted me up to be the person I am today. I cannot have achieved this without them.

1) INTRODUCTION

1.1 Spinal Muscular Atrophy

Spinal Muscular Atrophy (SMA) is characterized as a neurodegenerative disease that targets alpha-motoneurons in the anterior horn of the spinal cord (Liu, Jong, Chiang, & Yang, 1992; U R Monani, 2005). It is an autosomal recessive disease that has been found to show varying levels of severity in humans (D'Amico, Mercuri, Tiziano, & Bertini, 2011; U R Monani, 2005). SMA is the most prominent genetic cause of infantile death, estimated to affect one in every 6000-10,000 children born, depending on the region (D'Amico et al., 2011; Pearn, 1980). The disease results in the death of lower alpha-motoneurons, and due to the lack of innervation to the associated muscles, causing muscle weakness and eventual atrophy (D'Amico et al., 2011; U R Monani, 2005). The associated phenotypes consist of deteriorating muscle control, and thus inability to perform daily tasks without aid. The aforementioned continuum of the level of severity of the disease has been characterized and sub-grouped into five clinical subcategories of SMA, as follows:

Type 0: Prenatal onset of the disease. Characterized by respiratory failure at birth. Patient generally survives, at most, a few weeks following birth (W. D. Arnold, Kassar, & Kissel, 2015; Dubowitz, 1999; MacLeod, Taylor, Lunt, Mathew, & Robb, 1999; Zerres et al., 1997).

Type 1: This is the most common subcategory of diagnosed SMA accounting for roughly 45% of cases. Onset of the disease occurs between 0-6months after birth. Initially, the infant will appear normal, followed by the development of muscle weakness, and

difficulty breathing. Due to the overall weakness, these infants lack the postural strength require to sit independently. Affected individuals generally survive less than a year following birth (W. D. Arnold et al., 2015; Schroth, 2009; Zerres et al., 1997; Zerres & Rudnik-Schoneborn, 1995).

Type 2: Onset of the disease occurs between 6 and 18months of age. These infants are able to sit and initially develop relatively normally. However, as they age, these infants lack the ability to stand, and thus cannot walk without assistance. These individuals show general muscle weakness which is found to be most prominent in the lower limbs. Affected individuals generally survive around 25 years of age (W. D. Arnold et al., 2015; D'Amico et al., 2011; Zerres et al., 1997; Zerres & Rudnik-Schoneborn, 1995).

Type 3: This classification of SMA is characterized by late onset of the disease, occurring between 18months of age and early adulthood. Initial infant development is normal, allowing these individuals to stand and walk without any assistance. Due to the vast time frame in which the onset of the disease occurs, symptoms show more variability in terms of severity. Generally, progressive muscle weakness eventually makes it difficult to walk or climb stairs to a point that affected patients may eventually become impaired. These individuals tend to live a normal lifespan (W. D. Arnold et al., 2015; Moosa & Dubowitz, 1973; Zerres et al., 1997; Zerres & Rudnik-Schoneborn, 1995).

Type 4: This is the least severe classification of SMA, characterized by onset during adulthood. Symptoms are similar to that of Type 3 SMA, however, due to the later onset (during adulthood), symptoms are much less severe. These individuals live a normal lifespan with minimal impairments in comparison to the other categories of SMA (W. D.

Arnold et al., 2015; Piepers et al., 2008; Zerres et al., 1997; Zerres & Rudnik-Schoneborn, 1995).

In 1995, the survival motor neuron (*SMN*) gene was found to be the "SMA-determining gene" (Lefebvre et al., 1995). Homozygous mutations or deletions in this gene, now known as the *SMN1* gene, have been discovered as the root cause behind the disorder. The *SMN1* gene is found in chromosome 5q13, and encodes for a 38kDa protein termed the SMN protein (Brzustowicz et al., 1990; Lefebvre et al., 1995). Thus the lack of sufficient functional levels of SMN protein results in the aforementioned SMA phenotype.

1.2 *SMN*2

In humans however, there is an almost identical inverted duplication of the *SMN*1 gene found in the same chromosome. This inverted duplication, termed *SMN*2, is centromeric to the *SMN*1 gene, and differs by only 5 nucleotides (Lefebvre et al., 1995). These five nucleotides do not change the amino acid sequence of the protein (Lefebvre et al., 1995; Umrao R. Monani et al., 1999). Notably, a cytosine to thymine (C840T) substitution in a splicing regulatory element of the *SMN*2 gene causes the production of ~90% protein product that lacks exon 7 (and ~10% full length SMN) as a result of alternative splicing defects in the associated mRNA (Kashima & Manley, 2003; Lorson, Hahnen, Androphy, & Wirth, 1999; J. Vitte et al., 2007). These protein products formed from alternatively spliced mRNAs are truncated, unstable, and degraded, and therefore a single *SMN*2 transcript cannot compensate for the lack of *SMN*1 protein product (Burnett et al., 2009; Umrao R. Monani et al., 1999; J. Vitte et al., 2007). Studies have shown that

patients have varying numbers of copies of the *SMN*2 gene, and that the presence of more *SMN*2 copies is correlated with delayed SMA onset, or a decreased SMA phenotype (Harada et al., 2002; Lefebvre et al., 1997; McAndrew et al., 1997; Prior, Swoboda, Scott, & Hejmanowski, 2004). Mouse studies have revealed that deletion of the *SMN*1 gene results in embryonic death. When inserting the *SMN*2 transgene into an SMA mouse model, increasing copy numbers decreased the severity of the disease, and it was found that ~8 *SMN*2 copies was sufficient to fully rescue the phenotype (U R Monani et al., 2000).

1.3 SMN Function

The exact role and function of the ubiquitously expressed 38kDa (294 amino acids in length) SMN protein has yet to be fully understood. However, it is known that some level of SMN protein expression is required for the viability of cells (Lefebvre et al., 1995; Owen, 2000; Paushkin et al., 2000; Schrank et al., 1997; J. Wang & Dreyfuss, 2001). The protein consists of several domains, including the well-studied Tudor domain found in the central region of the protein that is necessary for its binding to different target proteins (Hubers et al., 2011; Sabra, Texier, El Maalouf, & Lomonte, 2013; Selenko et al., 2001). It is established that SMN is abundantly expressed in the motoneurons of the spinal cord, and it is localized both in the nucleus and cytoplasm of cells (Coovert et al., 1997). Research groups have been gradually resolving different mechanisms in which the SMN protein plays an important role. To date, the most accepted and understood role of the SMN protein is its functional role in mediating the formation of small nuclear ribonucleoproteins (snRNPs) through the assembly of Sm

proteins with small nuclear RNAs (snRNAs) (Carissimi et al., 2005; Meister, Eggert, & Fischer, 2002). SMN forms a complex with Gemins2-7, known as the SMN complex. This complex has been shown to act as a chaperone as it binds Sm proteins as well as snRNAs, facilitating their interaction while ensuring binding specificity, thereby enabling the formation of the corresponding snRNPs (Carissimi et al., 2005; Meister et al., 2002; Terns & Terns, 2001; Yong, Wan, & Dreyfuss, 2004). snRNPs are of great importance due to their role in the formation of the spliceosome, a complex structure which mediates the splicing of pre-mRNA. Indeed it has been shown that in SMA model mice that some gemins display aberrant expression patterns which lead to a decrease in the levels of snRNPs (Gabanella et al., 2007). It has further been shown that SMN mutants found in SMA patients show defects in Sm protein binding affinity, confirming impairments in snRNP biogenesis (Pellizzoni, Charroux, & Dreyfuss, 1999). This deficiency of snRNPs in SMA has been linked to widespread pre-mRNA splicing defects in many different genes. (Fox-Walsh & Hertel, 2009; Huo et al., 2014; Z. Zhang, Lotti, Dittmar, Younis, Wan, Kasim, & Dreyfuss, 2008). The issue remains that such splicing defects would affect all cells due to the ubiquitous nature of SMN protein expression, yet SMA seems to mainly affect motoneurons and their associated muscles. The jury is still out as to whether the defects seen in SMA patients are due to aberrant snRNP biogenesis, or whether minimal SMN levels are sufficient for the spliceosome functionality and that the SMA phenotype is caused by other, less well defined, roles of SMN.

More recently, researchers have begun to steer their focus towards different roles of SMN, other than snRNP biogenesis and pre-mRNA splicing, in hopes of revealing novel mechanisms that may better explain the etiology behind SMA. Increases in SMN

protein levels have been reported within differentiating neurons in culture, indicating a possible key role during cellular differentiation and embryonic development (Shafey, MacKenzie, & Kothary, 2008; van Bergeijk, Rydel-Könecke, Grothe, & Claus, 2007). One of the important, more novel discovered roles of SMN is its function in axonal transport. Through motoneuron isolation from an SMA mouse model, it was discovered that the cell survival was unaltered, however, there was impaired axonal growth, suggesting a putative role of SMN in growth cones during differentiation. Furthermore, the SMN complex localizes into granules, which are actively transported bi-directionally along axons during axonal elongation (Rossoll et al., 2003; Setola et al., 2007; H. Zhang et al., 2006). Through the use of SMNΔ7 (SMN lacking exon 7) in cultured motoneurons, it has further been elucidated that the exon7 is essential for efficient granule formation and transport along the axon (H. L. Zhang et al., 2003). Moreover, SMN has been shown to form a complex in granules that interacts and co-localizes with β-Actin mRNA, profilin II, and hnRNP-R in the neurite extensions in culture during the outgrowth phase (Giesemann et al., 1999; Rossoll et al., 2002, 2003). These findings, taken together with the notion that β-actin mRNA and protein levels show reduced axon and growth cone expression in SMA motoneurons, highlight the importance of SMN in axonal transport as another possible mechanism at the root of the SMA phenotype (Rossoll et al., 2003). Through live cell imaging of primary neurons, SMN was found to be transported bidirectionally along axons in a speed consistent with that of motor protein dependent fast axonal transport (Fallini, Bassell, & Rossoll, 2010; H. L. Zhang et al., 2003). More recently this was confirmed in SMA mouse primary motoneurons where it was found that mRNA localization and local translation in the growth cones were disrupted (Akten et al.,

2011; Fallini, Donlin-Asp, Rouanet, Bassell, & Rossoll, 2016). The lack of proper localization and distribution of mRNAs essential for actin dynamics would result in an impairment in the formation of the axonal cytoskeleton. Through an impaired ability for efficient outgrowth of axons, motoneurons would lack the ability to form or maintain healthy neuromuscular junctions (NMJs), which may lead to the documented downstream muscle atrophy seen in patients. Indeed, it is well characterized that a deficiency in SMN results in an impairment in cytoskeletal dynamics of motoneurons (Mélissa Bowerman, Beauvais, Anderson, & Kothary, 2010; Mélissa Bowerman, Shafey, & Kothary, 2007; Nölle et al., 2011; Sharma et al., 2005; Wen et al., 2010). SMN has also been shown to interact with several different RNA-binding proteins, which could play a key part in the localization and transport of both proteins and RNAs involved in many different cellular processes including RNA transport, editing, translation, stabilization and splicing (Fallini et al., 2011, 2014; Fallini, Bassell, & Rossoll, 2012; Hubers et al., 2011; Mourelatos, Abel, Yong, Kataoka, & Dreyfuss, 2001; Piazzon et al., 2008; Tadesse, Deschênes-Furry, Boisvenue, & Côté, 2008; I.-F. Wang, Reddy, & Shen, 2016). Interestingly, SMN protein was found to co-localize and interact with HuD within RNA granules (Akten et al., 2011; Hubers et al., 2011). Furthermore, SMN was found to be required for the proper recruitment of HuD and its target mRNAs, as well as the RNA granule marker KSRP, into the dense neuronal RNA granules (Hubers et al., 2011). Importantly, these mobile SMN granules were absent of Sm proteins, suggestive of a role other than snRNP biogenesis (Fallini et al., 2011; H. Zhang et al., 2006). Taken together with the aforementioned axonal transport movement patterns of the SMN protein, it is evident that SMN is necessary for proper recruitment of mRNA and RNA-binding proteins in these

RNA granules, as well as their axonal transport. Much research remains to be done before a conclusive picture is drawn regarding the exact role of SMN and the mechanism by which a deficiency of SMN would cause the SMA phenotype.

1.4 SMA and skeletal muscle

One of the most obvious symptoms of SMA is the well characterized muscle weakness. Skeletal muscles of SMA mice have been shown to express small cross sectional area, weaker force production, a shift towards smaller average myofiber size, aberrant myofibril morphology, reduced motor endplate size, and reduced levels of fully occupied endplates (Boyer et al., 2013; Eshraghi, McFall, Gibeault, & Kothary, 2016; Y. il Lee, Mikesh, Smith, Rimer, & Thompson, 2011; Walker et al., 2008). Among these studies, some have shown that the muscles are developmentally impaired in that they do not mature far past the early postnatal stages. More specifically, it has been shown that in an *SMN*Δ7 mouse model, the myofiber cross sectional area ceased to increase past post-natal day 5 (Dachs et al., 2011; Y. il Lee et al., 2011). Similarly, in a more severe $Smn^{-/-}$;*SMN*2 mouse model, the muscles did not seem to develop farther past post-natal day 1 (Dachs et al., 2011). This lack of muscle development precedes the onset of motoneuron loss. Two schools of thought exist on the matter of muscular defects in SMA: one suggests that muscular defects are a direct outcome of upstream neuronal defects, whereas the other suggests that muscles are affected independently as well, through intrinsic mechanisms (ie. muscle intrinsic defects in the lack of SMN protein levels, independent of motoneuron functionality and innervation status).

Recent research has begun to elucidate muscle intrinsic defects, independent of neural death, found in SMA models. This is often difficult to study given the cooperative

interplay between muscles and motoneurons. It is well known that the denervation of skeletal muscle results in muscle atrophy, however, with intrinsic muscle defects, therapeutic correction of the phenotype in motoneurons may not completely cure patients due to ongoing muscle atrophy. One initial study demonstrated that SMN functions as a sarcomeric Z-disc protein as it co-localizes and interacts with α-actinin (Rajendra et al., 2007; Walker et al., 2008). To further elucidate muscle intrinsic roles of SMN, many research groups have attempted tissue specific knockouts of SMN using promoters specific to the tissue of interest. In skeletal muscle, this was initially performed using a Cre-recombinase knockout, where it was recorded that the muscle specific knockout results in a dystrophic phenotype and muscle paralysis followed by death (Cifuentes-Diaz et al., 2001). However, it was later suggested that a complete knockout of SMN may be lethal to any tissue, seemingly due to SMN's base ubiquitous function in snRNP assembly (J. M. Vitte et al., 2004). Using the same Cre system with the Myf5 promoter, SMN was depleted in skeletal muscle. These mice also harbor *SMN*2 and *SMN*Δ7 transgenes as to prevent the tissue toxicity that was noted in a full SMN knockout. Despite the reduction of SMN protein levels, it was shown that muscle specific deletion or rescue had no significant phenotypic effect (C C Iyer et al., 2015). However, when looking at the level of SMN reduction, it is evident that the transgenic mice still maintain roughly 25-30% normal SMN protein levels. Such levels of SMN may be sufficient for the efficient fulfillment of SMN's role in muscle. As described in an $Smn^{2B/-}$ model, higher levels of SMN (~15% of control mice) result in a less severe phenotype, suggesting the possibility of a critical threshold upon which the muscle seems phenotypically normal (M Bowerman, Murray, Beauvais, Pinheiro, & Kothary, 2012). To

attempt to answer this discrepancy, a full-length SMN rescue was performed in SMA model mice, using the human skeletal actin (HSA) promoter. No overt phenotypic improvements were observed, suggesting that the muscle is more of a downstream target rather than directly affected (Gavrilina et al., 2008). However, in this case, the effects of a beneficial muscular effect may be undermined by the fact that the upstream motoneurons are still impaired due to the SMA phenotype. Furthermore, a contradictory study focused on pre-symptomatic stages argues that muscle weakness is instigated in the pre-symptomatic phase, prior to any perceivable denervation (as all neuromuscular junctions remained intact and seemingly healthy), or motoneuron death (Boyer et al., 2013). In agreement, another study published that increased SMN expression in muscle improved myofiber size, but had no impact on synaptic function (Martinez et al., 2012). One of the main issues with tissue specific control of protein expression is the fact that promoters are often found to show signs of leakage into other tissues, which may skew the observed results. Given the contradictory nature of the aforementioned studies, an analysis of the mechanistic basis behind the possible muscle specific defects could shed some light on the issue.

In 2005, it was revealed that a deficiency of SMN protein in C2C12 myoblasts results in defects in myogenesis and decreased cellular proliferation. The severity of such defects were shown to be directly correlated to the level of SMN knockdown in the cells (Shafey, Côté, & Kothary, 2005). Similar results were seen using human derived primary skeletal muscle cells from SMA patients, where it was presented that there was an impairment in myogenic fusion upon initiation of differentiation. Further to these findings, an impairment in the clustering of acetylcholine receptors (AChR), as well as a

delayed switch to its adult isoform was also noted (A.-S. Arnold et al., 2004; Kong et al., 2009). This could account for the documented post-synaptic impairment in neuromuscular junction maturation (Kariya et al., 2008). To solidify that these post-synaptic defects are independent of motoneuron health, an earlier study had previously shown that healthy motoneurons co-cultured with SMN deficient satellite cells derived from SMA patients resulted in the degradation and disorganization of innervated fibers, occurring between 1 and 3 weeks of incubation (Braun, Croizat, Lagrange, Warter, & Poindron, 1995).

Given that muscle development has been shown to be limited in SMA mice, perhaps SMN is somehow involved in myogenesis. Studies in mouse models of SMA revealed a misregulation of the myogenic markers during myogenesis and development. More specifically, in primary *Smn*$^{2B/-}$ mouse myoblasts, it was shown that Pax7 and MyoD protein levels were reduced in proliferating cells, and that Myogenin and myosin heavy chain (MHC) levels were reduced in differentiated cells. Furthermore, hind limb muscles of the *Smn*$^{2B/-}$ mouse showed a delay in the expression of Pax7, MyoD and Myogenin during post-natal development, while the more severe *Smn*$^{-/-}$;*SMN*2 demonstrated deficiencies in protein levels of the same myogenic factors. Developmentally, the muscles of these mice exhibit smaller myofiber size along with a greater presence of immature myofibers, suggesting the lack of proper muscle development (Boyer et al., 2014). Further to these findings, misregulations within the isoform switch of ryanodine receptor and voltage gated sodium channel were noted, as well as decreased levels of SERCA1a protein (an essential protein to the formation of Ca^{2+} pumps in muscle) (Boyer et al., 2013). In addition, the muscles in SMA model mice

express greater levels of the immature MHC isoform in comparison to control counterparts which may also play part in the morphological immature muscle fibers observed (Kong et al., 2009; Y. il Lee et al., 2011). Together, these findings suggest that in an SMN deficiency, the intrinsic molecular processes that take place during myogenesis show evident signs of impairment, independently of innervation by a motoneuron. Interestingly, satellite cells derived from SMA model mice (*Smn*$^{-/-}$;*SMN*2) seemed comparable in proliferation and survival to that of control mice. Analysis of differentiation markers upon initiation of differentiation demonstrated premature expression of myogenic markers, along with an associated reduction of efficiency in myotube formation (Hayhurst, Wagner, Cerletti, Wagers, & Rubin, 2012). Though the exact mechanistic source underlying the defects caused by an SMN deficiency still remains unclear, it seems tangible that the source of the muscle specific issues resides with muscle cell differentiation, and further development.

1.5 SMN and the Tudor domain

Of key importance to SMN's functionality is its central Tudor domain. The Tudor domain is characterized as a domain which binds methylated arginines of interacting proteins (Côté & Richard, 2005; Westley J. Friesen, Massenet, Paushkin, Wyce, & Dreyfuss, 2001). This Tudor domain of SMN protein encoded from exon 3 and is comprised of a highly conserved string of about 50 amino acids (Ponting, 1997). This domain is formed of a tertiary structure of five β-pleated sheets that form a barrel-like pocket with a hydrophobic core surrounded by several negatively charged amino acid residues forming an overall charged surface. This surface is said to be the site of protein-

protein interactions through the binding of dimethyl-arginines in arginine and glycine rich areas of the interacting proteins (Côté & Richard, 2005; Selenko et al., 2001; Sprangers, Groves, Sinning, & Sattler, 2003). Several Tudor domain mediated protein-protein interactions have been confirmed to date, including fibrillarin, Sm D1 and D3 proteins, KSRP, and HuD (Westley John Friesen & Dreyfuss, 2000; Hubers et al., 2011; Jones et al., 2001; Selenko et al., 2001; Tadesse et al., 2008). Furthermore, it has been confirmed that the Tudor domain is necessary for snRNP biogenesis to occur (Bühler, Raker, Lührmann, & Fischer, 1999). One of the known point mutations within the Tudor domain that results in SMA is known as the E134K point mutation. This mutation results in a single amino acid substitution from a glutamic acid to a lysine, which abrogates the binding capacity of the Tudor domain by altering the charge distribution within the binding site, without modifying the structure of the domain (Bühler et al., 1999; Côté & Richard, 2005; Selenko et al., 2001). Collectively, these findings highlight the importance of SMN's interactions with proteins harboring methylated arginine residues. This warrants further research into these interacting partners as it may further elucidate the downstream mechanisms by which a single amino acid substitution, and consequently the abolishment of a protein-protein interaction, could result in such a traumatic disease.

1.6 Protein arginine methyltransferases

As previously mentioned, SMN's Tudor domain preferentially binds to methylated arginine residues on target proteins, thus emphasizing the importance of post-translational methylation. There exists a family of 9 confirmed protein members termed protein arginine methyltransferases (PRMTs). As the name describes, these proteins are

involved in catalyzing the post-translational modification of methylation through transfer of a methyl group to their target proteins (Bedford & Clarke, 2009; Cha & Jho, 2012). PRMTs can catalyze such reactions due to the presence of a highly conserved methyltransferase domain which allows the transfer of a methyl group, from the methyl donor S-adenosylmethionine (SAM), to the guanidino nitrogen atoms of an arginine residue within the target protein (Blanc & Richard, 2017). This post-translational methylation has the potential to not only locally changing the structure of the protein, but can also prevent the formation of hydrogen bonds with other amino acids (Bedford & Clarke, 2009; Yang & Bedford, 2012). There are three types of methylated arginines in eukaryotes based on the methylation pattern: ω-N^G-monomethylarginine (MMA), ω-N^G,N^G-asymmetric dimethylarginine (aDMA), and ω-N^G,N'^G-symmetric dimethylarginine (sDMA) (Blanc & Richard, 2017). PRMTs are classified as per the type of methylation in which they are capable of performing. All three types (Type I: PRMTs 1, 2, 3, 4, 6 and 8, Type II: PRMTs 5 and 9, Type III: PRMT 7) of PRMTs are capable of generating a monomethylarginine, however, only Type I and Type II are capable of dimethylation. As shown in Figure 1, Type I PRMTs catalyze the formation of the asymmetrical dimethylarginine, whereas Type II PRMTs catalyze the formation of the symmetrical dimethylarginine arrangement (Bedford & Clarke, 2009; Blanc & Richard, 2017; Yang & Bedford, 2012). PRMTs have many known protein targets that are involved in several different cellular processes including splicing, gene regulation, subcellular localization, cellular signaling and protein stability (Auclair & Richard, 2013; Blanc & Richard, 2017; Yang & Bedford, 2012).

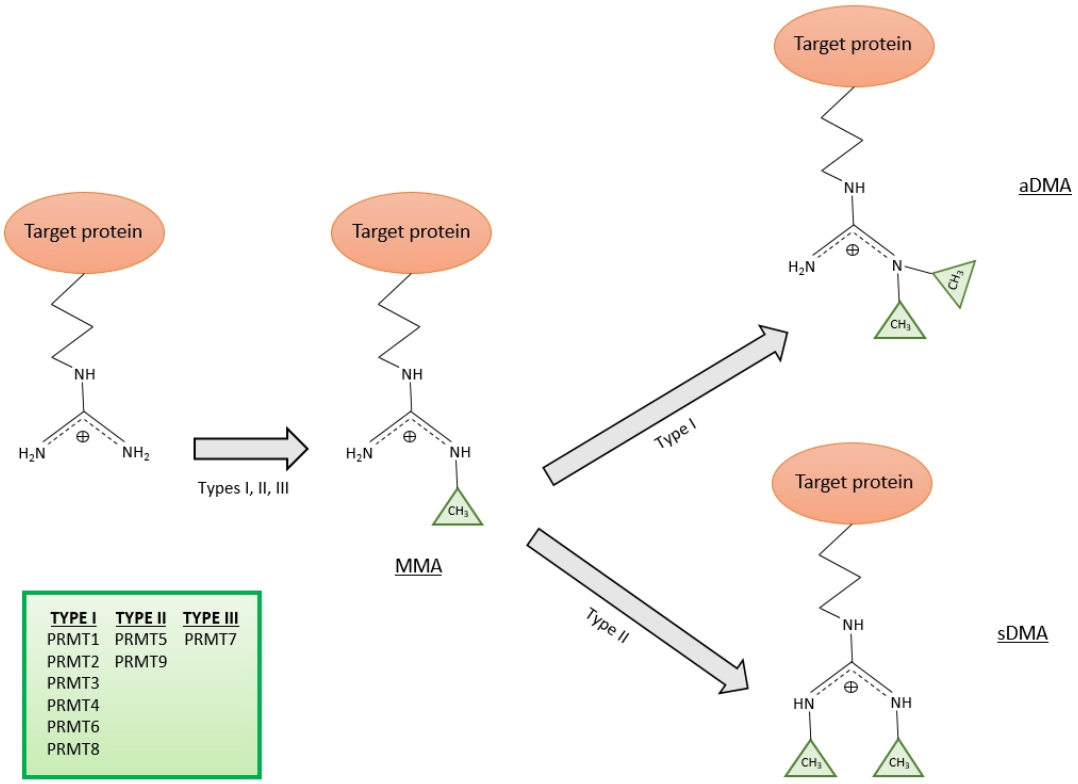


Figure 1: PRMTs and methylation. Protein arginine methyltransferases (PRMTs) transfer a methyl group from S-adenosyl-L-methionine (SAM) to the arginine of a target protein. All PRMTs are capable of monomethylation (MMA). Only Type I PRMTs are further capable of producing asymmetrical dimethyl arginines (aDMA) and only Type II are capable of producing symmetrical dimethyl arginines (sDMA). Type III is limited to MMA alone.

1.7 Co-activator associated arginine methyltransferase 1 (PRMT4)

Among the aforementioned family of PRMTs is PRMT4, also known as co-activator associated arginine methyltransferase 1 (CARM1). This 63kDa protein was first discovered through a yeast two-hybrid screening due to its interaction with the C-terminal of GRIP1, a member of the p160 family of coactivators. CARM1 protein was further found to be able to bind all three of the p160 coactivators. Due to its homology with other methyltransferases, CARM1's methyltransferase ability was put to the test, and it was found that CARM1 favorably methylates histone H3 at arginine 17 and 26 (D. Chen et al., 1999; Stallcup et al., 2000). Mutations within the highly conserved region of CARM1 not only abolished its ability to methylate histone H3, but also resulted in reduced transcriptional activation of genes (U. M. Bauer, Daujat, Nielsen, Nightingale, & Kouzarides, 2002; D. Chen et al., 1999; Chevillard-Briet, Trouche, & Vandel, 2002; H. Ma et al., 2001).

Mice exhibiting a knockout of CARM1 were much smaller than their littermates, and would die perinatally, emphasizing the importance of the protein (Yadav et al., 2003). Other studies had begun to unravel the wide range of newfound targets that are subject to methylation by CARM1, affecting post-transcriptional regulation. Included among these newfound targets are various RNA-binding proteins and splicing factors (Cheng, Côté, Shaaban, & Bedford, 2007; Fujiwara et al., 2006; J. Lee & Bedford, 2002; Li et al., 2002). Remarkably, CARM1 was shown to be involved in cell cycle regulation. By means of methylating the RNA-binding protein HuD, CARM1 is able to indirectly affect the levels of cyclin-dependent kinase inhibitor 1 (known as p21) through the modulation of the stability of p21 mRNA. Knockdown of CARM1 in PC12 cells results

in an upregulation of p21 protein levels, which was correlated with slower cell proliferation, and healthy neuritogenesis in response to nerve growth factor (Fujiwara et al., 2006). It was later confirmed that, mechanistically, the methylation of HuD changes its binding affinity for the target mRNAs in which it stabilizes, thus regulating the abundance of these mRNAs. Moreover, the same group showed that CARM1 is necessary for maintaining the proliferative state of MN-1 cells, and that upon initiation of differentiation, CARM1 is rapidly downregulated (Hubers et al., 2011). Interestingly, CARM1 knockout in mice had shown hyperproliferation of pulmonary epithelial cells of the mice during the embryonic stages. This hyperproliferation was accompanied by increased levels of immature alveolar cells, and concurrently a lack of mature alveolar cells, suggesting a dependence on CARM1 for cell cycle regulation. Furthermore, gene expression analysis also revealed misregulation of the cell cycle genes, as well as misregulation of differentiation markers (O'Brien et al., 2010). It has also been shown in satellite cells that CARM1 methylates Pax7 regulatory factor. This methylation is necessary towards the induction of myogenic factor 5 (Myf5) expression towards the initiation of the myogenic program (Kawabe, Wang, McKinnell, Bedford, & Rudnicki, 2012). Further to this, CARM1 has also been found to play a role in the transcriptional regulation of late myogenic genes in myoblasts (Dacwag, Bedford, Sif, & Imbalzano, 2009). Taken together, these studies suggest that the multifaceted CARM1 protein plays an important role in transcriptional gene regulation, as well as a role in cell cycle regulation through modulating the binding of RNA-binding proteins, or modulation of myogenic factors.

Interestingly, in 2012, an interplay between SMN protein and CARM1 mRNA was discovered. Through translational repression, SMN downregulates levels of CARM1, and thus it was found that CARM1 levels are upregulated in the spinal cords of an SMA mouse model, as well as in SMA patient derived primary fibroblasts (both of which are deficient in SMN levels) (Sanchez et al., 2013). Thus a potential role for misregulation of the downstream targets of CARM1 could play a mechanistic role in the SMA.

1.8 Hu family of proteins

The Hu family of proteins are a family of four RNA-binding proteins known as HuR (or HuA), HuB, HuC, and HuD. This family of proteins is also referred to as the ELAV family of proteins, due to their homology with the embryonic lethal abnormal vision RNA-binding protein found in drosophila (Good, 1995; Hinman & Lou, 2008). These RNA-binding proteins have been known to play roles in mRNA stabilization, pre-mRNA splicing and alternative splicing, nucleocytoplasmic shuttling, translational regulation, and polyadenylation (Abdelmohsen & Gorospe, 2010; Fan & Steitz, 1998b; Hinman & Lou, 2008; K Kasashima, Terashima, Yamamoto, Sakashita, & Sakamoto, 1999; Szabo et al., 1991). The Hu proteins are known to mainly be expressed neuronally, with the exception of HuR exhibiting a more ubiquitous nature as it is expressed in all tissues (Barami, Iversen, Furneaux, & Goldman, 1995; W. J. Ma, Cheng, Campbell, Wright, & Furneaux, 1996; Szabo et al., 1991). The Hu proteins are well known to be expressed early on in neuronal cells as they play an important role in the regulation of cell cycle and the initiation of cellular differentiation (W Akamatsu et al., 1999; Anderson et al., 2000; Barami et al., 1995; Katsumi Kasashima, Terashima, Yamamoto,

Sakashita, & Sakamoto, 1999; Marusich, Furneaux, Henion, & Weston, 1994; Yano, Okano, & Okano, 2005).

Hu proteins are comprised of 3 highly conserved RNA recognition motifs (RRMs) through which they bind RNA transcripts (Okano & Darnell, 1997). As shown in Figure 2, RRM1 and RRM2 cooperate to bind an AU-rich element (ARE) in the target mRNA, whereas RRM3 binds the polyA tail and maintain the RNA-protein complex stability (Beckel-Mitchener, Miera, Keller, & Perrone-Bizzozero, 2002; Inoue, Muto, Sakamoto, & Yokoyama, 2000; W.-J. Ma, Chung, & Furneaux, 1997). The best established and studied role of these RNA-binding proteins is their role in stabilizing mRNA transcripts through the binding of the aforementioned AREs that are typically found in the 3' untranslated region (3'UTR) of the transcripts (Hinman & Lou, 2008). Through binding these AREs, Hu proteins stabilize the transcripts by preventing access to the ARE-binding proteins that recruit the exosome for mRNA degradation. This binding also prevents cleavage by an endonuclease known to target these AREs (C. Y. Chen et al., 2001; Zhao, Chang, & Furneaux, 2000). In so doing, Hu proteins successfully prolong the half-life of the targeted mRNAs in which they bind. Such a stabilizing effect generally results in a greater transcript abundance, and concomitantly, an upregulation of the translated protein. For example, in RKO cells, HuR has been shown to stabilize p21 mRNA in response to the onset of cellular stress using ultraviolet (UV) light, thus resulting in increased p21 levels due to prolonged mRNA half-life. This increase in stability was reported alongside an increase in HuR cytoplasmic localization (W. Wang et al., 2000). HuD has also been found to bind the 3'UTR of p21 mRNA transcripts within neuronal cells (Hubers et al., 2011; Joseph, Orlian, & Furneaux, 1998).

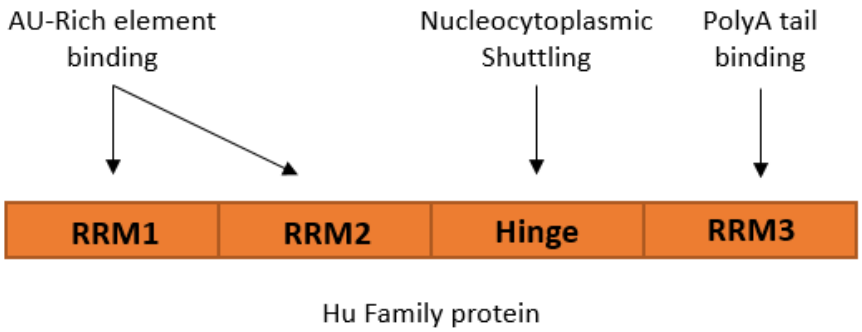


Figure 2: Domains of the Hu family of proteins. All members of the Hu family have 3 RNA-recognition motifs (RRM1-3), and a hinge region between RRM2 and RRM3.

Much research to date has implicated HuD playing a role in neuritogenesis. It was initially found that inhibition of HuD in PC12 cells resulted in the failure of neurites to grow in response to nerve growth factor stimulation, and that overexpression of HuD alone resulted in the growth of short spontaneous neurites (Katsumi Kasashima et al., 1999; Mobarak et al., 2000). This effect was said to be mediated through the stabilization of the mRNA transcripts of a neuronal growth-associated protein essential for neuritogenesis (Mobarak et al., 2000). A study of a HuD deficiency in mice revealed impaired embryonic development of the cranial nerve, as well as a decreased number of neurons produced by primary neurospheres. In adult stages, these mice reveal hind-limb reflex deficits, as well as poor rotarod performance (Wado Akamatsu et al., 2005). A more recent study revealed that HuD knockout mice exhibit defects in the dendritic outgrowth phase, as well as defective dendritogenesis in the CA3 region of the hippocampus. These same mice were more susceptible to auditory seizures, often leading to their death (DeBoer et al., 2014). Evidently, as depicted in these studies, the protein function of HuD is essential for proper development of neurons. HuD is methylated by CARM1 at and arginine residue within its hinge region. This methyl-HuD directly interacts with the SMN protein at the Tudor domain, leading to the question as to whether SMA neuronal defects may act, in part, through a mechanism involving this interaction (Hubers et al., 2011). It has been published that the SMN-HuD complex interacts with cpg15 (a protein that promotes axon branching and neuromuscular junction formation) mRNA in the axons of motoneurons of the spinal cord. Further to these finding, it was shown that an SMN deficiency reduced the levels of cpg15 mRNA, and that the overexpression of cpg15 can partially rescue the axonal defects of the SMA phenotype, in

a zebrafish model (Akten et al., 2011). In agreement with these findings, our lab has previously shown that SMA-like defects (ie. neurite extension, RNA granule recruitment, and mRNA localization defects) in MN-1 motoneurons exhibiting an SMN knockdown were rescued through the overexpression of HuD (Hubers et al., 2011). These findings provide strong evidence for the possibility that HuD may mechanistically play a key role in the motoneuron defects seen in SMA. However, HuD cannot be accountable for the aforementioned muscle intrinsic defects seen in SMA as HuD is specific to neurons. This brought our interest to the HuD homologue, HuR due to its ubiquitous expression.

1.9 HuR and skeletal muscle

HuR is the only member of the Hu family of proteins that is known to be expressed in skeletal muscle. HuR is a 36 kDa protein known to play an important role in the differentiation and development of muscle cells, through its characterized role in mRNA stabilization, as previously described (Figure 3). HuR protein levels were found to be quickly upregulated upon initiation of differentiation in C2C12 myoblasts. This upregulation is accompanied by a translocation of HuR into the cytoplasm, where the majority of the protein increase is measured. Additionally, this activation of HuR has been linked to the regulation of genes that are essential to muscle differentiation, through the prolongation of their half-lives (Figueroa et al., 2003). Among these stabilized transcripts are Myogenin, MyoD, AChE, AChR-B, and p21, all of which have been shown to be essential to muscle cell differentiation (Deschenes-Furry et al., 2005; Figueroa et al., 2003; Füchtbauer & Westphal, 1992; Joassard et al., 2015; Rudnicki & Jaenisch, 1995; van der Giessen, Di-Marco, Clair, & Gallouzi, 2003). Moreover, the

knockdown of HuR completely inhibited myogenesis in C2C12 cells. This effect could then be rescued through the overexpression of HuR (van der Giessen et al., 2003).

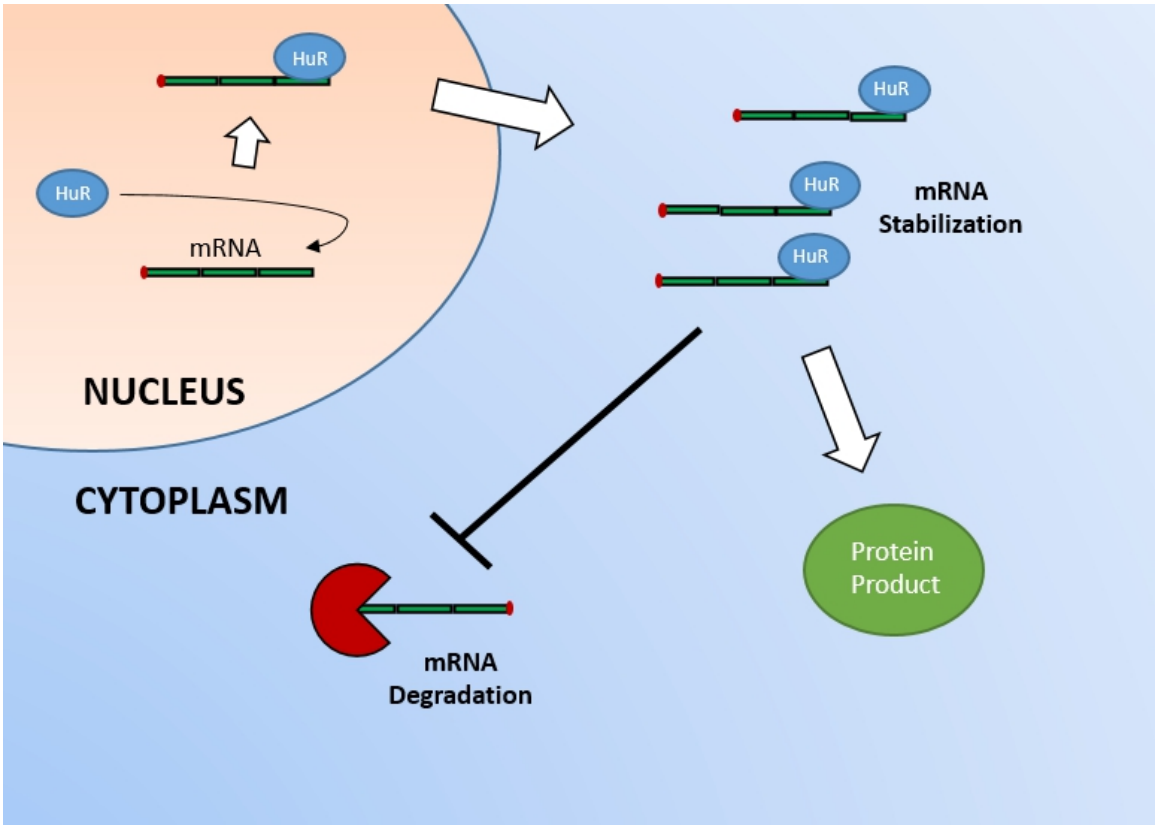


Figure 3: HuR in mRNA stabilization. Once activated through upstream signaling, HuR accumulates in the cytoplasm. This translocation may be activated through various different post-translational modifications in the HuR nucleocytoplasmic shuttling sequence (HNS). Cytoplasmic accumulation of HuR results in increased binding to mRNA targets on their AU-rich elements (AREs) in the 3'UTR.This binding stabilizes the associated mRNA by occupying the ARE and thus inhibiting ARE-mediated decay. mRNA half-life is then increased, resulting in a greater abundance of mRNA transcripts, thereby leading to increased protein levels.

Localization of HuR seems to be key during myogenesis. Studies have shown that HuR plays a role in aiding mRNA nuclear export through a CRM-1 dependent manner (I.-E. Gallouzi & Steitz, 2001; I. E. Gallouzi, Brennan, & Steitz, 2001). In muscle, HuR is predominantly expressed in the nucleus, and its translocation is correlated with muscle repair, regeneration or development (Figueroa et al., 2003; Joassard et al., 2015). Notably, between RRM2 and RRM3 is a hinge region which is less conserved amongst the Hu family members. It is well established that within this region, HuR contains a "HuR nucleocytoplasmic shuttling sequence" (HNS) housing both a nuclear localization signal as well as a nuclear export signal (Fan & Steitz, 1998a). Post-translational modifications within the hinge region of HuR have been shown to trigger the translocation of the protein, as summarized in Figure 4. For example, it was shown in HeLa and human mesangial cells that protein kinase C (PKC) phosphorylates HuR at S221, and this post-translational modification was found to be essential for the cytoplasmic translocation of the protein (Doller, Schlepckow, Schwalbe, Pfeilschifter, & Eberhardt, 2010; Kim, Yang, Kuwano, & Gorospe, 2008). It has further been shown that during muscle repair in mice, HuR levels and cytoplasmic localization are increased, and target mRNA levels are upregulated due to increased half-life as a result of HuR stabilization (Figueroa et al., 2003; Joassard et al., 2015). Mechanistically, these effects were shown to be regulated by the activation of p38 MAPK, which has previously been confirmed to phosphorylate HuR at T118 (with other possible targets at S202 and S221) (Joassard et al., 2015; Lafarga et al., 2009). How exactly these modifications result in translocations is not fully understood.

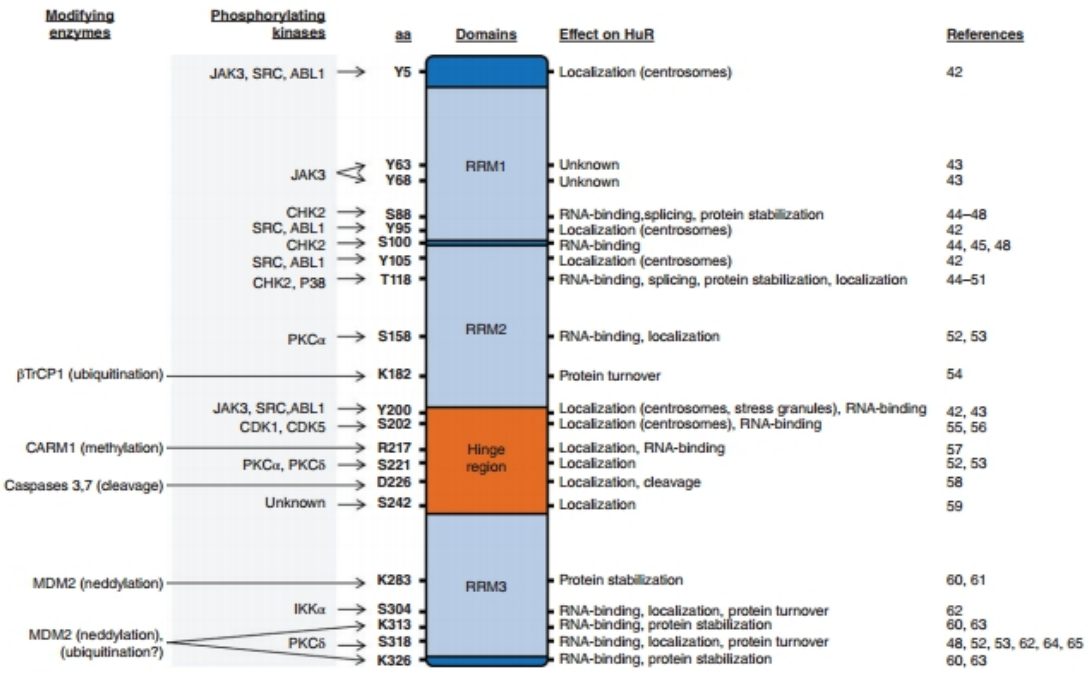


Figure 4: Post-translational modifications of HuR. Schematic depicting post-translational modifications and their proposed or hypothesized effect on HuR. (© Grammatikakis, Abdelmohsen, & Gorospe, 2017 by permission)

One proposed mechanism involves the cleavage of HuR by Caspase 3 and 7 (at site D226) to generate the cleavage product 1 (CP1, 24kDa) and the cleavage product 2 (CP2, 8kDa). The CP1 fragment has then been shown to bind and interfere with transportin-2 (a protein involved in the nuclear import of HuR), resulting in the accumulation of HuR in the cytoplasm. However, HuR-CP1 alone is not sufficient for the myogenic role that HuR plays, but rather HuR as well as its cleavage was required for myogenesis (Beauchamp et al., 2010). Another possible mechanism underlying HuR's nucleocytoplasmic shuttling is through protein-protein interactions with different import and export proteins. Such interactions may be governed through post-translational modifications as well. We cannot eliminate the possibility that both suggested mechanisms play a synergistic role to surmount to the overall observed cytoplasmic translocation of HuR. Further to these findings, HuR has been found to bind its own mRNA at the 3'UTR, which may possibly play part in the upregulation of total HuR levels seen during myogenesis and muscle repair (Srikantan & Gorospe, 2012; Yi et al., 2009).

Notably, HuR directly binds and stabilizes the SMN mRNA in neurons. SMN protein levels were found to increase in response to p38 activation, through the stabilization of SMN mRNA by HuR (F Farooq, Balabanian, Liu, Holcik, & MacKenzie, 2009; Faraz Farooq et al., 2013). Furthermore, activation of p38 in SMA mice, through administration of celecoxib by intraperitoneal injection (IP injection), lead to an increase in SMN protein levels in the spinal cord and brain. These treated mice exhibit an improved phenotype as reported through increased survival time, and improved motor function (Faraz Farooq et al., 2013). Together, these studies highlight the importance of HuR in the induction and regulation of SMN protein in neurons. However, the improved

phenotype in the mice cannot be attributed solely to the drug's effect within neurons. Through IP injection, many other tissues will have been targeted by the drug administration as well, insinuating the possibility that HuR also plays a similar key role in skeletal muscle. Furthermore, the aforementioned motor function improvements (that were tested through righting time experiments) could be attributed to both neuronal and muscular improvements in the treated mice.

1.10 *Smn*$^{2B/-}$ mouse line

Many different mouse models of SMA have been generated to date. Given that the mouse genome lacks the *SMN*2 gene, many of these models have an inserted *SMN*2 transgene as to produce base levels of SMN, resulting in a phenotype similar to that in human patients with severe SMA (U R Monani et al., 2000). In this study, we make use of the *Smn*$^{2B/-}$ mouse model on a mixed background, which has a less severe phenotype than the *Smn*$^{-/-}$;*SMN*2. These mice are generated through crossing an *SMN*$^{+/-}$ (C57BL/6) mouse with an *SMN*$^{2B/2B}$ (C57BL/6 x CD1) (M Bowerman et al., 2012). The 2B mutation is a 3 nucleotide substitution within the exon splicing enhancer region of *SMN* exon 7 (DiDonato et al., 2001; Hammond et al., 2010). These mice produce approximately 15% full length SMN protein in comparison to control mice. With a median life span of roughly 28 days, and initial visible phenotype at postnatal day 10, these mice display a slightly less severe phenotype than other severe models, allowing a more prolonged analysis of the disease progression (M Bowerman et al., 2012). *Smn*$^{2B/-}$ mice are well characterized to show reduced body weight, weaker muscle force during early development, reduced muscle cross-sectional area, progressive death of motoneurons,

defects in NMJ remodeling, and impairments in motor function (M Bowerman et al., 2012; Boyer et al., 2013; Murray, Beauvais, Bhanot, & Kothary, 2013).

1.11 Rationale and hypothesis

The SMN protein was previously shown to bind directly to the RNA-binding protein HuD in motoneurons. This interaction occurs through the Tudor domain of the SMN protein, and is thus abolished in SMA. It is facilitated by the methylation of arginine 248 (in the hinge region) by CARM1. Notably, this methylation is known to alter the RNA-binding affinity of certain HuD mRNA targets and plays a role in the capability of neurons to differentiate (Fujiwara et al., 2006; Hubers et al., 2011). SMN was further shown to co-localize and interact with SMN in the neurites of motoneurons, and is suggested to play a key role in the axonal transport and regulation of mRNAs essential for neuritogenesis (Akten et al., 2011; Fallini et al., 2011, 2016; Hubers et al., 2011; Sanchez et al., 2013). Strikingly, the overexpression of HuD was able to rescue SMA-like defects in SMN-knockdown motoneurons (Hubers et al., 2011). These studies suggest an important role for the regulation of the RNA-binding protein HuD in the mechanisms underlying SMA defects. However, such findings fail to explain the presence of motoneuron independent muscle intrinsic defects in SMA.

Given the high degree of conservation between HuD and HuR, and the fact that HuR is also methylated by CARM1 in the hinge region, we propose that HuR may play a similar key role in SMA. Furthermore, muscle intrinsic defects have been characterized, and HuR is the only member of the Hu family of proteins expressed in muscle. For these reasons, we postulate that HuR may be involved in the aforementioned muscle intrinsic

defects found in SMA. In muscle cells, HuR cytoplasmic translocation was found to be essential towards efficient myogenic gene regulation and concomitantly, efficient differentiation (Figueroa et al., 2003). Thus, we hypothesize that an SMN deficiency results in HuR functional defects in skeletal muscle, thereby affecting HuR's role in muscle development and repair. Since we suspect that, like HuD, HuR interacts with SMN through the Tudor domain at HuR's methylated arginine within the hinge region, we proceed in scrutinizing this methylation in myoblasts to uncover its potential role.

AIM I: Determining the functional role of the methylation of HuR by CARM1

Objectives:

- Confirm that there is an interaction between SMN and HuR.
- Characterize the impact of CARM1 methylation on HuR functionality in muscle cells.

We next progress towards analyzing HuR in an SMA mouse model. CARM1 levels are known to be upregulated in motoneurons of SMA mice, and as such, we propose that CARM1 may also be upregulated in SMA mice (Sanchez et al., 2013). Such an upregulation would suggest increased HuR methylation, possibly affecting HuR function in skeletal muscle. Previous findings have shown that HuR levels are upregulated in response to sciatic nerve denervation. HuR is activated and translocated to the cytoplasm where it has been found to bind mRNA targets, thereby increasing their stability (Joassard et al., 2015). We make use of this paradigm to explore HuR levels in its basal state during development, as well as its capacity to activate and translocate in response to injury via sciatic nerve denervation.

AIM II: Revealing whether HuR is impaired in *Smn*$^{2B/-}$ skeletal muscle

Objectives:

- Determine if CARM1 and HuR levels are altered in *Smn*$^{2B/-}$ mice
    - Changes in CARM1 would hint towards changes in methylation of HuR
- Determine if HuR's function during denervation is impaired

Such findings will provide a good indication as to whether HuR may be involved in the etiology of the SMA phenotype, and whether it is worth pursuing early postnatal studies on HuR functionality.

2) MATERIALS AND METHODS

2.1 Cell Culture

Mouse C2C12 myoblasts (ATCC/CEDARLANE Corporation - Burlington, Ontario) were plated in either 25mm, 100mm, 150mm, or 6well culture plates (Corning/VWR - Mississauga, Ontario). Cells were grown in growth medium which was comprised of Dulbecco's modified Eagle's medium (DMEM; Wisent Bioproducts - St.Bruno, Quebec) with 10% fetal bovine serum (Wisent Bioproducts - St.Bruno, Quebec), 1% penicillin/streptomycin (Hyclone/Fisher Scientific - Ottawa, Ontario), and 1% L-glutamine (Fisher Scientific/Hyclone - Ottawa, Ontario). Plated cells were maintained and incubated in a humidified incubator kept at 37^{O}C with 5% CO_2. Cell medium was replaced with fresh medium at least every 48h.

2.2 Cell differentiation

Myoblast differentiation was induced by incubating cells with differentiation medium comprised of Dulbecco's modified Eagle's medium (DMEM; Wisent Bioproducts - St.Bruno, Quebec) with 2% horse serum (Wisent Bioproducts - St.Bruno, Quebec), 1% penicillin/streptomycin (Hyclone/Fisher Scientific - Ottawa, Ontario), and 1% L-glutamine (Fisher Scientific/Hyclone - Ottawa, Ontario). For healthy myotube formation, culture plates were coated with Matrigel (VWR - Mississauga, Ontario). Differentiation medium was introduced to cells at ~80-90% confluency, after which they were incubated as previously described, and replaced with fresh medium every 48h.

2.3 Transfection

Using Lipofectamine with Plus reagent (Invitrogen/Fisher Scientific - Ottawa, Ontario), transfections were performed as per the provided instructions. Transfections were performed on cells at 50-70% confluency for a 6hour incubation period, using 1.5µg transfection reagent per 1µg of plasmid. Medium was then changed to regular growth medium followed by an overnight incubation.

2.4 Stable cell line

Stable cell lines expressing shCARM1 were generated by transfecting C2C12 myoblasts with an shRNA against CARM1 inserted within a pRS vector backbone (OriGene - Rockville, Maryland, United States). Cells that have uptaken the shCARM1 plasmid were then selected for using 2µg/mL puromycin (Sigma Aldrich - Oakville, Ontario) for

greater than 48hours. Individual clones were then isolated and grown to form colonies which were then tested and used for experimentation.

2.5 Animal Care, surgical denervations and dissections

Smn$^{2B/-}$ mice (provided by Dr. Kothary, OHRI, University of Ottawa), were bred and maintained at the University of Ottawa animal care facilities, according to the guidelines provided by the University of Ottawa, as well as the Canadian Council on Animal Care. Surgical denervations were performed on weaning day (day 21), on mice that were anaesthetized with 2-3% isoflurane. A 2-3mm portion of the left sciatic nerve was sectioned and removed as to prevent re-innervation. The open skin was then glued back together post-surgery. Dissections were performed 3 days post-denervation, and at various time points between P12 and P26 in non-denervated mice (as described in the results). Gastrocnemius muscles were dissected and quickly frozen down using liquid nitrogen for protein and RNA extraction. For immunofluorescent probing, muscles were covered in Tissue-Tek OCT Compound (VWR - Mississauga, Ontario), and frozen down in melting isopentane that was pre-cooled using liquid nitrogen. All samples were stored at -80ºC.

2.6 Protein extraction, purification and quantification for cell culture

Cells grown in culture plates were washed in sterile phosphate buffered saline (PBS; Sigma Aldrich - Oakville, Ontario). Cells were scraped in RIPA lysis buffer made of 50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP40, 0.1% SDS, and 0.5% sodium deoxycholate. A complete protease inhibitor cocktail (Sigma Aldrich - Oakville, Ontario)

was added to inhibit protein degradation. Scraped lysates were then freeze thawed between liquid nitrogen and a 37ºC water bath a minimum of 3 times, followed by centrifugation at 12,000 RPM for 10 minutes. Supernatants were transferred to new 1.5mL tubes, and stored at -20ºC. Proteins were then quantified using the BCA protein assay kit (Pierce/Fisher Scientific - Ottawa, Ontario) as per the provided instructions. Bovine serum albumin at varying concentrations was used to form a standard curve. Using a spectrophotometer, optical densities of samples were read at wavelength 562nm, followed by calculating the protein concentration of samples by interpolating values into the standard curve.

2.7 Protein extraction, purification and quantification for tissue samples

Dissected tissues were frozen, then crushed using a tissue pulverizer on dry ice, and collected into a 1.5mL tube. Protein was extracted using a muscle UREA extraction buffer made of 7M UREA, 2M Thiourea, 4M CHAPS, 100mM DTT, 125mM Tris-HCl, and a protease inhibitor cocktail (Sigma Aldrich - Oakville, Ontario). Samples were then pulse vortexed for 30min at room temperature, followed by a 20 minute centrifugation at 20,000g. Supernatant was transferred to a new tube and stored at -80ºC. Proteins were quantified using CB-X protein assay kit (G-Biosciences/Fisher Scientific - Ottawa, Ontario) as per the provided instructions. Bovine serum albumin at varying concentrations was used to form a standard curve. Optical density was read at wavelength 595nm using a spectrophotometer, and associated concentrations were calculated using the standard curve.

2.8 RNA extraction, RT-PCR and qPCR

RNA was extracted for both plated cells, and crushed muscle tissue using TRIzol extraction buffer (Sigma Aldrich - Oakville, Ontario) as per provided instructions. Samples were then quantified using a spectrophotometer. Samples were treated with DNAse I (Fisher Scientific - Ottawa, Ontario), followed by a reverse transcription (RT) PCR using All-in-one RT MasterMix (ABM/Applied Biological Materials - Richmond, British Columbia). Quantitative PCR was then performed on the formed cDNA using a SYBR Green PCR kit (Qiagen Canada - Toronto, Ontario). Primers used are shown below. All samples were normalized to 18S.

AChE
Fwd: 5'-CGGAGGCTCTCATCAATACTGG-3'
Rev: 5'-GGGACCCCGTAAACCAGAAAG-3'

AChR β-subunit
Fwd: 5'-CATCATCGCTCACCCCAC-3'
Rev: 5'-ACGGTCCACAACCATGGC-3'

Myogenin
Fwd: 5'-TCACATAAGGCTAACACCCAG-3'
Rev: 5'-GGAATTCGAGGCATATTATGA-3'

18S
Fwd: 5'-CGCCGCTAGAGGTGAAATC-3'
Rev: 5'-CCAGTCGGCATCGTTTATGG-3'

MyoD
Fwd: 5'-CCACTATGCTGGACAGGCAGT-3'

Rev: 5’-TGGCATGATGGATTACAGCG-3’

MAFbx
Fwd: 5'-AGCGACCTCAGCAGTTACTGC-3'
Rev: 5'-CTTCTGGAATCCAGGATGGC-3'

MuRF1
Fwd: 5'-TGTCTGGAGGTCGTTTCCG-3'
Rev: 5'-ATGCCGGTCCATGATCACTT-3'

p21
Fwd: 5’-CAGCGATATCCAGACATTCAGA-3’
Rev: 5’-CTCAGACACCAGAGTGCAAGAC-3’

2.9 Western blots

Quantified protein samples were boiled in loading buffer, and run on a 10% polyacrylamide gel. Once separated, the proteins were then transferred to a nitrocellulose membrane (GE Healthcare/VWR - Mississauga, Ontario) for either 2h at 100V, or overnight at 30V. This is followed by a full membrane stain using Ponceau S (Sigma Aldrich - Oakville, Ontario) to confirm successful transfer and similar loading in every lane. Membranes were then blocked in 5% milk in PBST (PBS with 0.1% tween) for a minimum of 30min. Next, membranes are incubated on a shaker with 1% milk in PBST, including our antibody of interest; HuR (1:500; Santa Cruz Biotechnology - Mississauga, Ontario), CARM1 (1:2000; Bethyl/CEDARLANE Corporation - Burlington, Ontario), SMN (1:2000; Bethyl/CEDARLANE Corporation - Burlington, Ontario), B-Actin (1:40000; Santa Cruz Biotechnology - Mississauga, Ontario), B23 (1:1000; Sigma

Aldrich - Oakville, Ontario), tubulin (1:1000; Sigma Aldrich - Oakville, Ontario). Antibodies were incubated either 1-2hours at room temperature, or overnight at -4ºC. Blots were washed several times in PBST, followed by a 1 hour incubation with the corresponding secondary antibody of interest, conjugated to horse radish peroxidase (HRP) or a fluorescent marker. ECL (Pierce/Fisher Scientific - Ottawa, Ontario) was used to expose and capture protein levels of the HRP conjugated secondary antibodies, whereas fluorescent imaging was scanned using the Odyssey Fc imaging system (LiCor – Lincoln, Nebraska, United States).

2.10 Immunofluorescent staining in cell culture

Cultured cells were washed in sterile PBS, then fixed in 1% formaldehyde, followed by another 3 PBS washes before storage at 4ºC. Shortly after, fixed cells were permeabilized for 10 minutes in PBS with 0.5% TX-100, then blocked for 10 minutes in a PTS blocking solution of PBS with 0.1% TX-100, and 1% BSA (Sigma Aldrich - Oakville, Ontario). Cells were then incubated for 1hr at room temperature with PTS solution containing the antibody of interest; HuR (1:50; Santa Cruz Biotechnology - Mississauga, Ontario), CARM1 (1:200; Bethyl/CEDARLANE Corporation - Burlington, Ontario), MF20 (1:1). Cells were washed 3 times for 10 minutes each using PBS, followed by a 1 hour incubation with a fluorescent secondary antibody while being shielded from light; Alexa 594 (1:500; Sigma Aldrich - Oakville, Ontario), Alexa 488 (1:500; Sigma Aldrich - Oakville, Ontario). Cells were washed in PBS 3 more times. Cells were then mounted with Vectashield with DAPI (MJS Biolynx/Cedarlane -

Burlington, Ontario), and covered with a cover slip of which the edges were sealed. Images were taken using an upright fluorescent microscope.

2.11 Immunofluorescent staining in tissue sections

Frozen gastrocnemius muscle samples were sectioned and placed on a microscopy slide (VWR - Mississauga, Ontario). Slides were then fixed in 4% paraformaldehyde, and washed in sterile PBS (Sigma Aldrich - Oakville, Ontario). Immunofluorescent probing was then done on the slides using a Mouse on Mouse kit (MJS Biolynx/Cedarlane - Burlington, Ontario), as per the provided instructions. Primary antibodies used are as follows: HuR (1:50; Santa Cruz Biotechnology - Mississauga, Ontario), Laminin (1:500; Sigma Aldrich - Oakville, Ontario). Sections were then mounted with Vectashield with DAPI (MJS Biolynx/Cedarlane - Burlington, Ontario) and covered with a cover slip of which the edges were sealed. Images were taken using an upright fluorescent microscope.

2.12 Immunoprecipitation

Cultured cells were washed with sterile PBS (Sigma Aldrich - Oakville, Ontario), scraped in fresh PBS. Samples were centrifuged at 800rpm and then resuspended and lysed for 30min (4ºC) in 1mL NP40 buffer made of 50mM Tris HCl pH 7.5, 150mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate. Lysed cells were then centrifuged, then added to precleared Protein A or G beads (Pierce/Fisher Scientific - Ottawa, Ontario) in NP40 buffer, along with either 4µg of HuR antibody (Sigma Aldrich - Oakville, Ontario), 3µg of SMN antibody (Bethyl/CEDARLANE Corporation -

Burlington, Ontario), or 3µg of IgG antibody (Sigma Aldrich - Oakville, Ontario). Samples were tumbled overnight. Beads were then centrifuged and washed several times in NP40 buffer before being boiled in loading dye and run on a 10% SDS-Page gel for western blot analysis.

2.13 Generation of HuR mutants

We made use of pcDNA3 myc-HuR as a backbone upon which we performed site directed mutagenesis to substitute arginine 217 to lysine or tryptophan. Primers for the mutation were designed using QuikChange Primer Design Program, and the mutagenesis itself was performed using the QuikChange Lightning mutagenesis kit (Agilent Technologies Canada - Mississauga, Ontario) as per provided instructions. Through PCR amplification, mutated HuR plasmids were generated and confirmed via sequencing at StemCore Laboratories (OHRI, Univeristy of Ottawa) for integrity.

2.14 Statistical Analyses and Randomization

Graphical data shown as mean values ±SEM. Statistical analysis performed using unpaired t-test as well as ANOVA methods. Newman-Keuls ANOVA post hoc test used for multiple comparisons. All analyses were performed in GraphPad Prism with an α-level of significance set to 0.05 for all comparisons.

Images were taken at random unless otherwise stated. Image analysis was confirmed through the blind analysis, of sample images, by a third party. Several fields from various different replicates of each experiment were confirmed to account for bias.

3) RESULTS

Given that HuD is methylated by CARM1, and this methylation seemingly plays a role in the defects seen in SMA motoneurons, we believe the RNA-binding protein HuR may also play an important role underlying the SMA defects, similar to that of HuD (Fujiwara et al., 2006; Hubers et al., 2011). Moreover, since HuR is a ubiquitously expressed protein, and not specific to motoneurons as is HuD, we hypothesize that HuR may be the culprit for the muscle intrinsic defects seen in the SMA pathology. Based on the interplay between HuD, CARM1 and SMN, and the fact that HuR is known to be methylated within the hinge region as well, we first set our focus to understand the relevance of the methylation of HuR in muscle cells.

3.1 AIM I: Determining the functional role of HuR methylation

3.1.1 SMN interacts with HuR in C2C12 myoblasts

Before delving into understanding the impact of HuR methylation in muscle, we first needed to confirm that HuR interacts with the SMN protein in muscle. To test this interaction, we performed co-immunoprecipitation experiments where we pulled down either HuR or SMN from C2C12 myoblasts, and probed for the interacting partner through western blot analysis. As shown in Figure 5, both reciprocal immunoprecipitations show the presence of the interacting partner, confirming that indeed, HuR and SMN do interact within C2C12 myoblasts. Through the pulldown of a GST-fused Tudor domain, it was confirmed that the binding occurs at the Tudor domain.

This binding was abolished with the SMA causing mutation E134K (Supplementary Figure 1).

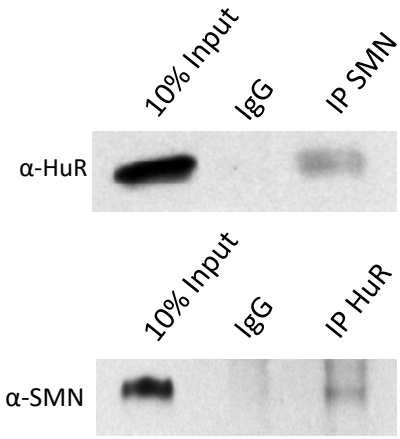


Figure 5: SMN and HuR interact in C2C12 myoblasts. Co-immunoprecipitation experiments were performed pulling down both HuR and SMN in proliferating C2C12 myoblasts. Western blot analyses were performed depicting 10% input in lane 1, IgG control IP in lane 2, and the protein of interest IP in lane 3. (N=3).

3.1.2 CARM1 knockdown has no impact on HuR levels or localization in C2C12 myoblasts

HuR is known to be methylated by CARM1, with the major methylation site at arginine 217, within the hinge region of the protein (Li et al., 2002). This methylation has further been shown to influence the ability of HuR to regulate the turnover of several mRNA targets in HeLa cells (Pang et al., 2013). It is also established that HuR localization is mainly nuclear in the myoblast phase, and during initiation of differentiation, HuR shuttles towards the cytoplasm of C2C12 cells (Figueroa et al., 2003). We proceeded to determine whether the methylation of HuR by CARM1 plays a possible role in the initiation of differentiation of C2C12s, specifically through modulation of HuR levels and localization as has been seen during differentiation. To do this, an shRNA targeting CARM1 was used to generate a stable shCARM1 cell line, through puromycin antibiotic selection in the C2C12 parental line. We generated an empty pRS vector stable cell line alongside the shCARM1 line, as a control. Protein lysates were collected and purified from these cultured myoblasts. We began by analyzing our cells in the myoblast proliferating phase to view if CARM1 has an impact on HuR basal levels during proliferation. Through western blot analyses, the knockdown of CARM1 was confirmed at a >90% knockdown, as shown in Figure 6A. Probing for HuR levels revealed unchanged levels of HuR in response to the CARM1 knockdown (Figure 6B), suggesting that CARM1 levels has no impact on HuR levels in C2C12 myoblasts.

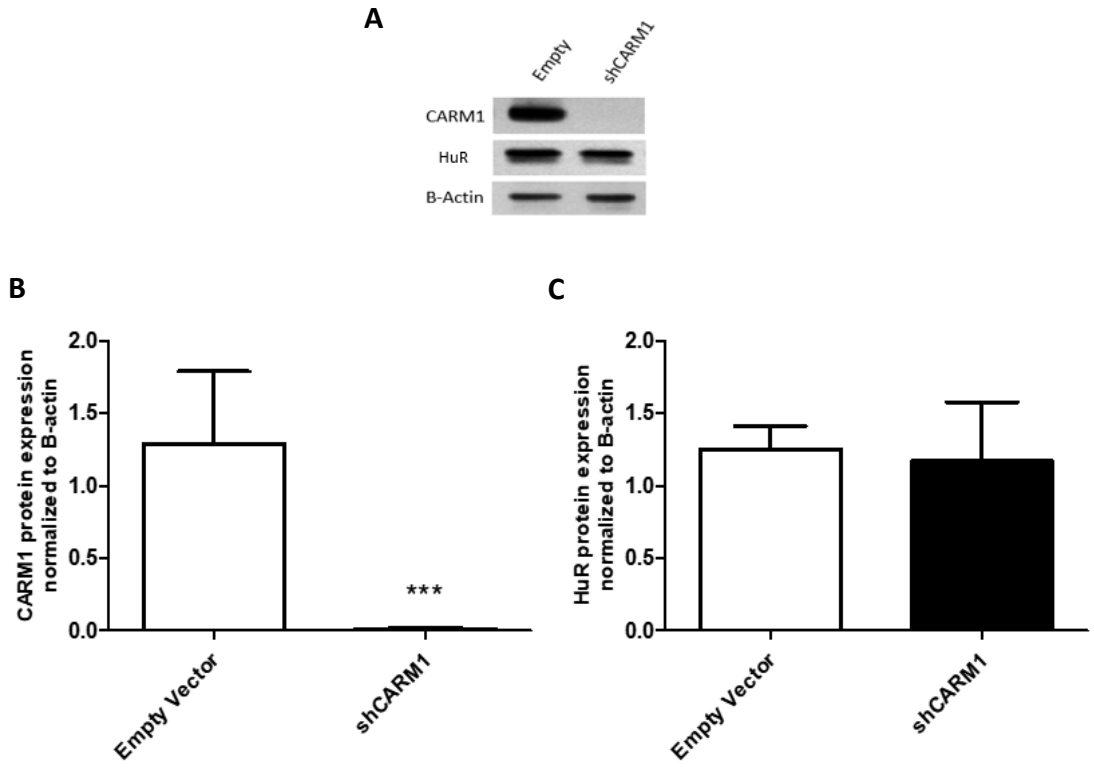


Figure 6: Knockdown of CARM1 has no effect on HuR protein levels in proliferating myoblasts. Protein extracts were collected from a stable line of C2C12 myoblasts expressing shCARM1, alongside our empty vector control. Through western blot analysis, HuR and CARM1 levels were measured. [A] Representative western blots are shown. [B] Quantitative expression of CARM1 levels are shown in a column graph confirming the successful knockdown of CARM1. [C] Graphical representation of HuR protein level quantifications. ($p<0.05$, $n=3$).

This is consistent with what was previously observed in HeLa cells where HuR levels were unchanged, however, levels of methyl-HuR were found to decrease by 80% (Pang et al., 2013).

To test if HuR levels remain constant but localization is changed, we used the same stable cell lines and fixed them in formaldehyde during the exponential proliferative stage. Immunofluorescent probing for both HuR and CARM1 were performed, allowing to depict whether the knockdown of CARM1 affects the HuR localization in C2C12 myoblasts. The CARM1 fluorescent signal was weaker in the shCARM1 line, as expected due to the knockdown, however, no difference was seen in HuR localization in the shCARM1 cells with respect to the empty vector control (Figure 7). In both the shCARM1 and the control, HuR localization was mainly nuclear and indistinguishable from one another. Thus, CARM1 levels do not directly affect HuR levels or subcellular localization in proliferating C2C12 myoblasts.

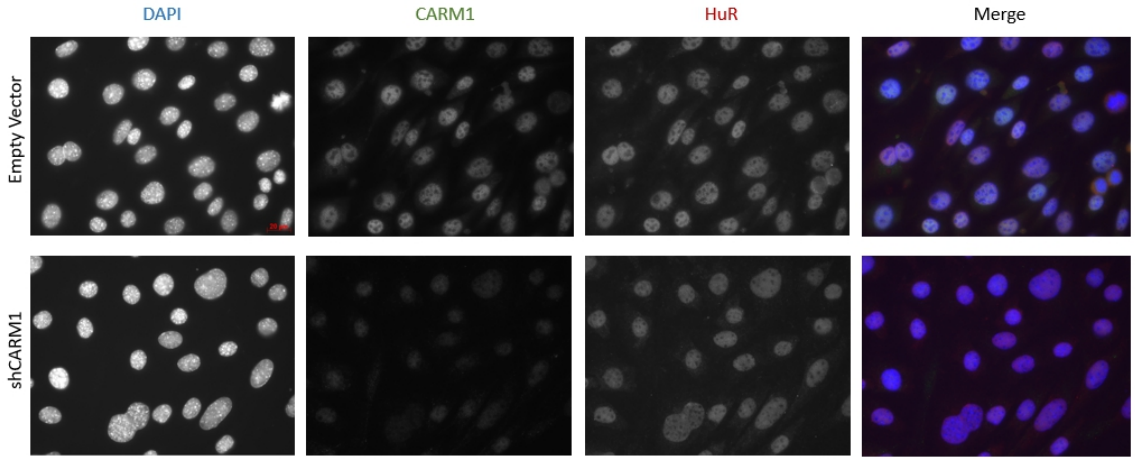


Figure 7: Knockdown of CARM1 has no effect on HuR localization in proliferating myoblasts. Immunofluorescent analysis of C2C12 myoblasts in the proliferative stage was performed for both our shCARM1 and empty vector control stable lines. DAPI staining was used to show the nuclei of cells in blue, CARM1 shown in green, and HuR localization shown in red. Representative images shown above. Experiment was replicated 3 separate times, each of which 5-7 random field of views were captured.

3.1.3 CARM1 is necessary for cytoplasmic localization of HuR during myogenesis

HuR is known to be mainly nuclear in proliferating cells, such as C2C12 myoblasts. However, in differentiating cells, HuR protein levels are known to be increased and shuttled cytoplasmically at 6h, followed by a gradual decrease to baseline levels (Figueroa et al., 2003). To test the possibility that CARM1 may play a key role during differentiation of myoblasts, we made use of the same stable shCARM1 cell line, along with its empty vector counterpart. Cells were plated and grown to ~90% confluency, upon which differentiation was initiated. Cells were harvested for protein at 0h, 6h, and 48h post initiation of differentiation. In agreement with the literature, the empty vector stable line shows a significant increase of HuR protein levels after 6hrs of differentiation, followed by a return to steady state levels at 48h of differentiation (Figure 8). In contrast, the deficiency of CARM1 prevented such an upregulation of HuR from taking place as our shCARM1 stable line maintained the same levels of HuR throughout the three time points.

It has previously been shown through subcellular fractionation that the major increase in HuR protein levels occurs in the cytoplasm. In order to test whether CARM1 plays a role in the subcellular localization of HuR, we performed immunofluorescent probing for HuR on 1 day (24h) differentiated myoblasts. In the shCARM1 stable line, HuR remains mainly nuclear with little accumulation in the cytoplasm (Figure 9). In the control line however, cytoplasmic accumulation of HuR protein is evident along the full length of the bipolar cell. In confirmation, Supplementary Figure 2 depicts that HuR-CP1 (expressed following initiation of differentiation) is only detectable in the presence of

CARM1. These results suggest that CARM1 is necessary for HuR cytoplasmic shuttling during differentiation.

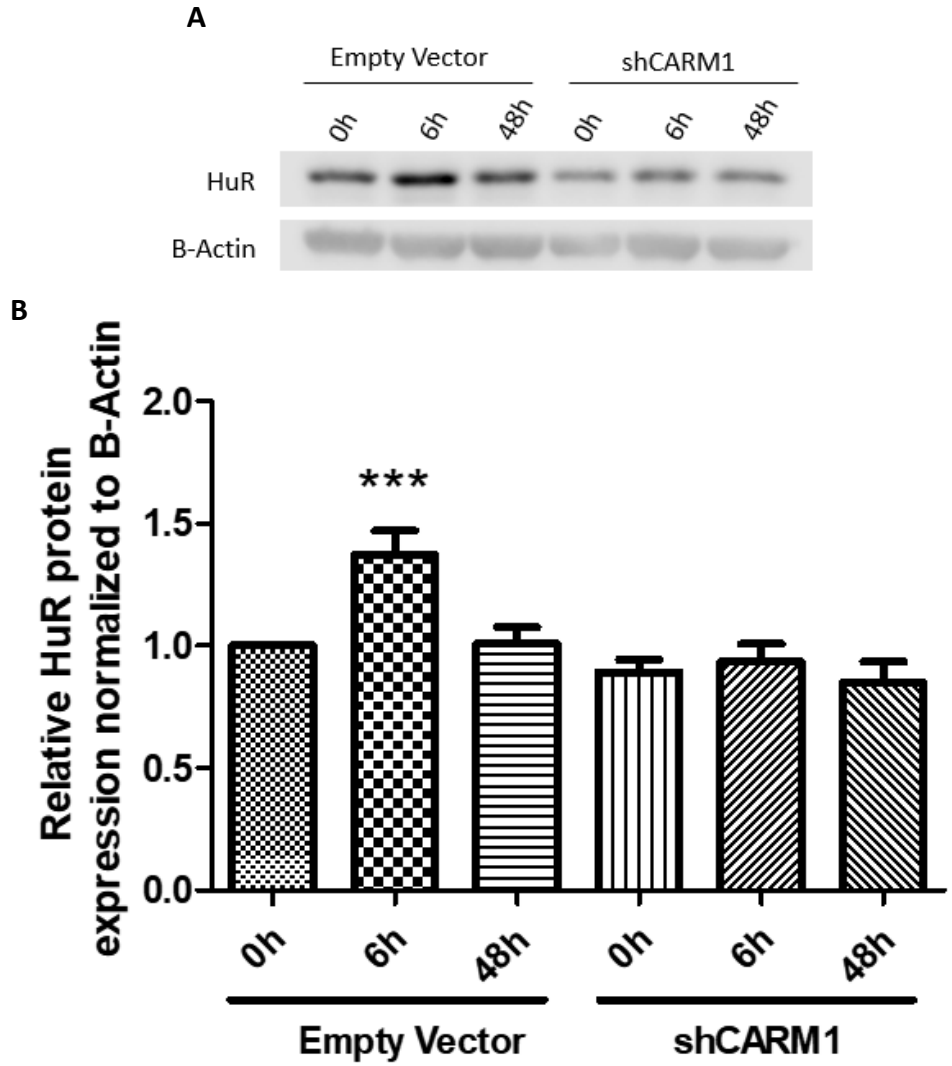


Figure 8: Knockdown of CARM1 impairs HuR upregulation during myogenic differentiation. Using our stable shCARM1 cell line, alongside our empty vector control, differentiation was induced for 0h, 6h or 48h. Protein extracts were collected and purified from each time point. Through western blot analysis, HuR protein levels were quantified. [A] A representative blot of HuR levels throughout differentiation is shown. [B] Graphical representation of quantified western blots is shown. (Statistical analysis performed using ANOVA, $p<0.05$, $n=6$).

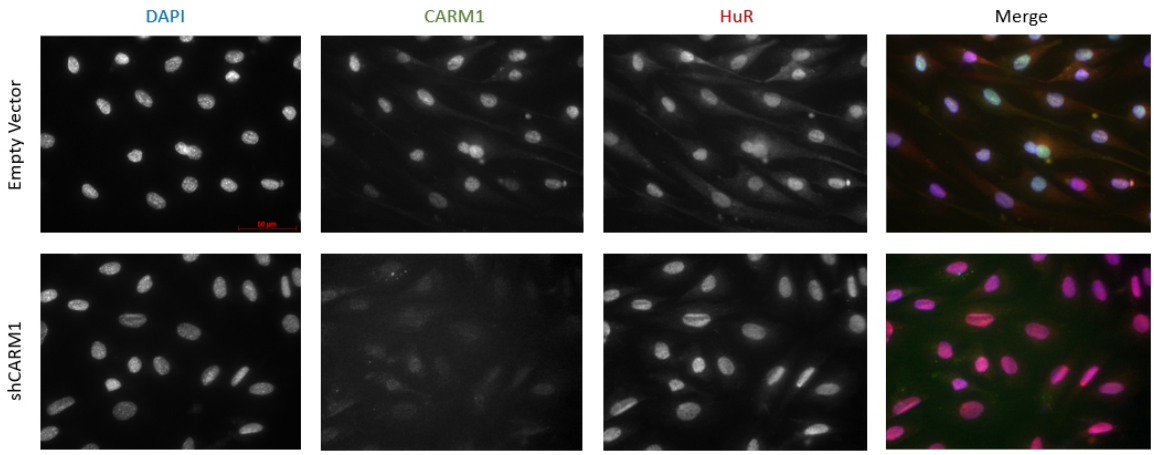


Figure 9: Impairment in HuR cytoplasmic translocation in response to knockdown of CARM1. Using our stable shCARM1 cell line, alongside our empty vector control, differentiation was induced for 24h. Cells were fixed, followed by immunofluorescent probing for CARM1 (green), HuR (red), along with DAPI staining to label the nuclei (blue). Representative images shown above. Experiment was replicated 4 separate times, each of which 5-7 random field of views were captured.

3.1.4 CARM1 is necessary for proper myotube formation

Given that CARM1 was shown to be necessary for both the upregulation and cytoplasmic localization of HuR, we continued to confirm that the knockdown of CARM1 results in defective myogenesis. It has previously been shown that by using the methyltransferase inhibitor adenosine dialdehyde that differentiation of C2C12 cells was impaired, and associated with deficient expression of Myogenin, MHC and MyoD (S. L. Chen, Loffler, Chen, Stallcup, & Muscat, 2002). We confirm here that indeed, after 4 days (96h) of differentiation, the shCARM1 stable line failed to form healthy myotubes in comparison to the control line (Figure 10A). The differentiation index (total percent of nuclei within the MHC staining) and fusion index (total percent of nuclei within the MHC staining, with 3 or more nuclei per myotube) were measured in both cell lines (Figure 10B). On both fronts, significant impairments were seen in the CARM1 deficient cell line. Therefore, sufficient CARM1 protein expression is necessary towards the formation of healthy myotubes.

CARM1 is known to methylate a great number of different targets, including histones. Furthermore, CARM1 is known for its role in transcriptional regulation of many genes. For this reason, it cannot yet be concluded that the regulation of HuR by CARM1 is the source of the myogenic defects noted. However, given that CARM1 is known to methylate HuR, that a deficiency in CARM1 prevents the upregulation of HuR and cytoplasmic localization following induction of differentiation, and the fact that a CARM1 knockdown inhibits myoblast differentiation, we postulate a causative role of the specific methylation of HuR.

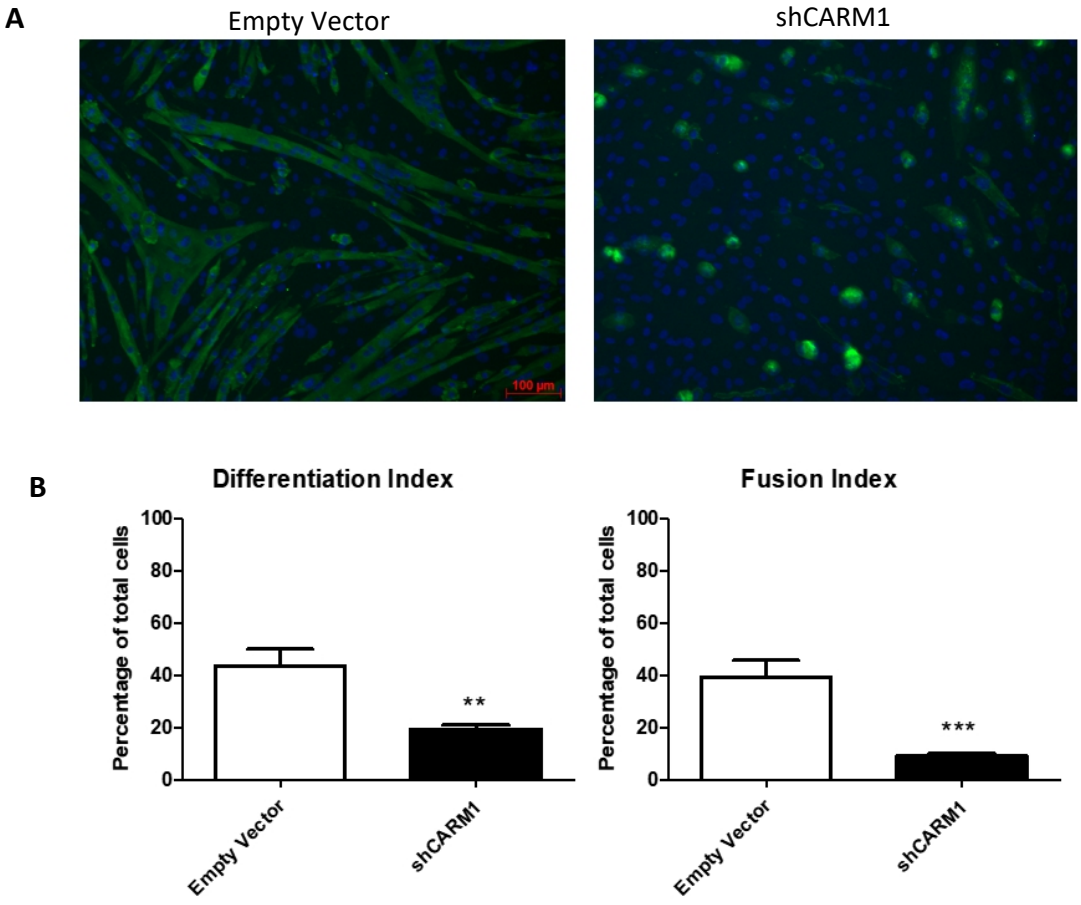


Figure 10: CARM1 is necessary for the fusion of myoblasts. We differentiated our shCARM1 stable cell line, alongside our empty vector line, for 4 days. Cells were then fixed followed by immunofluorescent probing for myosin heavy chain (MHC), marking the formation of myotubes. Images of 10 random fields of view were taken per plate of cells as to depict an unbiased and accurate representation of the overall plate. [A] Representative images of 4 day differentiated cells showing DAPI staining (depicting nuclei) in blue, and MHC in green. [B] Differentiation (1 cell or more within MHC staining) and fusion (3 or more cells within MHC staining) indices were counted and plotted graphically. (Statistical analysis using t-test, $p>0.05$, $n=6$).

3.1.5 HuR methylation is necessary for its cytoplasmic translocation during differentiation

In order to determine whether HuR methylation plays part in the myogenic defects seen in the previous experiments, we created HuR mutants that mimic the post-translational methylation (of arginine 217, in the hinge region), or the lack thereof. To do this, we cloned the mouse HuR gene into a pcDNA3 backbone as a control plasmid. We then generated two more variants of the vector, housing 1 to 2 point mutations to substitute the arginine 217 with either a tryptophan (methyl mimic) or a lysine (non-methylated mimic) as depicted in Figure 11A. The tryptophan substitution mimics the methyl group due to the bulky side chain and local hydrophobicity, similar to that of two methyl groups, whereas the lysine substitution prevents methylation while maintaining a positive charge (Hubers et al., 2011). The generated mimics contain a Myc-tag, allowing for mutant specific probing. As shown in Figure 11B, transfected C2C12 myoblasts successfully expressed our HuR mutants.

Through the use of these methyl mutants, we set forth to confirm the localization effects of HuR with respect to the methylation status. To do this, we overexpressed both methyl mutants of HuR in C2C12 myoblasts, alongside the overexpression of wild type (WT) HuR, and an empty vector. Following one day of differentiation, cells were fixed and probed for HuR. As shown in Figure 12, immunofluorescent images clearly indicate a drastic increase in cytoplasmic HuR levels in the HuR methyl mimic (R217W), whereas there was no perceivable change in the localization of the non-methyl mimic (R217K) when compared to controls.

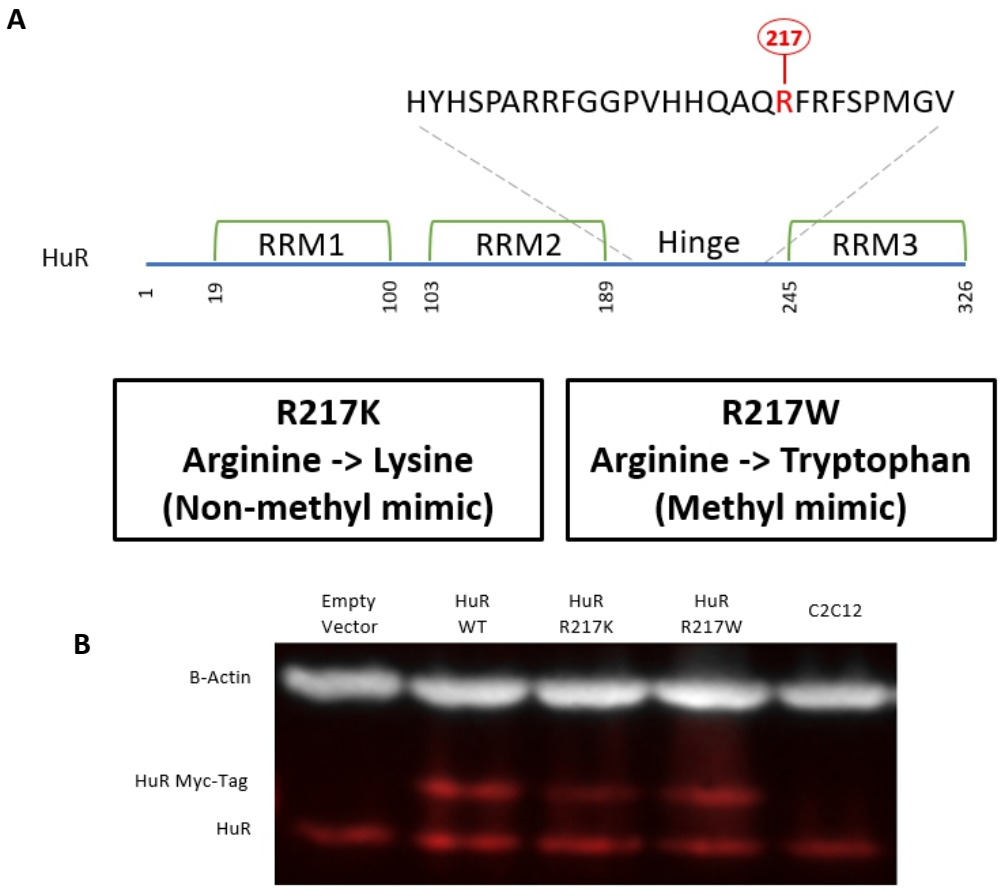


Figure 11: Generation of HuR methyl mutants. [A] HuR was cloned and mutated by substituting arginine 217 for either a tryptophan (R217W; methyl mimic) or a lysine (R217K; non-methyl mimic). Produced plasmids were sequenced to ensure accuracy of substitution. C2C12 cells were transfected with the generated mutants, alongside a wild type HuR (HuR WT) and an empty vector control. A myc tag was inserted into the sequence to allow for mutant probing. [B] Western blot analysis confirms the successful expression of HuR mutants in the transfected cells.

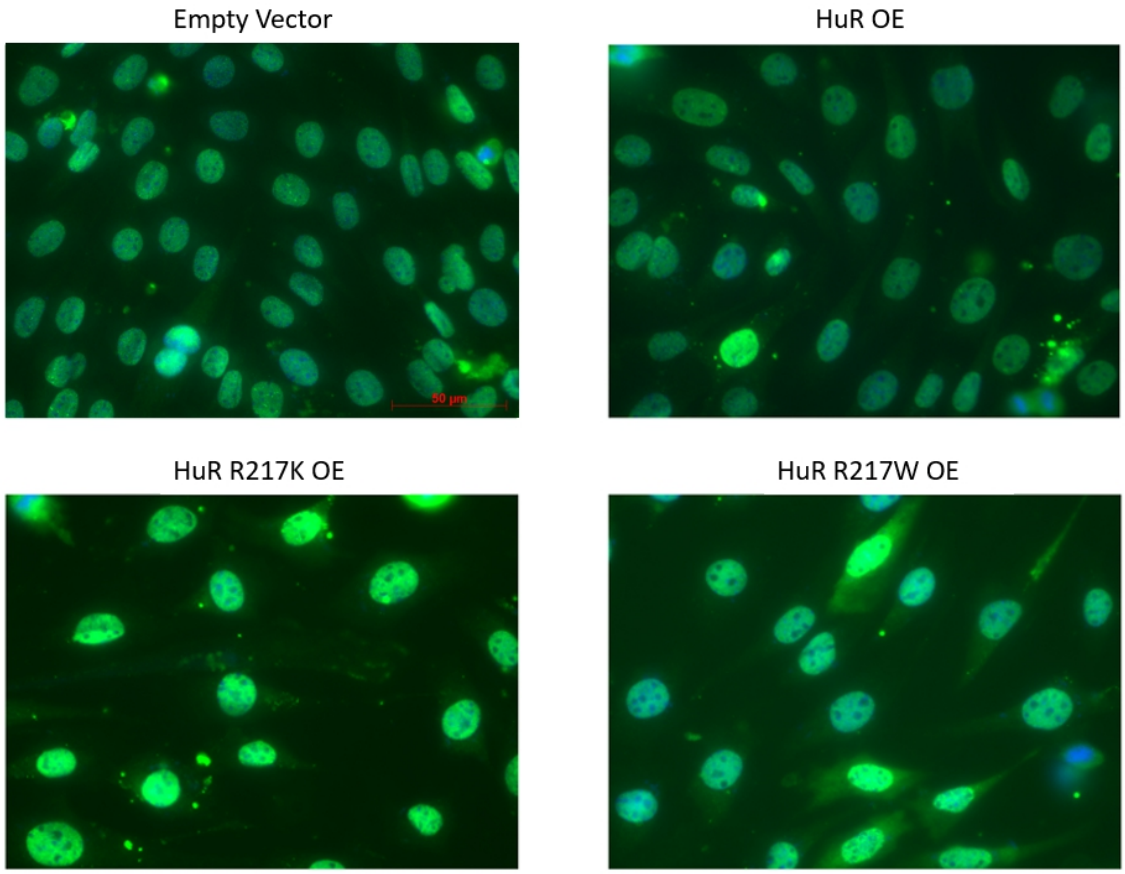


Figure 12: Methylation of HuR promotes its cytoplasmic localization during myogenic differentiation. C2C12 cells were transfected with our generated methyl-mimic HuR (HuR R217W), our non-methyl mimic (HuR R217K), a wild type HuR as control (HuR WT), and an empty vector control. Cells were then differentiated for 1 day, followed by fixation and immunofluorescent probing for HuR (green). Representative images are shown depicting the localization of HuR relative to its methylation state. Experiment was replicated 5 separate times, each of which 6-8 fields of view were captured.

Furthermore, upon four days of differentiation (Supplementary Figure 3), cells overexpressed with R217K revealed more patchy areas of inefficient myotube formation, whereas the R217W overexpression, as well as the two controls, show a greater distribution of fused cells forming healthier and longer myotubes. These results point towards the confirmation that HuR methylation plays a direct role in the translocation potential of the protein, and consequently, affecting the ability of cells to differentiate.

Finally, to confirm that the effects seen are due to the mutant HuR, and not a byproduct of unforeseen collateral experimental effects, we took advantage of the Myc-tag that has been engineered onto the HuR mutants. Cells were transfected with the mutants, followed by one day of differentiation before being fixed and probed for Myc-tag. Figure 13 confirms that indeed, methyl mimic HuR is expressed robustly in the cytoplasm, whereas non-methyl mimic HuR remains nuclear. In agreement with these findings, the WT HuR overexpression depicts the presence of both cytoplasmic expression, and lack thereof. Using line overlay fluorescent intensity quantification, we measured the expression levels of HuR along the length of the differentiating myoblasts. In red we measure the mutant HuR intensity, and marked in blue is the nucleus through fluorescent DAPI staining (Figure 14).

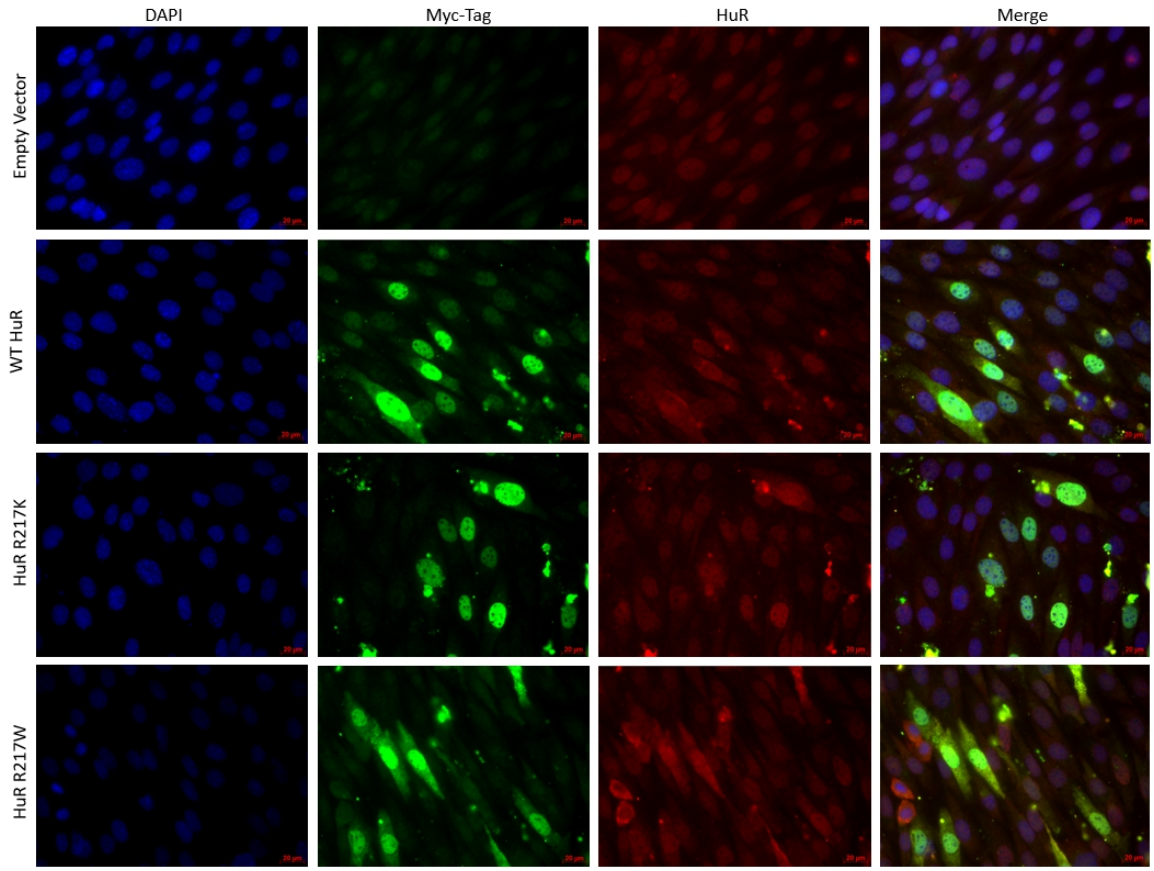


Figure 13: HuR methylation is necessary for cytoplasmic translocation. C2C12 cells were transfected with our generated methyl-mimic HuR (HuR R217W), our non-methyl mimic (HuR R217K), a wild type HuR as control (HuR WT), and an empty vector control. Cells were then differentiated for 1 day, followed by fixation and immunofluorescent probing for Myc-tag (green) as well as HuR (red). DAPI staining of nuclei is shown in blue. Localization of mutant protein expression is shown in the representative images. Experiment was replicated 3 separate times, each of which 7-10 random fields of view were captured.

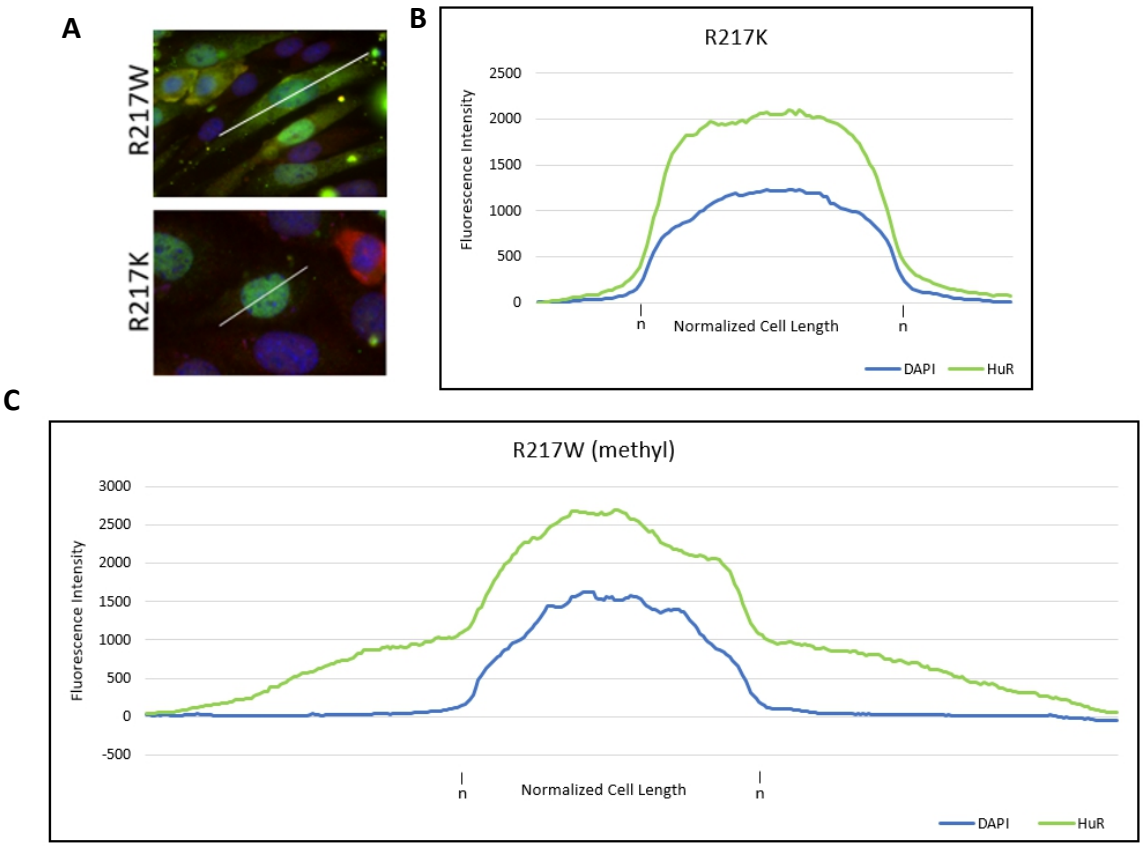


Figure 14: Quantification of cytoplasmic translocation of HuR in differentiating myoblasts. C2C12 cells were transfected with our generated methyl-mimic HuR (HuR R217W), our non-methyl mimic (HuR R217K), a wild type HuR as control (HuR WT), and an empty vector control. Immunofluorescent images were quantified using line overlay fluorescent intensity quantification. A line was drawn through each Myc-tag expressing cell in the field of view. We ensured the line traversed the middle of the nucleus and cytoplasm, along the length of the differentiating myoblast. The line ends slightly past where there was no longer any fluorescent intensity reading remaining (ie. end of Myc-HuR signal). Experiment was replicated 3 separate times, each of which 7-10 random fields of view were captured. Every Myc-expressing cell was measured in each field from the replicated experiments (~20 measured cells per experimental replicate). In green we show Myc-Tag HuR, and in blue we show DAPI to signify the bounds of the nucleus (depicted as "n" on the graphs). Intensity plots were normalized along the length of the nucleus, ensuring accurate depictions of intensity per subcellular region. Intensity readings were normalized to DAPI staining intensity in cytoplasmic region (ie. DAPI background fluorescence). Plots were merged together to form a total average plot depiction of fluorescent intensity per condition. [A] Representation of line draw in both the R217K and R217W transfected cells. [B] Graphical representation of line intensity quantification of the R217K transfected cells. [C] Graphical representation of line intensity quantification of the R217W transfected cells.

The length of each cell was normalized with respect to the length of the nuclear membrane, and the intensity was normalized as to ensure that the extracellular regions emit an intensity reading of zero (as to account for background staining). The intensity profile for each Myc-expressing cell per field of view (~20 cells per experimental replicate) were merged together from all 3 separate replications, to form an average intensity plot for each mutant. Quantitatively, it is evident that the R217W transfected cells exhibit a spread of HuR in the nucleus as well as the cytoplasm, with a fading intensity farther into the cytoplasm. In the R217K cells however, a drastic drop on the border of the nuclear fraction is observed, with negligible cytoplasmic fluorescent intensity. To more accurately quantify this, we performed subcellular fractionation under the same condition as previously described. Western blot analyses for the HuR mutants were performed and quantified as a ratio of cytoplasmic Myc-HuR over nuclear Myc-HuR, thus providing a measure of relative cytoplasmic distribution (Figure 15). It is evident that R217K mutants are less cytoplasmically localized, whereas R217W mutants show enhanced cytoplasmic localization. In 4 day differentiated cells using myc-tag staining, we show that R217W expressing cells group up and fuse together to form thick healthy myotubes, whereas the R217K expressing cells were few in number, and not found within myotubes (Figure 16).

Altogether, these results suggest an important role of the regulation of HuR by CARM1, likely through its methylation. We show not only that decreased CARM1 levels impairs the upregulation of HuR protein levels during differentiation, but also that the methylation of HuR is necessary for the cytoplasmic localization of HuR. Thus, we show that the methylation of HuR plays an important role in muscle cell differentiation. Given

the shown interaction between HuR and SMN at the Tudor domain, we further postulate the possibility of an intricate system where the interplay between HuR, CARM1, and SMN is essential for muscle development.

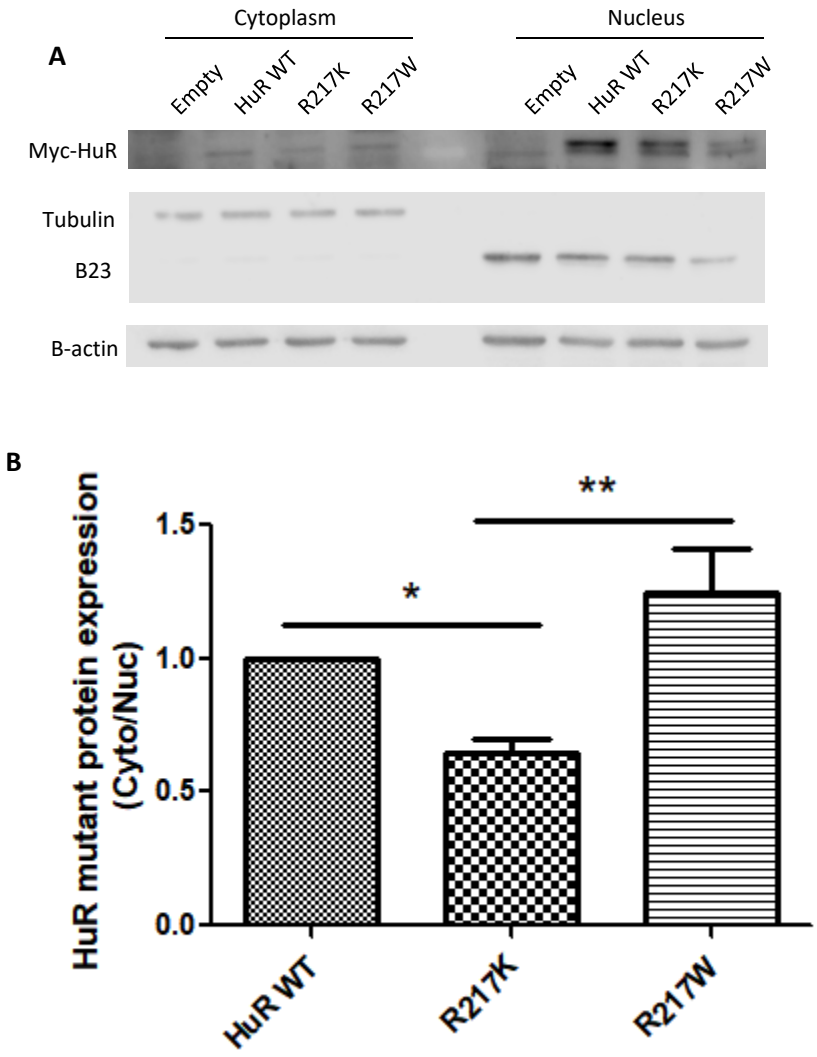


Figure 15: Subcellular fractionation of HuR mutants in 1 day differentiated myoblasts. C2C12 cells were transfected with our generated methyl-mimic HuR (R217W), our non-methyl mimic (R217K), a wild type HuR as control (HuR WT), and an empty vector control (Empty). Cells were differentiated for one day, followed by subcellular fractionation of the cells. Myc-HuR was quantified in both nuclear and cytoplasmic fractions through western blot analysis. [A] Representative blots shown above. B23 was used as a nuclear marker, and tubulin used as a cytoplasmic marker. B-actin shown to confirm equal loading in each well. [B] Graphical representation of the average mutant HuR expression shown as a ratio of cytoplasmic over nuclear expression. Statistical analysis performed using ANOVA. ($p<0.05$, $n=4$).

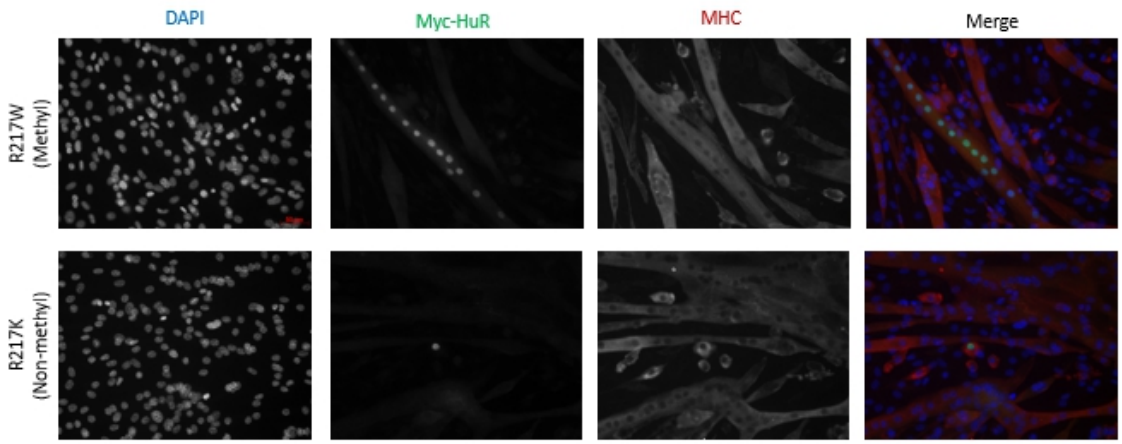


Figure 16: Methyl-HuR overexpression results in fusion and formation of healthy myotubes. C2C12 cells were plated and transfected with both R217K (non-methyl mimic) and R217W (methyl mimic) mutants of HuR. Cells were then differentiated for 4 days, followed by immunofluorescent probing for Myc-tag (green) and myosin heavy chain (MHC, red; depicting myotubes). DAPI (blue) was used to show the nuclei of cells. (n=2).

3.2 AIM II: Revealing whether HuR is impaired in *Smn*$^{2B/-}$ skeletal muscle

3.2.1 CARM1 levels are upregulated in *Smn*$^{2B/-}$ mice

Given our findings that HuR methylation is essential for myoblast differentiation, and that HuR interacts with SMN, we sought to explore the possibility that HuR is misregulated in SMA, possibly resulting in the muscle developmental defects that have previously been characterized. To do so, we made use of a mouse model of severe SMA known as the *Smn*$^{2B/-}$ mouse with a median survival time of ~28 days (M Bowerman et al., 2012). Previous studies have shown an increase in CARM1 levels in SMA mouse motoneurons (including *Smn*$^{2B/-}$) as well as SMA patient derived primary fibroblasts. This was suggested to be resultant of translational de-repression due to the lack of SMN protein (Sanchez et al., 2013). To test whether a similar upregulation of CARM1 protein levels is present in skeletal muscle of *Smn*$^{2B/-}$ mice, we harvested the proteins from the gastrocnemius muscles of the mice and probed for CARM1 through western blot analysis. We selected the gastrocnemius muscle to work with due to its comparatively large size relative to the other hind limb skeletal muscles, allowing us to easily harvest sufficient amounts of protein as well as RNA. Muscles were dissected at postnatal day 22, 24 and 26 as the disease has visibly progressed. As presented in Figure 17B, CARM1 levels at all time points analyzed were significantly upregulated (by more than 7 fold) in *Smn*$^{2B/-}$ mice in comparison to their control counterparts. Such an increase may have drastic effects on the regulation of CARM1 regulated genes, as well as CARM1-mediated methylation.

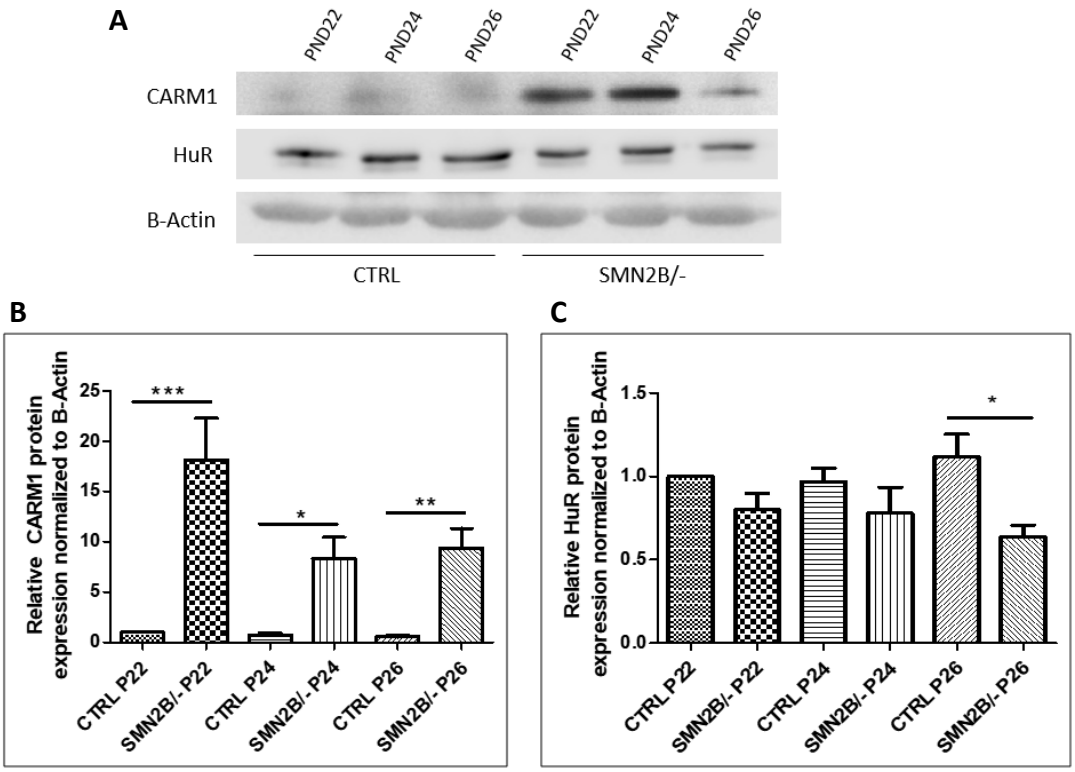


Figure 17: Upregulation of CARM1 levels, and no drastic change in HuR levels in Smn$^{2B/-}$ skeletal muscle. Gastrocnemius muscles of *Smn*$^{2B/-}$ mice and their controls were dissected at postnatal day 22, 24, and 26. Protein was extracted from the skeletal muscle, and subject to western blot analysis. We probed for both CARM1 and HuR protein levels. Quantifications were normalized to B-actin. [A] Representative blots shown. [B] CARM1 graphical representation of the western blot quantification. [C] HuR graphical representation of the western blot quantification. (Statistical analysis completed using ANOVA, $p<0.05$, n=5-7).

3.2.2 Lack of major change in HuR protein levels in *Smn*$^{2B/-}$ mice

In light of the upregulated CARM1 levels in *Smn*$^{2B/-}$ skeletal muscle, and the fact that CARM1 methylation had an impact on HuR upregulation, cytoplasmic localization and myogenic differentiation in C2C12 cells, we sought to assess whether the increase in CARM1 had an impact on HuR levels in the *Smn*$^{2B/-}$ mouse model as well. To do this, we performed western blot analyses on the same gastrocnemius muscles from before, this time probing for HuR. No significant changes in HuR levels were observed at 22 or 24 day time points, however a significant downregulation was observed at day 26 (Figure 17C). The issue remains that this down-regulation of HuR protein levels occurs only at the end stages of the disease, and as such cannot be attributable to the defects in muscle development that have previously been characterized. To ensure that an overt effect of HuR during earlier disease development is not missed, we performed a preliminary timewise analysis of *Smn*$^{2B/-}$ muscles at varying time points from postnatal day 12 – 20 (Figure 18). Western blot analysis revealed no major discrepancies between any of the time points, suggesting that no major event is being missed. Thus, a downregulation of SMN levels seemingly has no effect on HuR protein levels in skeletal muscle.

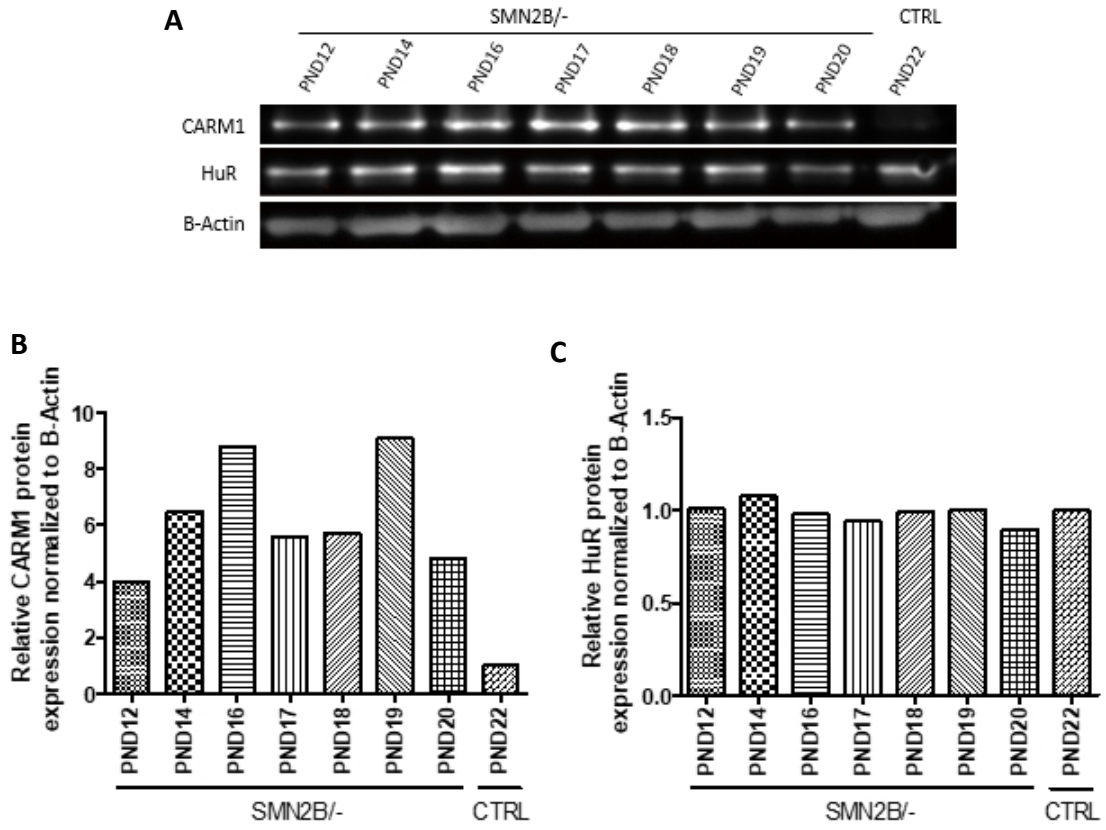


Figure 18: Temporal analysis of CARM1 and HuR in Smn$^{2B/-}$ mice. *Smn*$^{2B/-}$ gastrocnemius muscles were dissected at postnatal day 12, 14, 16, 17, 18, 19, and 20. Muscles were crushed and harvested for protein. Through western blot analysis (including a 22day control sample for comparison), protein levels of HuR and CARM1 were measured, and normalized to B-Actin. [A] Representative western blot shown. [B] Quantification of CARM1 protein levels throughout the various time points is shown graphically. [C] Quantification of HuR protein levels throughout the various time point is shown graphically. (No statistical analysis was performed. n=1).

3.2.3 HuR upregulation and translocation upon denervation is impaired in *Smn*$^{2B/-}$ mice

No overt changes in HuR levels were observed in these mice that could explain the muscle intrinsic defects during development. However, in culture we had determined that HuR levels were only affected by CARM1 levels after the initiation of differentiation. Accordingly, we must not neglect the possibility that baseline levels of HuR are unaffected, but the activation of HuR during development or denervation may be impaired in *Smn*$^{2B/-}$ mice. To test for this, we make use of a model of induced sciatic nerve denervation which has been shown to result in the upregulation of HuR, a shift towards cytoplasmic localization of HuR, and the regulation of HuR targets that are involved in the neuromuscular junction and muscle repair (Eftimie, Brenner, & Buonanno, 1991; Figueroa et al., 2003; Joassard et al., 2015; Michel, Vu, Tetzlaff, & Jasmin, 1994; van der Giessen et al., 2003). First, a small portion of the sciatic nerve of the left hind limb of both *Smn*$^{2B/-}$ and control mice was surgically excised (preventing re-innervation) at postnatal day 21 (at the time of weaning). The right leg was left intact, and was used as an innervated control. Denervation was visually confirmed in the following days as the mice were paralyzed in the left hind limb. We then dissected the gastrocnemius muscles of the mice at postnatal day 24 (or 3 days post denervation). Proteins were harvested from the muscles, followed by the comparative analysis of HuR protein levels through western blot analysis. Quantification of the blots demonstrated a clear upregulation of HuR protein levels in response to denervation in the control mice (Figure 19). Strikingly, the *Smn*$^{2B/-}$ muscles show no change in HuR protein levels in response to denervation, despite our aforementioned findings of upregulated CARM1 levels in these mice.

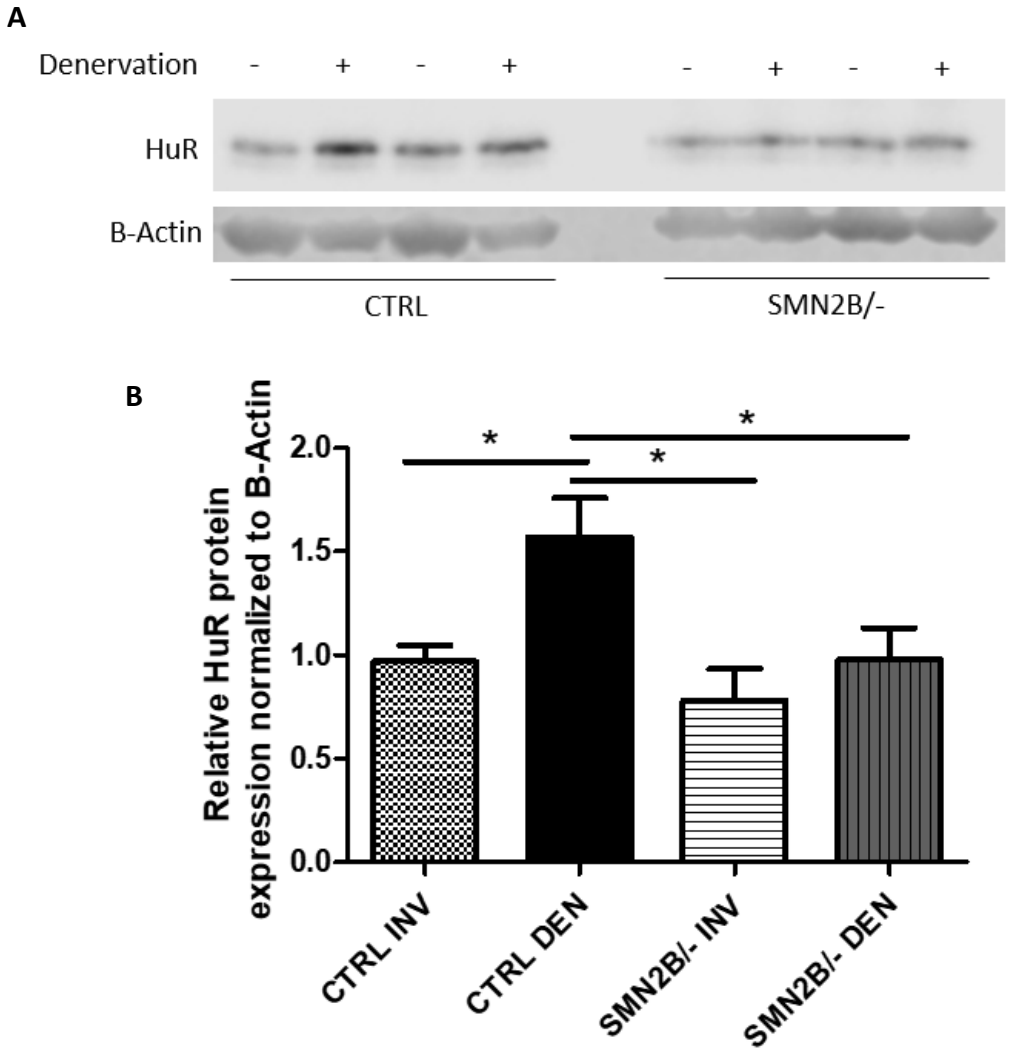


Figure 19: Impaired response of HuR to sciatic nerve denervation in Smn$^{2B/-}$ mice. Surgical sciatic nerve denervation was performed on the left hind limb of both control and *Smn*$^{2B/-}$ mice at day 21 post birth. Gastrocnemius muscles were then dissected 3 days post-denervation. Protein was extracted from the skeletal muscle, and subject to western blot analysis. We probed for both HuR protein levels, normalized to B-actin. [A] Representative western blot is shown. [B] HuR graphical representation of the western blot quantifications is shown. (Statistical analysis completed using ANOVA, $p<0.05$, n=6-8).

Given the defective upregulation of HuR in response to denervation, we predicted that HuR cytoplasmic translocation would also demonstrate signs of impairment. In order to assess this, denervations were surgically performed as previously described, followed by dissections 3 days post denervations. Dissected muscles were then sectioned and subjected to immunofluorescent probing for HuR protein (green). Laminin staining (red) was used to depict the cytoplasmic membrane, and DAPI (blue) staining to depict the nuclei (Figure 20A and zoomed in shown in Figure 20B). While the control denervated muscle expressed increased levels of cytoplasmic HuR, the *Smn*$^{2B/-}$ showed no visible difference between innervated and denervated expression patterns (both of which resemble the innervated control). The average fluorescence intensity of cytoplasmic HuR expression was counted from 5 fields of view per muscle cross section (3 sections per muscle). The average of these values were taken and plotted graphically. This analysis was repeated for all 3 experimental replicates. Figure 21 quantitatively depicts the overall average cytoplasmic HuR intensity of muscle fibers (n=3). Control denervated muscles show a significant increase in cytoplasmic translocation, whereas no change was seen within the *Smn*$^{2B/-}$ muscles. These results are in agreement with the lack of increased HuR protein levels in response to denervation.

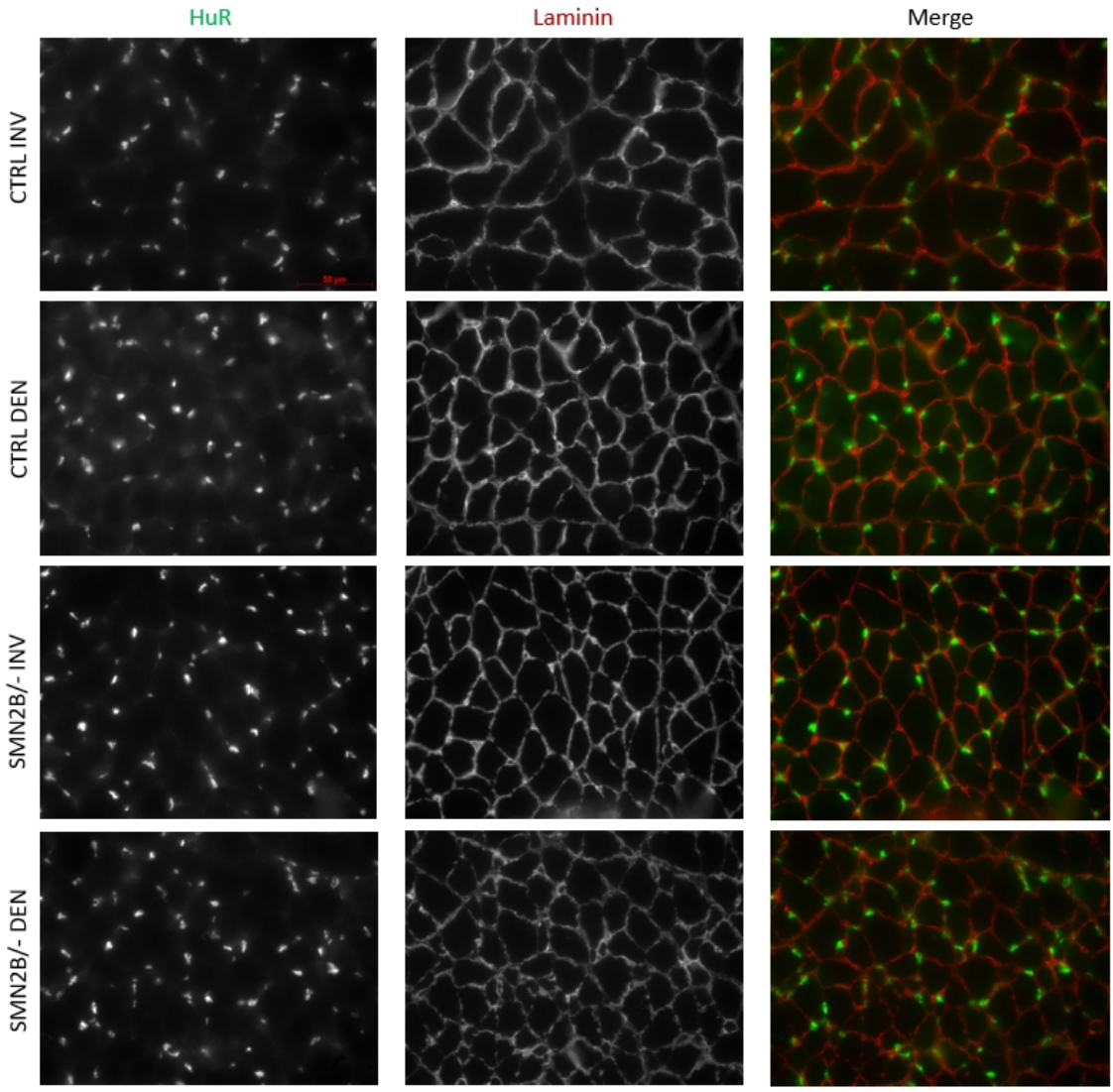


Figure 20A: Smn$^{2B/-}$ mice show impaired HuR cytoplasmic translocation in response to denervation. Surgical sciatic nerve denervation was performed on the left hind limb of both control and *Smn*$^{2B/-}$ mice at day 21 post birth. Gastrocnemius muscles were then dissected 3 days post-denervation. Cross-sections were cut, followed by immunofluorescent probing for HuR (green) and Laminin (red, to depict the outer membrane of muscle fibers). Images of 5 different fields of view were taken. 3 separate experimental replicates were performed. Representative images shown.

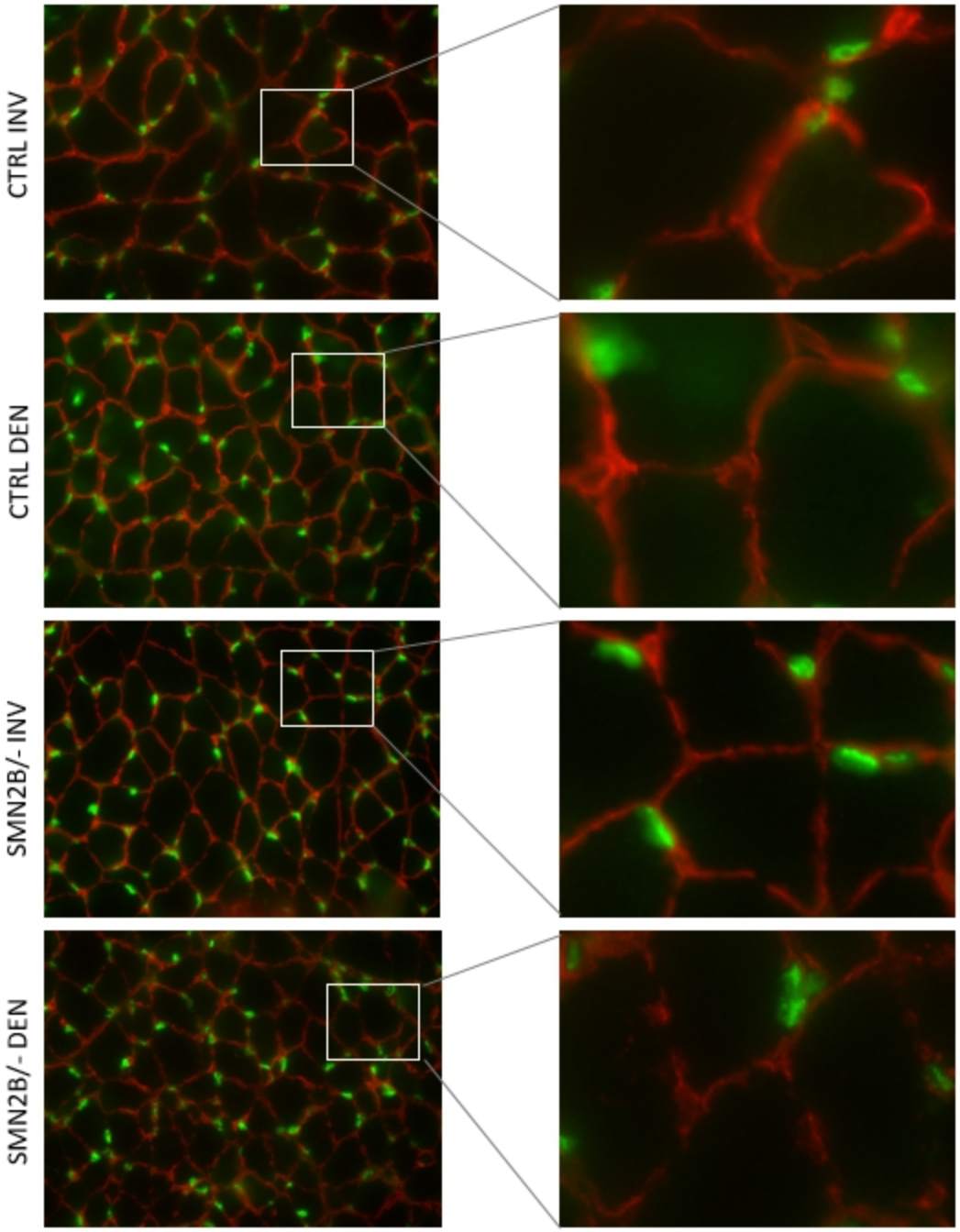


Figure 20B: *Smn*$^{2B/-}$ mice show impaired HuR cytoplasmic translocation in response to denervation. (Zoom). Surgical sciatic nerve denervation was performed on the left hind limb of both control and *Smn*$^{2B/-}$ mice at day 21 post birth. Gastrocnemius muscles were then dissected 3 days post-denervation. Cross-sections were cut, followed by immunofluorescent probing for HuR (green) and Laminin (red, to depict the outer membrane of muscle fibers). Images of 5 different fields of view were taken. 3 separate experimental replicates were performed. Representative images shown.

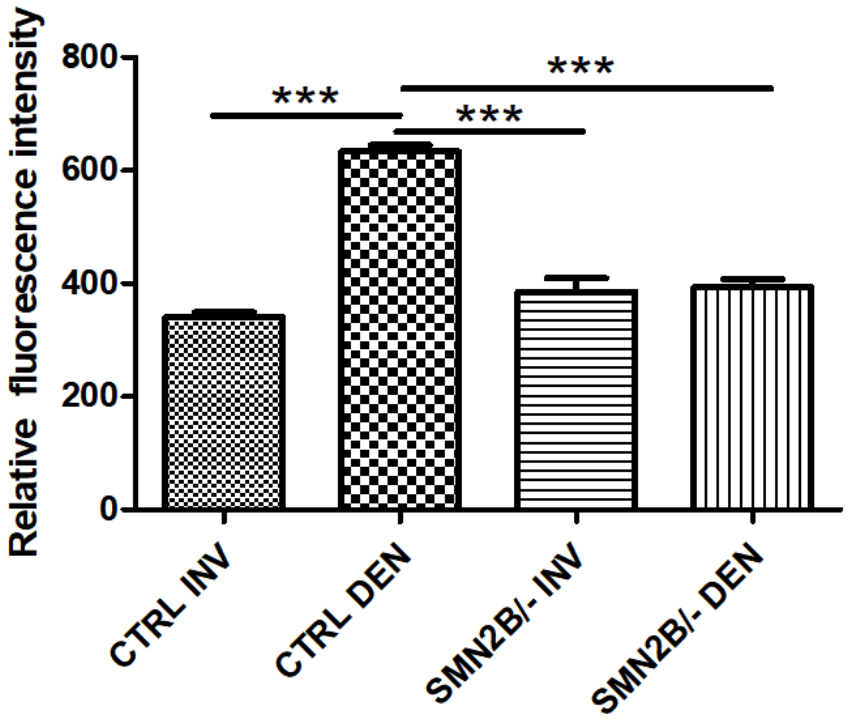


Figure 21: Smn$^{2B/-}$ muscles show no increase in HuR cytoplasmic translocation in response to denervation. Surgical sciatic nerve denervation was performed on the left hind limb of both control and *Smn*$^{2B/-}$ mice at day 21 post birth. Gastrocnemius muscles were then dissected 3 days post-denervation. Cross-sections were cut, followed by immunofluorescent probing for HuR (green) and Laminin (red, to depict the outer membrane of muscle fibers). Average fluorescence intensity of cytoplasmic HuR expression (from ~20 cells) was analyzed from 5 fields of view from 3 sections per muscle. The average of these values were taken and plotted graphically. This analysis was repeated for all 3 experimental replicates. (Statistical analysis using ANOVA, $p<0.05$).

3.2.4 HuR mRNA targets show signs of misregulation in *Smn*$^{2B/-}$ mice

HuR was previously found to bind and stabilize the AChR-β subunit in denervated muscles, thereby resulting in an overall increase in AChR-β mRNA (Joassard et al., 2015). Given HuR's role in mRNA stabilization upon denervation, along with our results showing defects in HuR function in the *Smn*$^{2B/-}$ mice, we set forth to explore the possibility that HuR mRNA targets are also misregulated in response to denervation. We selected genes that are known to be bound at the 3'UTR by HuR, and that are known to be regulated in response to injury. Two well established E3 ubiquitin ligases that are key players in skeletal muscle atrophy were also analyzed. mRNA transcript levels of the myogenic factors MyoD, Myogenin, as well as acetylcholinesterase (AChE), p21, AChR-β, and the two ubiquitin ligases MaFbx, and MuRF1 were assessed through qPCR analysis. The myogenic factors Myogenin and MyoD have both been shown to be upregulated in response to denervation, along with p21, AChR-β and the two ubiquitin ligases, and finally AChE has been shown to be downregulated (Sue C Bodine & Baehr, 2014; Eftimie et al., 1991; Ishido, Kami, & Masuhara, 2004; Joassard et al., 2015; Michel et al., 1994).

First, to see how atrogene mRNA levels compare to previous literature, we measured both MAFbx and MuRF1 at postnatal day 21. It has been found in whole hind limb lysates that mRNA levels of both atrogenes are upregulated in *Smn*$^{2b/-}$ mice (Deguise et al., 2016). Shown in Figure 22, gastrocnemius muscles lysates of the same mice show a trend towards increased MAFbx levels, and significantly increased MuRF1 levels.

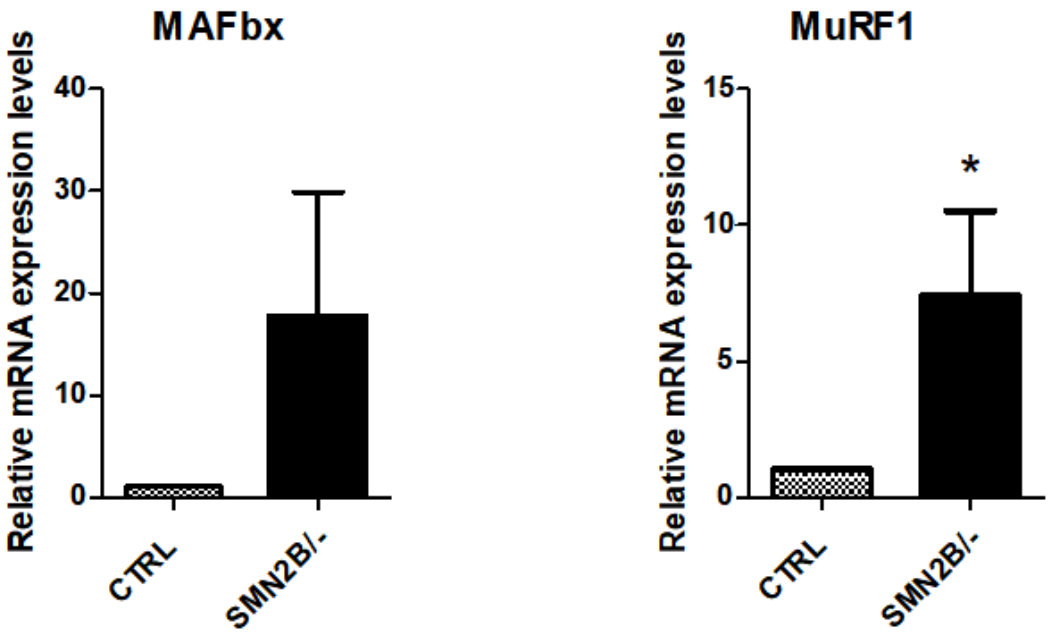


Figure 22: Increased mRNA expression of atrogenes in $Smn^{2B/-}$ mice. Gastrocnemius muscles were dissected at postnatal day 21. Following RNA extraction, purification and RT-PCR, qPCR analyses of the atrogenes MAFbx and MuRF1 were performed. Graphical representation of results shown. Values normalized to 18S. (Statistical analyses performed using t-test, $p<0.05$, n=3-5).

Next, qPCR analysis on muscles dissected 3 days post denervation (surgical sciatic nerve denervation at PND21) revealed defective regulation of certain mRNA targets in *Smn*$^{2B/-}$ mice. Myogenin and MaFbx mRNA transcripts responded normally to denervation in *Smn*$^{2B/-}$ mice as transcript levels are significantly upregulated in the denervated muscles of the mice, to the same level as that of control denervated muscles (Figure 23). However, denervated muscles of control mice show significant increases in MyoD, p21, AChR-β, MyoD, and a significant decrease in AChE, whereas the denervated *Smn*$^{2B/-}$ muscles show no significant change in these transcript levels with respect to their innervated control leg. These findings are suggestive of some level of impairment in the regulation of key skeletal muscle mRNA transcripts in the *Smn*$^{2B/-}$ mice, implying that the lack of HuR response to denervation may indeed play an essential role in this misregulation. The resultant impaired gene regulation during times of muscle repair or remodeling provides a plausible explanation for the phenotypic gradual muscle atrophy observed.

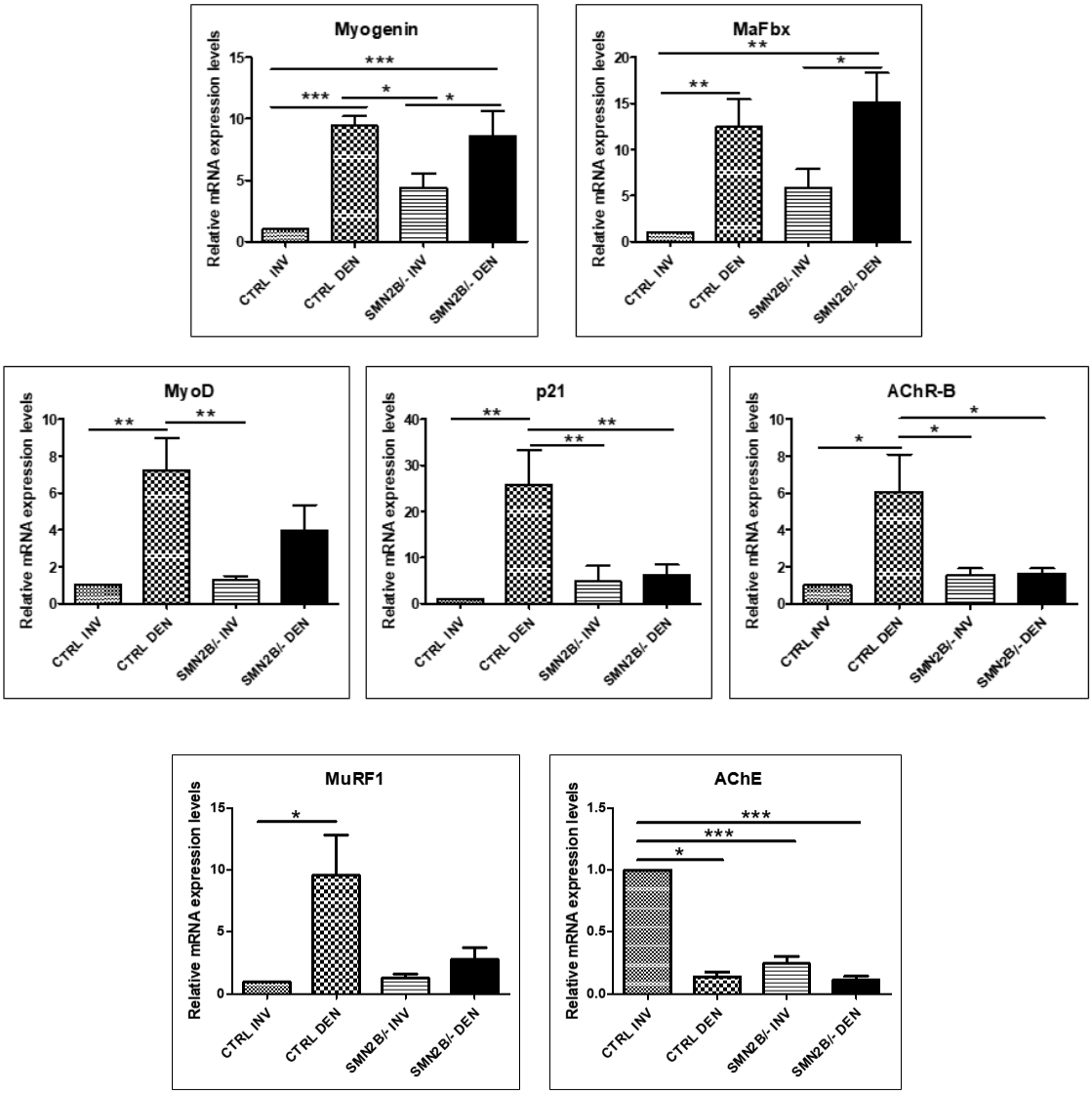


Figure 23: Aberrant expression of mRNA targets in Smn$^{2B/-}$ mice in response to denervation. Surgical sciatic nerve denervation was performed on the left hind limb of both control and *Smn*$^{2B/-}$ mice at day 21 post birth. Gastrocnemius muscles were then dissected 3 days post-denervation. Following RNA extraction, purification and RT-PCR, qPCR analyses of Myogenin, MyoD, AChE, AChR-β, p21, MaFbx and MuRF1 were performed. Graphical representation of results shown. Values normalized to 18S. (Statistical analyses performed using ANOVA, $p<0.05$, n=4-6).

4) DISCUSSION

We have discovered that CARM1 is necessary for HuR's myogenic activity during muscle cell differentiation. Knockdown of CARM1 resulted in the impairment of HuR's upregulation and cytoplasmic translocation upon initiation of myoblast differentiation. Furthermore, we have shown specifically that the methylation of HuR, enhanced HuR's cytoplasmic shuttling during differentiation. Moreover, we provided evidence for an interaction between HuR and SMN proteins in myoblasts. Importantly, in the skeletal muscles of $Smn^{2B/-}$ mice we revealed that CARM1 levels were upregulated, suggestive that the previously published translational de-repression of CARM1 is not limited to motoneurons within the spinal cord (Sanchez et al., 2013). We also observed defects in HuR upregulation and translocation in response to denervation, as well as aberrant expression of HuR mRNA targets, suggestive of defects in muscle repair and remodeling. Taken together, this study provides evidence towards the possibility of a key role of HuR in muscle intrinsic defects within an SMA model.

4.1 Methylation of HuR by CARM1 and its implications in myoblasts

4.1.1 Cooperation between CARM1 and HuR in muscle cells

In this study, we began by demonstrating that myoblasts in the proliferative stage displayed no overt signs of defective functionality in the expression or localization of HuR given a knockdown of CARM1. This is in agreement with the literature as no relationship between CARM1 levels and the steady state health of proliferating muscle

cells has been established to date. One study had revealed that the methylation of the regulatory factor Pax7 in satellite cells, by CARM1, directly regulated Pax7 binding activity. Pax7 was then found to result in downstream transcriptional activation of Myf5, promoting the initiation of the myogenic program (Kawabe et al., 2012). Other studies have placed the focus on myoblast differentiation, as it has previously been described that CARM1 is implicated in the transcriptional regulation of late myogenic genes (Dacwag et al., 2009). In 2002, it was determined that CARM1 plays an essential role in the differentiation of myoblasts. More specifically, CARM1 was shown to possess the ability to directly regulate myogenic factors through the methylation of its targets. This was suggested due to the discovery that blocking methyltransferase activity would inhibit myoblast differentiation, and that CARM1 interference caused the misregulation of certain essential myogenic factors (S. L. Chen et al., 2002). In our study, we confirmed the inhibition of muscle cell fusion in response to a knockdown of CARM. We further revealed that given a deficiency of CARM1 levels, the function of the RNA-binding protein HuR was defective during differentiation.

It has been shown that the RNA-binding protein HuR is upregulated upon initiation of differentiation in muscle cells, followed by a cytoplasmic accumulation. This is accompanied by the increased of activity in stabilizing target mRNAs that are necessary for myogenesis through ARE-mediated stabilization (Figueroa et al., 2003). We propose that during a deficiency of CARM1 protein levels in myoblasts, the link between the misregulated myogenic factors throughout differentiation is due, at least in part, to the defective HuR functionality. HuR depletion leads to a reduction in Myogenin, and MyoD mRNA expression levels, alongside the inhibition of muscle cell

differentiation (van der Giessen et al., 2003). Here, we found that the knockdown of CARM1 results in the impairment of the upregulation of HuR protein levels in response to the induction of differentiation. This defect in HuR regulation was associated with the lack of cytoplasmic translocation as well, suggestive of the overall impairment of HuR's myogenic role within these cells. A lack of HuR myogenic activation would result in decreased stability of the mRNAs of myogenic genes, and thus decreased protein expression of the essential myogenic factors, ultimately resulting in impaired myogenic differentiation as was observed through the knockdown of CARM1 (Buckingham et al., 1994). However, there remains the possibility that the knockdown of CARM1 is simply slowing down the differentiation process, resulting in aberrant HuR expression and translocation. Inhibition knockdown of CARM1 or HuR, or the inhibition of methyltransferase activity have all been shown to result in impaired myogenesis (S. L. Chen et al., 2002; Figueroa et al., 2003; van der Giessen et al., 2003). Furthermore, CARM1 knockdown has been shown to directly affect HuR's ability to stabilize mRNAs, without altering HuR levels (Pang et al., 2013). To make a causative link between HuR regulation by CARM1, we turned our focus to methyl-mutants of HuR to specifically target the post-translational regulation of HuR by CARM1.

4.1.2 Methylation of HuR is necessary for proper HuR function during differentiation

No mechanism has been resolved to fully clarify whether CARM1 directly regulates transcription of early myogenic genes or rather if it is by means of post-transcriptional regulation. We believe that CARM1 is implicated in both pre- and post-transcriptional regulation of myogenic genes. One of CARM1's well known targets of

methylation is histone H3, and it has been shown that some form of gene regulation at the transcriptional level is present as a result of this methylation (U.-M. Bauer, Daujat, Nielsen, Nightingale, & Kouzarides, 2002; Wu, Cui, Wang, Li, & Wong, 2012). Aside from histone modifications resulting in downstream gene regulation, CARM1 has also been shown to interact directly with Myogenin, MEF2C and MEF2D transcription factors, suggesting a possible synergistic role in transcriptional activation of different gene targets (S. L. Chen et al., 2002; Dacwag et al., 2009). One early mechanism was suggested through a cooperative manner where CARM1 acts as a co-activator with MEF2C and GRIP1 in the transactivation of a MEF2 dependent reporter, suggesting that CARM1 does indeed act in direct synergy (as a coactivator) with different regulatory factors (S. L. Chen et al., 2002). Our study provides evidence towards a post-transcriptional mode of gene regulation as well, through the methylation of HuR. Through the use of methyl mutant variants of HuR, we have shown the necessity of HuR methylation at arginine 217 for the ability of HuR to activate during differentiation. Specifically, we have shown that the methyl mutant variant of HuR (R217W) was capable of translocating to the cytoplasm, whereas the non-methyl mutant (R217K) remained mainly in the nucleus. In MN-1 cells, it was observed that CARM1 methylation of HuD, a closely related family member of HuR, was acting as a form of binding switch that would alter the binding affinity of the RNA-binding protein for some of its mRNA substrates. HuD was further shown to directly bind with the SMN's Tudor domain, at HuD's methylated arginine within the hinge region. Overexpression of HuD in motoneurons was able to rescue SMA-like defects, suggesting a key role of HuD in SMA (Hubers et al., 2011). Due to the similar function and conserved functional domains

between HuR and HuD, it is fair to propose that HuR methylation in muscle cells may play a similar role to that of HuD in motoneurons. In fact, it has previously been shown in HeLa cells that the methylation of HuR does change its binding affinity and capability to stabilize its mRNA targets (Pang et al., 2013). Thus it is probable that in the knockdown of CARM1 in differentiating myoblasts, not only is HuR expression and translocation affected, but also the binding affinity for specific mRNA targets are altered, thus resulting in aberrant protein expression of essential genes to myogenic differentiation. Further experimentation with HuR targets (through RNA immunoprecipitation in a CARM1 knockdown model as well as with HuR methyl mutants) is required to confirm such a theory.

Interestingly, most studies to date regarding HuR post-translational modifications and translocation are suggestive of different sites of phosphorylation that mediate the cytoplasmic translocation. In fact, HuR has a HuR nucleocytoplasmic shuttling sequence (HNS) within its hinge region, housing different phosphorylation sites that result in translocation of the protein (Fan & Steitz, 1998a; Grammatikakis et al., 2017). HuR methylation by CARM1 occurs on arginine 217, which is unsurprisingly also found within the proteins HNS. One well established mechanism underlying the cytoplasmic shuttling (that may be initiated in response to the post-translational modifications in the HNS) is through the cleavage of HuR within the hinge region. The cleavage product 1 of HuR (CP1) then binds to the import factor transportin-2 and prevents subsequent import of HuR, thereby resulting in the cytoplasmic accumulation of the full length protein (Beauchamp et al., 2010). We introduce the methylation at arginine 217 as a necessary post-translational modification towards the upregulation and cytoplasmic translocation of

HuR in myoblasts. It remains possible that the methylation of HuR may be a cellular signal leading to its cleavage into the CP1 fragment as we show that CARM1 deficient myoblasts lack the HuR-CP1 fragment (Supplementary Figure 2). More work is needed towards determining the mechanistic function of this methylation in HuR's translocating ability.

Overexpression of HuR mutants in C2C12s, followed by 4 days of differentiation revealed areas of less healthy myotube formation in the R217K (non-methyl) mutant, whereas the methyl mutant continued to show the formation of long and healthy myotubes. This provides indication that it is indeed the lack of methylation specifically that is causing the noted myogenic defects. We presented preliminary data through Myc-tag staining showing that the R217W mutants clustered together and formed nice healthy myotubes following 4 days of differentiation, whereas the R217K mutants were absent in the myotubes (Figure 16). Interestingly, there was a shortage of cells expressing R217K mutants overall at 4 days of differentiation, despite an ample presence in 1 day differentiated cells. One speculative explanation for this is that the methylation of HuR is necessary for cell viability during differentiation of muscle cells, and thus overexpression of the R217K mutant was lethal upon initiation of differentiation. A more efficient way to test this would be to substitute the arginine on the endogenous HuR gene through the use of CRISPR, thus limiting the HuR population to our mutant of choice. Alternatively, a rescue experiment with the HuR mutants of interest could also shed some light on the issue. In other words, a knockdown of CARM1 or HuR rescued with the expression of HuR methyl-mutants. Furthermore, the addition of a GFP side group to the protein would

allow real time analysis of the protein expression throughout differentiation by using live cell imaging technology.

4.1.3 HuR interacts with SMN in muscle cells

It has previously been found that HuD interacts directly with SMN through SMN's Tudor domain in a methylation dependent manner. It was further shown that previously described SMA causing mutations in the Tudor domain of the SMN protein abrogate this interaction. Finally, HuD was found to co-localize and interact in the neurites of motoneurons (Akten et al., 2011; Hubers et al., 2011). Given the conserved nature of the methylatable arginine within the hinge region of the RNA-binding proteins, it comes to no surprise that we were also able to show an interaction between HuR and SMN in myoblasts. We suspect that this is a direct interaction, as was seen with HuD, however further analysis is required to confirm this (Hubers et al., 2011). SMN is thought to play a role in the transport of several essential mRNAs in RNA granules, throughout neurite extensions (Akten et al., 2011; Hubers et al., 2011; Rossoll et al., 2003; H. Zhang et al., 2006). SMN's exact role in its binding to RNA-binding proteins is not well understood. Given its known role as a chaperone for various RNP complexes, its interaction with HuR and HuD may be involved in the specific assembly of RNP complexes with their associated mRNAs, similar to SMNs function in snRNP biogenesis. This was previously suggested in motoneurons where SMN was found to be necessary for proper HuD and poly-A positive mRNA localization in axons (Fallini et al., 2011). Furthermore, an interaction between HuD and SMN was found to be necessary towards the recruitment of KSRP, HuD, and some of its mRNA targets in RNA granules of

neurons (Hubers et al., 2011). This suggests that the formation of a complex with the RNA-binding proteins allows for mRNA cytoplasmic transport in response to different stimuli. In the case of muscle cells, methylation of HuR is necessary for proper myogenesis, likely due to HuR's mRNA stabilization function which requires cytoplasmic translocation of the RNA-binding protein. SMN may play a part in this translocation through its interaction with HuR and the formation of mRNP complexes. This can be confirmed through the use of the SMA causing mutation (E134K) of the SMN Tudor domain, thereby abrogating its Tudor-mediated RNA-binding protein interaction (Fallini et al., 2011; Hubers et al., 2011; Jones et al., 2001; Selenko et al., 2001). By preventing a HuR-SMN interaction, we can then analyze whether the interaction is necessary for HuR cytoplasmic localization. To complement this, co-localization can be confirmed along with bimolecular fluorescence complementation (BiFC) to determine the spatial and temporal factors behind the HuR-SMN interaction.

Furthermore, HuR is known to bind the 3'UTR of SMN mRNA transcripts as well, thereby upregulating SMN levels (F Farooq et al., 2009). Thus, HuR activation during differentiation would be predicted to upregulate SMN levels, suggestive of a positive feedback mechanism by which HuR and SMN are both upregulated and work synergistically. Interestingly, the knockdown of HuR, SMN and CARM1 in C2C12 myoblasts have all revealed impaired myotube formation to some degree, emphasizing the possibility of an interplay between then three (S. L. Chen et al., 2002; Shafey et al., 2005; van der Giessen et al., 2003). Given that HuD overexpression was found to rescue SMA-like defects in SMN deficient motoneurons, it would be interesting to see if an over

expression of HuR would similarly rescue myogenic defects in an SMN deficient condition (Hubers et al., 2011).

4.2 HuR function in the skeletal muscle of SMA mice

4.2.1 Upregulated levels of CARM1 and unchanged levels of HuR in *Smn*$^{2B/-}$ mice

It has previously been shown that CARM1 protein levels are upregulated in the spinal cord tissue of SMA mice, as well as in SMA human patient cells. This upregulation was shown to arise at the translational level. Mechanistically, SMN represses CARM1 mRNA translation, and thus in a deficiency of SMN (i.e. in SMA), CARM1 mRNA is de-repressed and thereby upregulated at the protein level (Sanchez et al., 2013). We have also shown, in confirmation with previous literature, that CARM1 is necessary in muscle cells. Together, these findings led us to the proposition that CARM1 levels may also be upregulated in SMA muscles. Indeed, we have revealed that *Smn*$^{2B/-}$ skeletal muscles show a high level of CARM1 protein with respect to their control counterparts. This suggests that SMN's role in translational regulation is not limited specifically to the spinal cord. However, the level of upregulation that we observed is upwards of 7 fold in skeletal muscle, whereas in spinal cord motoneurons an upregulation of no more than 2 fold was observed (Sanchez et al., 2013). Therefore, it is a possibility that other factors are present in skeletal muscle to result in the greater levels of CARM1 in the skeletal muscle. To confirm SMN's translational role, it is essential to recapitulate the experiments showing translational de-repression in SMA motoneurons, this time in skeletal muscle rather than in motoneurons (Sanchez et al., 2013).

Given the numerous roles that CARM1 upholds, this upregulation alone may be responsible for some of the intrinsic skeletal muscle defects that have been previously proposed. For example, CARM1 has been shown to play part in the transcriptional activation of late myogenic genes (Dacwag et al., 2009). It was further shown that inhibition of CARM1 impaired the expression of both Myogenin and MEF2 transcription factors, emphasizing the need for CARM1 during differentiation (S. L. Chen et al., 2002). Thus it might be hypothesized that the observed upregulation of CARM1 levels may result in premature expression of myogenic factors. Such was the case in *Smn*$^{-/-}$;*SMN*2 mice satellite cells, however, this was not the case in the *Smn*$^{2B/-}$ muscles as there is an observed delay in the expression of myogenic factors during their development (Boyer et al., 2014; Hayhurst et al., 2012). Furthermore, higher levels of CARM1 would lead to increased methylation of CARM1 targets, including histone H3, as well as RNA-binding proteins HuR and HuD, among others (D. Chen et al., 1999; Hubers et al., 2011; Pang et al., 2013). Such methylation would lead to changes in gene regulation, as well as alterations in the binding capacity of RNA-binding proteins (Hubers et al., 2011; Pang et al., 2013; Wu et al., 2012). Ultimately, gene expression profiles of affected muscles may be altered, which may play part in the characterized SMA pathology. Further experimentation is required to fully understand the impact of upregulated CARM1 levels on the myogenic program. We steered our focus towards our aforementioned proposed mechanism by which CARM1, SMN and HuR may be involved in an intricate system of mRNA stability mediated gene regulation.

Increased CARM1 would suggest an overall increase in the methylation of CARM1 targets, including HuR. As we have shown, such methylation *in vitro* results in

the upregulation and translocation of HuR during differentiation. However, we also reveal that HuR levels are unchanged in the *Smn*$^{2B/-}$ mice, except for a decrease in HuR levels at day 26. Though, at 26 days, these mice show severe phenotype onset and are near the end of their disease lifespan, therefore such a downregulation of HuR cannot be accountable for the developmental defects that have previously been noted (Y. il Lee et al., 2011). Through a brief preliminary analysis of HuR levels in these mice from day 12 to day 20 inclusively, we ensured that no major spike or dip in HuR levels is being overlooked due to the later time point selections. Accordingly, it appears that no major change in the levels of HuR was overlooked. Studies have shown that HuR's role in mRNA stability of myogenic genes becomes active during differentiation of myoblasts, suggesting the possibility that the activation of HuR was already missed. A quick increase in levels of HuR is initially noted, followed by the return to baseline levels by 48h. Though overall HuR levels return to baseline levels, cytoplasmic accumulation of HuR remains throughout the duration of myotube formation (Figueroa et al., 2003; van der Giessen et al., 2003). *In vivo* induction of injury or denervation in mice (surgical sciatic nerve denervation, or direct cardiotoxin injection) has been shown to result in the upregulation of HuR protein levels, as well as its translocation to the cytoplasm, followed by the increase in active mRNA stabilization through ARE mediated binding (Figueroa et al., 2003; Joassard et al., 2015). Given that HuR has mainly been implicated in early muscle differentiation and during muscle repair, it is plausible that the active myogenic role of HuR is rather specific to early myogenesis and muscle repair rather than throughout the growth and development of the already formed individual fibers. As such,

it comes to no surprise that no effect of HuR was seen in the skeletal muscles of our SMA mouse model.

4.2.2 Defects in HuR function and target mRNA regulation upon denervation

It has previously been shown that surgical sciatic nerve denervation in mice results in the upregulation of HuR in the denervated muscle. The denervated muscles were further shown to express cytoplasmic localization of the RNA-binding protein, and that the protein was functionally active in stabilizing AChR-β mRNA transcripts, thereby increasing the mRNA's half-life and thus upregulating mRNA transcript levels (Joassard et al., 2015). The muscle response to denervation mimics some of the myogenic events during early development. Given that this model has been shown to result in the activation of HuR, we made use of the model in our *Smn*$^{2B/-}$ mice, allowing a more complete assessment of possible HuR defects in SMA. The observed increase in HuR levels, and cytoplasmic translocation in response to denervation of control mice was in agreement with the literature, revealing that our model was functional, and that this provides a sufficient method to assess HuR's activation. The lack of observed upregulation or translocation of the RNA-binding protein in the *Smn*$^{2B/-}$ mouse muscles suggests that indeed, as we had hypothesized, HuR shows signs of defective functionality in SMA. HuR's ability to accumulate in the cytoplasm is key towards its role in mRNA stabilization, suggesting that *Smn*$^{2B/-}$ muscles may exhibit defects in mRNA stabilization (Figueroa et al., 2003; van der Giessen et al., 2003).

The issue presides that the *Smn*$^{2B/-}$ muscles are already partially denervated due to disease progression, which may affect HuR's ability to activate upon experimentally

induced denervation. However, some degree of induced denervation response should still remain as the mice continue to use their muscles in a functional manner, signifying that partial innervation remains. It has been measured in a pure C57BL6 background of the *Smn*$^{2B/-}$ model that by PND19 there is a loss of ~25% of motoneurons in the anterior horn region of the spinal cord (Eshraghi et al., 2016). Furthermore, in the mix breed *Smn*$^{2B/-}$ model, the tibial nerve maintained over ~85% of axons, though ~65% were undergoing some level of degeneration when compared to controls (M Bowerman et al., 2012). Moreover, the gastrocnemius muscle has been shown to exhibit a lesser extent of denervation with respect to other hindlimb muscles, and thus a sizable, yet partial, sciatic nerve denervation response should remain (Boido & Vercelli, 2016; Valsecchi, Boido, De Amicis, Piras, & Vercelli, 2015). In confirmation with this claim, mRNA analysis of *Smn*$^{2B/-}$ muscles revealed the expected increase (similar to that of control levels) of both Myogenin and MaFbx, in response to denervation, suggestive that the denervation response is not saturated in *Smn*$^{2B/-}$ mice, and that a detectable change is present. Interestingly, AChE, MyoD, p21, AChR-β and MuRF1 mRNA transcripts all show the expected increase (or decrease in the case of AChE) in control denervated muscles, but not in our SMA muscles. Given that all these targets have confirmed (or putative in the case of MaFbx and MuRF1) AREs that are bound by HuR, our findings of abnormal mRNA regulation is suggestive of HuR's impaired function in mRNA stability following denervation, resulting in aberrant mRNA expression of myogenic genes. To fully confirm this notion, an mRNA target unaffected by HuR would need be analyzed side by side with HuR targets in mRNA stability assays and RNA-immunoprecipitations.

Interestingly, both Myogenin and MyoD protein levels, as well as mRNA levels, were found to be upregulated in *Smn*$^{2B/-}$ hind limb muscles (in comparison to control counterparts) at postnatal day 21 (Boyer et al., 2014). We have shown at postnatal day 23 that the mRNA levels of both Myogenin and MyoD in innervated *Smn*$^{2B/-}$ muscles are not significantly different than that of control mice. However, our data reveal a trend towards upregulated Myogenin as we record slightly under a 5-fold increase transcript levels in comparison to controls, whereas a 6-fold increase was previously shown at PND21. MyoD mRNA transcripts had been recorded to display a 4-fold increase at PND21 (Boyer et al., 2014). At PND23, we observed no change, suggestive of a transient upregulation of this transcript that had likely peaked just before PND21. Evidently, a span of 48h can have drastic effects on mRNA regulation, and as such, a more in depth time sensitive study is necessary to fully elucidate the temporal changes in RNA levels. Furthermore, it must be noted that our study specifically analyzed the gastrocnemius muscles, whereas the previous study on *Smn*$^{2B/-}$ mice used full hind limb muscles (Boyer et al., 2014). It has been recorded in different SMA mice that the gastrocnemius is generally less affected than other hind limb muscles (such as the quadriceps), and as such develops the phenotype in a slower manner (Boido & Vercelli, 2016; Valsecchi et al., 2015). This could further account for the discrepancies noted.

Previous studies have shown that the atrogenes (MuRF1, MAFbx) mRNA levels are upregulated in the hindlimb of PND21 *Smn*$^{2B/-}$ mice. *Smn*$^{-/-}$;*SMN*2 mice only exhibit an increase in MAFbx (PND5), and not in MuRF1 (Deguise et al., 2016). Here we have denoted a similar trend in MAFbx levels at PND23 *Smn*$^{2B/-}$ gastrocnemius muscle, whereas MuRF1 levels lack any upregulation at the same time point. Analysis at PND21

revealed a trend towards increased MAFbx transcript levels, to a similar extent as previously reported (~18 fold increase vs the previously recorded ~25 fold), along with a significant increase in MuRF1 to similar levels as previously described (~7 fold vs the previously described ~10 fold; Figure 22). Thus the trend in atrogene expression is in agreement with the literature, suggesting that the discrepancies at PND23 are due to a 48h gap between analyses. It has previously been shown that atrogene knockout in non-diseased mice spares the loss of muscle mass in response to denervation (S. C. Bodine et al., 2001). Strikingly, one study revealed that knocking down the two atrogenes (MAFbx, MuRF1) in *SMN*Δ7 mice did not result in any protection from muscle atrophy (Chitra C Iyer, McGovern, Wise, Glass, & Burghes, 2014). However, the upregulated atrogenes in SMA mice correspond with increased levels of ubiquitinated proteins, suggestive that atrogenes do play a role, but perhaps are not alone in mediating the muscle wasting given that atrogene deletion did not aid the muscle wasting (Deguise et al., 2016). A necessary follow up experiment would be to analyze changes in levels of ubiquitinated proteins in the atrogene knockout mice, thus revealing whether ubiquitin-mediated atrophy is occurring, perhaps mediated through other less studied E3 ligases. Moreover, it has been shown that autophagosomal degradation is present in *SMN2B/-* mice, which suggests alternative pathways of degradation occurring simultaneously (Deguise et al., 2016). It is difficult to discern impairments in muscle development from ongoing and gradual muscle atrophy, and as such we must not limit our hypothesis to muscle wasting alone, but must also include the lack of initial development.

Myogenin, MyoD, AChRs, AChE, and p21 are known to be essential genes during muscle development, and during muscle cell differentiation, HuR is recognized as

a key player in the regulation of these genes (Andrés & Walsh, 1996; Deschenes-Furry et al., 2005; Figueroa et al., 2003; Fuentes & Taylor, 1993; Simon & Burden, 1993; van der Giessen et al., 2003). Inability of HuR to regulate these transcripts during denervation points to the possibility of an overall impairment in HuR function due to the lack of SMN protein. Given SMN's suggested cooperative role with HuD in mRNA regulation and localization in motoneurons, we suggest a similar role for HuR by which a HuR-SMN interaction (through the methyl sensing Tudor domain) is necessary for the formation of an mRNP complex, followed by cytoplasmic translocation, and thereby the mRNA regulation of HuR targets (Figure 24). In an SMN deficient model, the CARM1-methylated HuR lacks the ability to translocate to the cytoplasm, and is thus unable to perform its role in stabilizing mRNA transcripts, and concomitantly causing downstream aberrant protein expression. If accurate, such a mechanism would likewise account for the defective muscle development and maturation postnatally. Similar studies focused on the early postnatal role of HuR would be interesting to see and could elucidate such a theory.

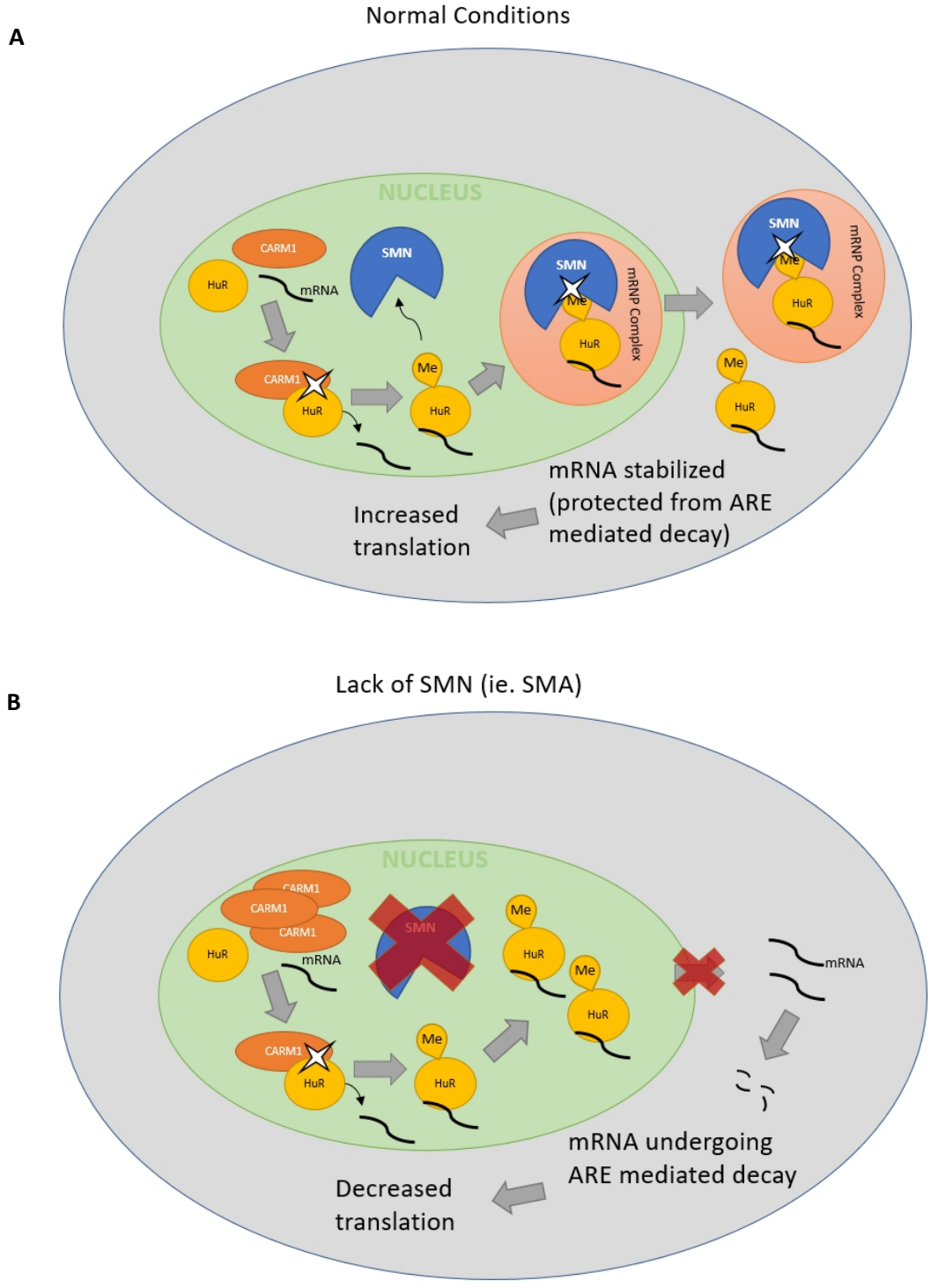


Figure 24: Suggested model for the cooperation between CARM1, HuR and SMN in myoblasts. The nucleus is shown in green, and the cytoplasm in blue-grey. Proposed mechanism shown in [A] normal conditions, [B] SMN deficient conditions (ie. SMA), and [C] CARM1 deficient conditions.

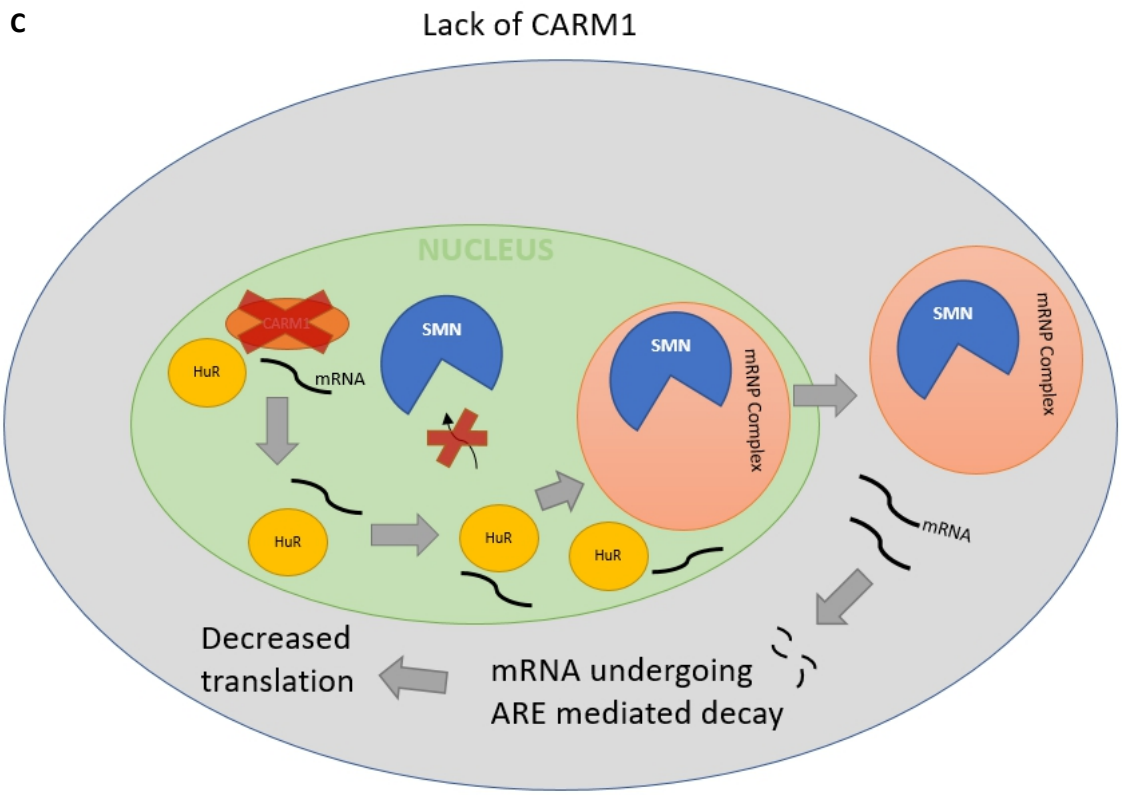


Figure 24 (continued): Suggested model for the cooperation between CARM1, HuR and SMN in myoblasts. The nucleus is shown in green, and the cytoplasm in blue-grey. Proposed mechanism shown in [A] normal conditions, [B] SMN deficient conditions (ie. SMA), and [C] CARM1 deficient conditions.

A relationship between p21 expression and muscle regeneration was established in 2015 where it was found that p21 knockout mice exhibit delayed muscle regeneration in response to intramuscular toxin injection, emphasizing the importance of p21 in muscle. In the same mice, it was noted that the expression of both MyoD and Myogenin mRNAs were delayed in response to the toxin injection in comparison to control muscles (Chinzei et al., 2015). Previous literature has shown increased p21 mRNA and protein levels in SMA mice motoneurons and spinal cords (Corti et al., 2008; Staropoli et al., 2015; Z. Zhang, Lotti, Dittmar, Younis, Wan, Kasim, Dreyfuss, et al., 2008). We observed a ~4-5 fold increase in the average recorded p21 mRNA levels in innervated $Smn^{2B/-}$ muscles, however, this difference was statistically insignificant in comparison with control mice. Moreover, we revealed that $Smn^{2B/-}$ muscles lack the ability to upregulate p21 mRNA levels in response to denervation, seemingly due to HuR defects. This would offset the cell cycle control, possibly leading to an impaired muscle injury response, as was seen with the p21 knockout mice.

Furthermore, we have shown a lack of MyoD upregulation upon denervation in the SMA model. Such defects would prevent the capacity for muscle repair or remodeling in skeletal muscles, and further point towards possible impairments in the postnatal development of the muscles as well. In agreement is the aberrant expression of the synaptic genes AChE and AChR-β in response to denervation. Though the lack of downregulation of AChE could be due to the already low levels in the SMA pathology, the lack of AChR-β upregulation is undoubtedly defective as it follows the exact same pattern as p21. Thus muscle specific defects pose the possibility of being as much responsible for neuromuscular junction defects as are motoneuronal defects. The

unaffected Myogenin upregulation in the deficiency of SMN could be as a result of alternative means of upregulation that over-rides the necessity of stabilization by HuR. Moreover, MaFbx and MuRF1 are E3 ubiquitin ligases that are normally paired together as they are both known to be upregulated upon denervation of skeletal muscle (Sue C Bodine & Baehr, 2014). Interestingly, here we find that only MaFbx mRNA transcript levels are upregulated in response to denervation, as opposed to the expected increase in both. As previously mentioned, MuRF1 levels were upregulated at PND21, and the levels had dropped down by PND23, possibly suggesting a temporal requirement for the ligase that is under the control of other upstream mechanisms, independent of MAFbx expression.

Myogenin had shown a vast upregulation in response to denervation, despite the lack of HuR upregulation or translocation. Although HuR has been shown to bind and stabilize Myogenin in muscle cells, this has not yet been confirmed during a denervation response. It is possible that the upregulation of Myogenin during denervation is independent of HuR, and by means of transcriptional regulation, or alternative forms of translational regulation. It would be interesting and beneficial to perform RNA stability assays alongside RNA-immunoprecipitations with HuR to fully understand the extent of the functional contribution of HuR towards the noted aberrant transcript expressions. Furthermore, we would clarify whether the putative AREs on MaFbx and MuRF1 are indeed being bound by HuR.

4.4 Concluding remarks and significance

We have uncovered the importance of CARM1 methylation on the regulation of HuR during myogenesis. Methylation of the RNA-binding protein is necessary for the upregulation of HuR, its cytoplasmic translocation, and thus important for HuR's functional role in mRNA stability. We have further uncovered that HuR interacts with SMN through the Tudor domain, leading us to the discovery that HuR activation (ie. upregulation and cytoplasmic translocation) and functional role in stabilizing target mRNAs show signs of impairment in SMA. We propose a model by which CARM1 methylates HuR, which then interacts with the SMN protein to translocate in the cytoplasm, allowing HuR to perform its role in stabilizing mRNAs. Such findings put an emphasis on muscle specific roles of SMN, highlighting the need for a system wide therapeutic intervention (accounting, at the bare minimum, for the motor unit) rather than motoneuron specific therapies. Furthermore, we provide evidence for the possibility of a functional involvement of the ubiquitously expressed RNA-binding protein HuR in the SMA etiology, complementing the previously uncovered role of HuD in motoneurons, and underscoring the Hu family of RNA-binding proteins as potential key players in the etiology of SMA (Hubers et al., 2011). Here we provide a step forward in attempting to understand the mechanism underlying the SMA etiology, with hopes of ultimately finding possible alternative, downstream methods for therapeutically treating SMA patients.

4.5 Future Directions

This study has laid out the foundation for a more in depth analysis of HuR's function in mRNA stabilization. We show that HuR methylation by CARM1 is necessary for its upregulation and translocation. One necessary step is to confirm that methylation is indeed decreased in our shCARM1 cell lines. We can further analyze the methylation of HuR during differentiation by using methyl-specific HuR antibodies. Luciferase binding assays, RNA immunoprecipitations (using both HuR and methyl-HuR antibodies), and RNA-stability assays for the known HuR mRNA targets would allow the determination of the effect of methylation on these targets. This will provide evidence revealing whether methylation of HuR modulates its binding to myogenic genes, thereby regulating their half-life through ARE-mediated binding. To further compliment this, and implicate SMN directly, BiFC can be used to determine the spatial and temporal parameters underlying the SMN-HuR interaction. GST-Tdr fusion protein pulldowns with the generated HuR mutants would allow the confirmation that the methylation of HuR is necessary for an SMN interaction. Next, either through the sole expression of non-methyl HuR (R217K) or the knockdown of SMN, HuR target mRNA binding and half-life measures will establish whether SMN plays a key role in HuR's function during myogenesis. Finally, as was done with HuD, an attempt to rescue SMN deficient cells will be made through the use of HuR mutants, as well as wildtype HuR. To complement this, the attempt to rescue CARM1 deficient cells with the generated HuR mutants would further solidify that CARM1 myogenic defects act through HuR specifically. Fully understanding the mechanism underlying the synergistic role between HuR, CARM1 and

SMN would provide key information in understanding the exact role of HuR in the SMA pathology.

In order to solidify the *in vivo* study, it is necessary to perform RNA stability assays following denervation in these $Smn^{2B/-}$ mice, alongside RNA-immunoprecipitation experiments, confirming binding of the targets by HuR. This would reveal whether or not the aberrant mRNA expression is in fact due to HuR misregulation. This analysis should be done side by side with another mRNA that HuR does not bind, as a negative control for comparison. Furthermore, the use of a methyl-HuR antibody would allow to quantify the levels of methylated HuR in these muscles, as well as its localization. Using the same antibody, as well as the regular HuR antibody, RNA immunoprecipitations in vivo would allow to determine how methylation of HuR affects its binding capacity to target mRNAs in muscle of SMA mice vs control. Moreover, a more in depth analysis using other muscles (soleus and extensor digitorium longus) to compare fast vs slow twitch muscles would be beneficial as our findings are strictly limited to the gastrocnemius muscle. A follow up study would then focus on the early development in the pre-symptomatic stages of these mice, thus determining if in fact developmental HuR misregulation may partially be responsible for the SMA pathology. Finally, using gene therapy, we could attempt to rescue SMA defects by overexpressing HuR, as well as its mutants, revealing whether or not HuR serves as a putative therapeutic target.

5) REFERENCES

Abdelmohsen, K., & Gorospe, M. (2010). Posttranscriptional regulation of cancer traits by HuR. *Wiley Interdisciplinary Reviews. RNA*, *1*(2), 214–29. https://doi.org/10.1002/wrna.4

Akamatsu, W., Fujihara, H., Mitsuhashi, T., Yano, M., Shibata, S., Hayakawa, Y., … Okano, H. (2005). The RNA-binding protein HuD regulates neuronal cell identity and maturation. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(12), 4625–30. https://doi.org/10.1073/pnas.0407523102

Akamatsu, W., Okano, H. J., Osumi, N., Inoue, T., Nakamura, S., Sakakibara, S., … Okano, H. (1999). Mammalian ELAV-like neuronal RNA-binding proteins HuB and HuC promote neuronal development in both the central and the peripheral nervous systems. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(17), 9885–90. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10449789

Akten, B., Kye, M. J., Hao, L. T., Wertz, M. H., Singh, S., Nie, D., … Sahin, M. (2011). Interaction of survival of motor neuron (SMN) and HuD proteins with mRNA cpg15 rescues motor neuron axonal deficits. *Proceedings of the National Academy of Sciences*, *108*(25), 10337–10342. https://doi.org/10.1073/pnas.1104928108

Anderson, K. D., Morin, M. A., Beckel-Mitchener, A., Mobarak, C. D., Neve, R. L., Furneaux, H. M., … Perrone-Bizzozero, N. I. (2000). Overexpression of HuD, but

not of its truncated form HuD I+II, promotes GAP-43 gene expression and neurite outgrowth in PC12 cells in the absence of nerve growth factor. *Journal of Neurochemistry*, *75*(3), 1103–14. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10936192

Andrés, V., & Walsh, K. (1996). Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. *The Journal of Cell Biology*, *132*(4), 657–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8647896

Arnold, A.-S., Gueye, M., Guettier-Sigrist, S., Courdier-Fruh, I., Coupin, G., Poindron, P., & Gies, J.-P. (2004). Reduced expression of nicotinic AChRs in myotubes from spinal muscular atrophy I patients. *Laboratory Investigation*, *84*(10), 1271–1278. https://doi.org/10.1038/labinvest.3700163

Arnold, W. D., Kassar, D., & Kissel, J. T. (2015). Spinal muscular atrophy: diagnosis and management in a new therapeutic era. *Muscle Nerve*, *51*(2), 157–167. https://doi.org/10.1002/mus.24497

Auclair, Y., & Richard, S. (2013). The role of arginine methylation in the DNA damage response. *DNA Repair*, *12*(7), 459–465. https://doi.org/10.1016/j.dnarep.2013.04.006

Barami, K., Iversen, K., Furneaux, H., & Goldman, S. A. (1995). Hu protein as an early marker of neuronal phenotypic differentiation by subependymal zone cells of the adult songbird forebrain. *Journal of Neurobiology*, *28*(1), 82–101.

https://doi.org/10.1002/neu.480280108

Bauer, U.-M., Daujat, S., Nielsen, S. J., Nightingale, K., & Kouzarides, T. (2002). Methylation at arginine 17 of histone H3 is linked to gene activation. *EMBO Reports*, *3*(1), 39–44. https://doi.org/10.1093/embo-reports/kvf013

Bauer, U. M., Daujat, S., Nielsen, S. J., Nightingale, K., & Kouzarides, T. (2002). Methylation at arginine 17 of histone H3 is linked to gene activation. *EMBO Reports*, *3*(1), 39–44. https://doi.org/10.1093/embo-reports/kvf013

Beauchamp, P., Nassif, C., Hillock, S., van der Giessen, K., von Roretz, C., Jasmin, B. J., & Gallouzi, I. E. (2010). The cleavage of HuR interferes with its transportin-2-mediated nuclear import and promotes muscle fiber formation. *Cell Death Differ*, *17*(10), 1588–1599. https://doi.org/10.1038/cdd.2010.34

Beckel-Mitchener, A. C., Miera, A., Keller, R., & Perrone-Bizzozero, N. I. (2002). Poly(A) tail length-dependent stabilization of GAP-43 mRNA by the RNA-binding protein HuD. *The Journal of Biological Chemistry*, *277*(31), 27996–8002. https://doi.org/10.1074/jbc.M201982200

Bedford, M. T., & Clarke, S. G. (2009). Protein Arginine Methylation in Mammals: Who, What, and Why. *Molecular Cell*. https://doi.org/10.1016/j.molcel.2008.12.013

Blanc, R. S., & Richard, S. (2017). Arginine Methylation: The Coming of Age. *Molecular Cell*, *65*(1), 8–24. https://doi.org/10.1016/j.molcel.2016.11.003

Bodine, S. C., & Baehr, L. M. (2014). Skeletal muscle atrophy and the E3 ubiquitin

ligases MuRF1 and MAFbx/atrogin-1. *American Journal of Physiology. Endocrinology and Metabolism*, *307*(6), E469-84. https://doi.org/10.1152/ajpendo.00204.2014

Bodine, S. C., Latres, E., Baumhueter, S., Lai, V. K., Nunez, L., Clarke, B. A., … Glass, D. J. (2001). Identification of Ubiquitin Ligases Required for Skeletal Muscle Atrophy. *Science*, *294*(5547), 1704–1708. https://doi.org/10.1126/science.1065874

Boido, M., & Vercelli, A. (2016). Neuromuscular Junctions as Key Contributors and Therapeutic Targets in Spinal Muscular Atrophy. *Frontiers in Neuroanatomy*, *10*, 6. https://doi.org/10.3389/fnana.2016.00006

Bowerman, M., Beauvais, A., Anderson, C. L., & Kothary, R. (2010). Rho-kinase inactivation prolongs survival of an intermediate SMA mouse model. *Human Molecular Genetics*, *19*(8), 1468–1478. https://doi.org/10.1093/hmg/ddq021

Bowerman, M., Murray, L. M., Beauvais, A., Pinheiro, B., & Kothary, R. (2012). A critical smn threshold in mice dictates onset of an intermediate spinal muscular atrophy phenotype associated with a distinct neuromuscular junction pathology. *Neuromuscul Disord*, *22*(3), 263–276. https://doi.org/10.1016/j.nmd.2011.09.007

Bowerman, M., Shafey, D., & Kothary, R. (2007). Smn depletion alters profilin II expression and leads to upregulation of the RhoA/ROCK pathway and defects in neuronal integrity. *Journal of Molecular Neuroscience*, *32*(2), 120–131. https://doi.org/10.1007/s12031-007-0024-5

Boyer, J. G., Deguise, M. O., Murray, L. M., Yazdani, A., De Repentigny, Y., Boudreau-

Lariviere, C., & Kothary, R. (2014). Myogenic program dysregulation is contributory to disease pathogenesis in spinal muscular atrophy. *Hum Mol Genet*, *23*(16), 4249–4259. https://doi.org/10.1093/hmg/ddu142

Boyer, J. G., Murray, L. M., Scott, K., De Repentigny, Y., Renaud, J. M., & Kothary, R. (2013). Early onset muscle weakness and disruption of muscle proteins in mouse models of spinal muscular atrophy. *Skelet Muscle*, *3*(1), 24. https://doi.org/10.1186/2044-5040-3-24

Braun, S., Croizat, B., Lagrange, M. C., Warter, J. M., & Poindron, P. (1995). Constitutive muscular abnormalities in culture in spinal muscular atrophy. *Lancet (London, England)*, *345*(8951), 694–5. https://doi.org/10.5555/URI:PII:S0140673695908692

Brzustowicz, L. M., Lehner, T., Castilla, L. H., Penchaszadeh, G. K., Wilhelmsen, K. C., Daniels, R., … Wood, D. (1990). Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2-13.3. *Nature*. https://doi.org/10.1038/344540a0

Buckingham, M., Schnegelsberg, P., Stead, R., Braun, T., Arnold, H., Jaenisch, R., & Pinset, C. (1994). Muscle differentiation. Which myogenic factors make muscle? *Current Biology : CB*, *4*(1), 61–3. https://doi.org/10.1016/S0960-9822(00)00014-2

Bühler, D., Raker, V., Lührmann, R., & Fischer, U. (1999). Essential role for the tudor domain of SMN in spliceosomal U snRNP assembly: implications for spinal muscular atrophy. *Human Molecular Genetics*, *8*(13), 2351–2357.

https://doi.org/10.1093/hmg/8.13.2351

Burnett, B. G., Muñoz, E., Tandon, A., Kwon, D. Y., Sumner, C. J., & Fischbeck, K. H. (2009). Regulation of SMN protein stability. *Molecular and Cellular Biology*, *29*(5), 1107–15. https://doi.org/10.1128/MCB.01262-08

Carissimi, C., Baccon, J., Straccia, M., Chiarella, P., Maiolica, A., Sawyer, A., … Pellizzoni, L. (2005). Unrip is a component of SMN complexes active in snRNP assembly. *FEBS Lett*, *579*(11), 2348–2354. https://doi.org/10.1016/j.febslet.2005.03.034

Cha, B., & Jho, E.-H. (2012). Protein arginine methyltransferases (PRMTs) as therapeutic targets. *Expert Opinion on Therapeutic Targets*, *16*(September), 651–664. https://doi.org/10.1517/14728222.2012.688030

Chen, C. Y., Gherzi, R., Ong, S. E., Chan, E. L., Raijmakers, R., Pruijn, G. J. M., … Karin, M. (2001). AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell*. https://doi.org/10.1016/S0092-8674(01)00578-5

Chen, D., Ma, H., Hong, H., Koh, S. S., Huang, S.-M., Schurter, B. T., … Stallcup, M. R. (1999). Regulation of Transcription by a Protein Methyltransferase. *Science*, *284*(5423). Retrieved from http://science.sciencemag.org/content/284/5423/2174.full

Chen, S. L., Loffler, K. A., Chen, D., Stallcup, M. R., & Muscat, G. E. O. (2002). The coactivator-associated arginine methyltransferase is necessary for muscle differentiation: CARM1 coactivates myocyte enhancer factor-2. *The Journal of*

Biological Chemistry, *277*(6), 4324–33. https://doi.org/10.1074/jbc.M109835200

Cheng, D., Côté, J., Shaaban, S., & Bedford, M. T. (2007). The Arginine Methyltransferase CARM1 Regulates the Coupling of Transcription and mRNA Processing. *Molecular Cell*, *25*(1), 71–83. https://doi.org/10.1016/j.molcel.2006.11.019

Chevillard-Briet, M., Trouche, D., & Vandel, L. (2002). Control of CBP co-activating activity by arginine methylation. *The EMBO Journal*, *21*(20), 5457–5466. https://doi.org/10.1093/emboj/cdf548

Chinzei, N., Hayashi, S., Ueha, T., Fujishiro, T., Kanzaki, N., Hashimoto, S., … Kurosaka, M. (2015). P21 deficiency delays regeneration of skeletal muscular tissue. *PLoS ONE*, *10*(5). https://doi.org/10.1371/journal.pone.0125765

Cifuentes-Diaz, C., Frugier, T., Tiziano, F. D., Lacene, E., Roblot, N., Joshi, V., … Melki, J. (2001). Deletion of murine SMN exon 7 directed to skeletal muscle leads to severe muscular dystrophy. *J Cell Biol*, *152*(5), 1107–1114. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11238465

Coovert, D. D., Le, T. T., McAndrew, P. E., Strasswimmer, J., Crawford, T. O., Mendell, J. R., … Burghes, A. H. M. (1997). The survival motor neuron protein in spinal muscular atrophy. *Human Molecular Genetics*, *6*(8), 1205–1214. https://doi.org/10.1093/hmg/6.8.1205

Corti, S., Nizzardo, M., Nardini, M., Donadoni, C., Salani, S., Ronchi, D., … Comi, G. P. (2008). Neural stem cell transplantation can ameliorate the phenotype of a mouse

model of spinal muscular atrophy. *The Journal of Clinical Investigation*, *118*(10), 3316–30. https://doi.org/10.1172/JCI35432

Côté, J., & Richard, S. (2005). Tudor domains bind symmetrical dimethylated arginines. *Journal of Biological Chemistry*, *280*(31), 28476–28483. https://doi.org/10.1074/jbc.M414328200

D'Amico, A., Mercuri, E., Tiziano, F. D., & Bertini, E. (2011). Spinal muscular atrophy. *Orphanet Journal of Rare Diseases*, *6*, 71. https://doi.org/10.1186/1750-1172-6-71

Dachs, E., Hereu, M., Piedrafita, L., Casanovas, A., Calderó, J., & Esquerda, J. E. (2011). Defective Neuromuscular Junction Organization and Postnatal Myogenesis in Mice With Severe Spinal Muscular Atrophy. *Journal of Neuropathology & Experimental Neurology*, *70*(6), 444–461. https://doi.org/10.1097/NEN.0b013e31821cbd8b

Dacwag, C. S., Bedford, M. T., Sif, S., & Imbalzano, A. N. (2009). Distinct protein arginine methyltransferases promote ATP-dependent chromatin remodeling function at different stages of skeletal muscle differentiation. *Molecular and Cellular Biology*, *29*(7), 1909–21. https://doi.org/10.1128/MCB.00742-08

DeBoer, E. M., Azevedo, R., Vega, T. A., Brodkin, J., Akamatsu, W., Okano, H., … Rasin, M.-R. (2014). Prenatal Deletion of the RNA-Binding Protein HuD Disrupts Postnatal Cortical Circuit Maturation and Behavior. *Journal of Neuroscience*, *34*(10), 3674–3686. https://doi.org/10.1523/JNEUROSCI.3703-13.2014

Deguise, M.-O., Boyer, J. G., McFall, E. R., Yazdani, A., De Repentigny, Y., & Kothary, R. (2016). Differential induction of muscle atrophy pathways in two mouse models

of spinal muscular atrophy. *Scientific Reports*, *6*(1), 28846. https://doi.org/10.1038/srep28846

Deschenes-Furry, J., Belanger, G., Mwanjewe, J., Lunde, J. A., Parks, R. J., Perrone-Bizzozero, N., & Jasmin, B. J. (2005). The RNA-binding protein HuR binds to acetylcholinesterase transcripts and regulates their expression in differentiating skeletal muscle cells. *J Biol Chem*, *280*(27), 25361–25368. https://doi.org/10.1074/jbc.M410929200

DiDonato, C. J., Lorson, C. L., De Repentigny, Y., Simard, L., Chartrand, C., Androphy, E. J., & Kothary, R. (2001). Regulation of murine survival motor neuron (Smn) protein levels by modifying Smn exon 7 splicing. *Human Molecular Genetics*, *10*(23), 2727–2736. https://doi.org/10.1093/hmg/10.23.2727

Doller, A., Schlepckow, K., Schwalbe, H., Pfeilschifter, J., & Eberhardt, W. (2010). Tandem phosphorylation of serines 221 and 318 by protein kinase Cdelta coordinates mRNA binding and nucleocytoplasmic shuttling of HuR. *Molecular and Cellular Biology*, *30*(6), 1397–410. https://doi.org/10.1128/MCB.01373-09

Dubowitz, V. (1999). Very severe spinal muscular atrophy (SMA type 0): an expanding clinical phenotype. *European Journal of Paediatric Neurology : EJPN : Official Journal of the European Paediatric Neurology Society*, *3*(2), 49–51. https://doi.org/10.1053/ejpn.1999.0181

Eftimie, R., Brenner, H. R., & Buonanno, A. (1991). Myogenin and MyoD join a family of skeletal muscle genes regulated by electrical activity. *Proceedings of the National*

Academy of Sciences of the United States of America, *88*(4), 1349–53. https://doi.org/10.1073/PNAS.88.4.1349

Eshraghi, M., McFall, E., Gibeault, S., & Kothary, R. (2016). Effect of genetic background on the phenotype of the *Smn* $^{2B/-}$ mouse model of spinal muscular atrophy. *Human Molecular Genetics*, *32*(20), ddw278. https://doi.org/10.1093/hmg/ddw278

Fallini, C., Bassell, G. J., & Rossoll, W. (2010). High-efficiency transfection of cultured primary motor neurons to study protein localization, trafficking, and function. *Molecular Neurodegeneration*, *5*(1), 17. https://doi.org/10.1186/1750-1326-5-17

Fallini, C., Bassell, G. J., & Rossoll, W. (2012). Spinal muscular atrophy: The role of SMN in axonal mRNA regulation. *Brain Research*. https://doi.org/10.1016/j.brainres.2012.01.044

Fallini, C., Donlin-Asp, P. G., Rouanet, J. P., Bassell, G. J., & Rossoll, W. (2016). Deficiency of the Survival of Motor Neuron Protein Impairs mRNA Localization and Local Translation in the Growth Cone of Motor Neurons. *The Journal of Neuroscience*, *36*(13), 3811–3820. https://doi.org/10.1523/JNEUROSCI.2396-15.2016

Fallini, C., Rouanet, J. P., Donlin-Asp, P. G., Guo, P., Zhang, H., Singer, R. H., … Bassell, G. J. (2014). Dynamics of survival of motor neuron (SMN) protein interaction with the mRNA-binding protein IMP1 facilitates its trafficking into motor neuron axons. *Developmental Neurobiology*, *74*(3), 319–332.

https://doi.org/10.1002/dneu.22111

Fallini, C., Zhang, H., Su, Y., Silani, V., Singer, R. H., Rossoll, W., & Bassell, G. J. (2011). The survival of motor neuron (SMN) protein interacts with the mRNA-binding protein HuD and regulates localization of poly(A) mRNA in primary motor neuron axons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(10), 3914–25. https://doi.org/10.1523/JNEUROSCI.3631-10.2011

Fan, X. C., & Steitz, J. A. (1998a). HNS, a nuclear-cytoplasmic shuttling sequence in HuR. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(26), 15293–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9860962

Fan, X. C., & Steitz, J. A. (1998b). Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the invivo stability of ARE-containing mRNAs. *The EMBO Journal*, *17*(12), 3448–3460. https://doi.org/10.1093/emboj/17.12.3448

Farooq, F., Abadía-Molina, F., MacKenzie, D., Hadwen, J., Shamim, F., O'Reilly, S., … MacKenzie, A. (2013). Celecoxib increases SMN and survival in a severe spinal muscular atrophy mouse model via p38 pathway activation. *Human Molecular Genetics*, *22*(17), 3415–3424. https://doi.org/10.1093/hmg/ddt191

Farooq, F., Balabanian, S., Liu, X., Holcik, M., & MacKenzie, A. (2009). p38 Mitogen-activated protein kinase stabilizes SMN mRNA through RNA binding protein HuR. *Hum Mol Genet*, *18*(21), 4035–4045. https://doi.org/10.1093/hmg/ddp352

Figueroa, A., Cuadrado, A., Fan, J., Atasoy, U., Muscat, G. E., Munoz-Canoves, P., …

Munoz, A. (2003). Role of HuR in skeletal myogenesis through coordinate regulation of muscle differentiation genes. *Mol Cell Biol*, *23*(14), 4991–5004. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12832484

Fox-Walsh, K. L., & Hertel, K. J. (2009). Splice-site pairing is an intrinsically high fidelity process. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(6), 1766–71. https://doi.org/10.1073/pnas.0813128106

Friesen, W. J., & Dreyfuss, G. (2000). Specific sequences of the Sm and Sm-like (Lsm) proteins mediate their interaction with the spinal muscular atrophy disease gene product (SMN). *Journal of Biological Chemistry*, *275*(34), 26370–26375. https://doi.org/10.1074/jbc.M003299200

Friesen, W. J., Massenet, S., Paushkin, S., Wyce, A., & Dreyfuss, G. (2001). SMN, the product of the spinal muscular atrophy gene, binds preferentially to dimethylarginine-containing protein targets. *Molecular Cell*, *7*(5), 1111–1117. https://doi.org/10.1016/S1097-2765(01)00244-1

Füchtbauer, E.-M., & Westphal, H. (1992). MyoD and myogenin are coexpressed in regenerating skeletal muscle of the mouse. *Developmental Dynamics*, *193*(1), 34–39. https://doi.org/10.1002/aja.1001930106

Fuentes, M. E., & Taylor, P. (1993). Control of acetylcholinesterase gene expression during myogenesis. *Neuron*, *10*(4), 679–87. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8386528

Fujiwara, T., Mori, Y., Chu, D. L., Koyama, Y., Miyata, S., Tanaka, H., … Tohyama, M.

(2006). CARM1 regulates proliferation of PC12 cells by methylating HuD. *Mol Cell Biol*, *26*(6), 2273–2285. https://doi.org/10.1128/MCB.26.6.2273-2285.2006

Gabanella, F., Butchbach, M. E. R., Saieva, L., Carissimi, C., Burghes, A. H. M., & Pellizzoni, L. (2007). Ribonucleoprotein Assembly Defects Correlate with Spinal Muscular Atrophy Severity and Preferentially Affect a Subset of Spliceosomal snRNPs. *PLoS ONE*, *2*(9), e921. https://doi.org/10.1371/journal.pone.0000921

Gallouzi, I.-E., & Steitz, J. A. (2001). Delineation of mRNA Export Pathways by the Use of Cell-Permeable Peptides. *Science*, *294*(5548). Retrieved from http://science.sciencemag.org/content/294/5548/1895?ijkey=ea9e08e28c19cf1af5d6c88fe39ae45616daa061&keytype2=tf_ipsecsha

Gallouzi, I. E., Brennan, C. M., & Steitz, J. A. (2001). Protein ligands mediate the CRM1-dependent export of HuR in response to heat shock. *RNA (New York, N.Y.)*, *7*(9), 1348–61. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11565755

Gavrilina, T. O., McGovern, V. L., Workman, E., Crawford, T. O., Gogliotti, R. G., DiDonato, C. J., … Burghes, A. H. M. (2008). Neuronal SMN expression corrects spinal muscular atrophy in severe SMA mice while muscle-specific SMN expression has no phenotypic effect. *Human Molecular Genetics*, *17*(8), 1063–75. https://doi.org/10.1093/hmg/ddm379

Giesemann, T., Rathke-Hartlieb, S., Rothkegel, M., Bartsch, J. W., Buchmeier, S., Jockusch, B. M., & Jockusch, H. (1999). A role for polyproline motifs in the spinal muscular atrophy protein SMN. Profilins bind to and colocalize with SMN in

nuclear gems. *Journal of Biological Chemistry*, *274*(53), 37908–37914. https://doi.org/10.1074/jbc.274.53.37908

Good, P. J. (1995). A conserved family of elav-like genes in vertebrates. *Proc Natl Acad Sci U S A*, *92*(10), 4557–4561. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7753842

Grammatikakis, I., Abdelmohsen, K., & Gorospe, M. (2017). Posttranslational control of HuR function. *Wiley Interdisciplinary Reviews: RNA*, *8*(1), e1372. https://doi.org/10.1002/wrna.1372

Hammond, S. M., Gogliotti, R. G., Rao, V., Beauvais, A., Kothary, R., & DiDonato, C. J. (2010). Mouse Survival Motor Neuron Alleles That Mimic SMN2 Splicing and Are Inducible Rescue Embryonic Lethality Early in Development but Not Late. *PLoS ONE*, *5*(12), e15887. https://doi.org/10.1371/journal.pone.0015887

Harada, Y., Sutomo, R., Sadewa, A. H., Akutsu, T., Takeshima, Y., Wada, H., … Nishio, H. (2002). Correlation between SMN2 copy number and clinical phenotype of spinal muscular atrophy: three SMN2 copies fail to rescue some patients from the disease severity. *J Neurol*, *249*(9), 1211–1219. https://doi.org/10.1007/s00415-002-0811-4

Hayhurst, M., Wagner, A. K., Cerletti, M., Wagers, A. J., & Rubin, L. L. (2012). A cell-autonomous defect in skeletal muscle satellite cells expressing low levels of survival of motor neuron protein. *Dev Biol*, *368*(2), 323–334. https://doi.org/10.1016/j.ydbio.2012.05.037

Hinman, M. N., & Lou, H. (2008). Diverse molecular functions of Hu proteins. *Cellular*

and Molecular Life Sciences. https://doi.org/10.1007/s00018-008-8252-6

Hubers, L., Valderrama-Carvajal, H., Laframboise, J., Timbers, J., Sanchez, G., & Cote, J. (2011). HuD interacts with survival motor neuron protein and can rescue spinal muscular atrophy-like neuronal defects. *Hum Mol Genet*, *20*(3), 553–579. https://doi.org/10.1093/hmg/ddq500

Huo, Q., Kayikci, M., Odermatt, P., Meyer, K., Michels, O., Saxena, S., … Schümperli, D. (2014). Splicing changes in SMA mouse motoneurons and SMN-depleted neuroblastoma cells: evidence for involvement of splicing regulatory proteins. *RNA Biology*, *11*(11), 1430–46. https://doi.org/10.1080/15476286.2014.996494

Inoue, M., Muto, Y., Sakamoto, H., & Yokoyama, S. (2000). NMR studies on functional structures of the AU-rich element-binding domains of Hu antigen C. *Nucleic Acids Research*, *28*(8), 1743–50. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10734193

Ishido, M., Kami, K., & Masuhara, M. (2004). In vivo expression patterns of MyoD, p21, and Rb proteins in myonuclei and satellite cells of denervated rat skeletal muscle. *American Journal of Physiology - Cell Physiology*, *287*(2). Retrieved from http://ajpcell.physiology.org/content/287/2/C484.long

Iyer, C. C., McGovern, V. L., Murray, J. D., Gombash, S. E., Zaworski, P. G., Foust, K. D., … Burghes, A. H. (2015). Low levels of Survival Motor Neuron protein are sufficient for normal muscle function in the SMNDelta7 mouse model of SMA. *Hum Mol Genet*, *24*(21), 6160–6173. https://doi.org/10.1093/hmg/ddv332

Iyer, C. C., McGovern, V. L., Wise, D. O., Glass, D. J., & Burghes, A. H. M. (2014). Deletion of atrophy enhancing genes fails to ameliorate the phenotype in a mouse model of spinal muscular atrophy. *Neuromuscular Disorders : NMD*, *24*(5), 436–44. https://doi.org/10.1016/j.nmd.2014.02.007

Joassard, O. R., Belanger, G., Karmouch, J., Lunde, J. A., Shukla, A. H., Chopard, A., … Jasmin, B. J. (2015). HuR Mediates Changes in the Stability of AChR beta-Subunit mRNAs after Skeletal Muscle Denervation. *J Neurosci*, *35*(31), 10949–10962. https://doi.org/10.1523/JNEUROSCI.1043-15.2015

Jones, K. W., Gorzynski, K., Hales, C. M., Fischer, U., Badbanchi, F., Terns, R. M., & Terns, M. P. (2001). Direct Interaction of the Spinal Muscular Atrophy Disease Protein SMN with the Small Nucleolar RNA-associated Protein Fibrillarin. *Journal of Biological Chemistry*, *276*(42), 38645–38651. https://doi.org/10.1074/jbc.M106161200

Joseph, B., Orlian, M., & Furneaux, H. (1998). p21(waf1) mRNA contains a conserved element in its 3'-untranslated region that is bound by the Elav-like mRNA-stabilizing proteins. *The Journal of Biological Chemistry*, *273*(32), 20511–6. https://doi.org/10.1074/JBC.273.32.20511

Kariya, S., Park, G. H., Maeno-Hikichi, Y., Leykekhman, O., Lutz, C., Arkovitz, M. S., … Monani, U. R. (2008). Reduced SMN protein impairs maturation of the neuromuscular junctions in mouse models of spinal muscular atrophy. *Hum Mol Genet*, *17*(16), 2552–2569. https://doi.org/10.1093/hmg/ddn156

Kasashima, K., Terashima, K., Yamamoto, K., Sakashita, E., & Sakamoto, H. (1999). Cytoplasmic localization is required for the mammalian ELAV-like protein HuD to induce neuronal differentiation, *4*(11). https://doi.org/10.1046/j.1365-2443.1999.00292.x

Kasashima, K., Terashima, K., Yamamoto, K., Sakashita, E., & Sakamoto, H. (1999). Cytoplasmic localization is required for the mammalian ELAV-like protein HuD to induce neuronal differentiation. *Genes to Cells : Devoted to Molecular & Cellular Mechanisms*, *4*(11), 667–83. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10620013

Kashima, T., & Manley, J. L. (2003). A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. *Nature Genetics*, *34*(4), 460–463. https://doi.org/10.1038/ng1207

Kawabe, Y., Wang, Y. X., McKinnell, I. W., Bedford, M. T., & Rudnicki, M. A. (2012). Carm1 Regulates Pax7 Transcriptional Activity through MLL1/2 Recruitment during Asymmetric Satellite Stem Cell Divisions. *Cell Stem Cell*, *11*(3), 333–345. https://doi.org/10.1016/j.stem.2012.07.001

Kim, H. H., Yang, X., Kuwano, Y., & Gorospe, M. (2008). Modification at HuR(S242) alters HuR localization and proliferative influence. *Cell Cycle (Georgetown, Tex.)*, *7*(21), 3371–7. https://doi.org/10.4161/cc.7.21.6895

Kong, L., Wang, X., Choe, D. W., Polley, M., Burnett, B. G., Bosch-Marce, M., … Sumner, C. J. (2009). Impaired Synaptic Vesicle Release and Immaturity of

Neuromuscular Junctions in Spinal Muscular Atrophy Mice. *Journal of Neuroscience*, *29*(3), 842–851. https://doi.org/10.1523/JNEUROSCI.4434-08.2009

Lafarga, V., Cuadrado, A., Lopez de Silanes, I., Bengoechea, R., Fernandez-Capetillo, O., & Nebreda, A. R. (2009). p38 Mitogen-activated protein kinase- and HuR-dependent stabilization of p21(Cip1) mRNA mediates the G(1)/S checkpoint. *Molecular and Cellular Biology*, *29*(16), 4341–51. https://doi.org/10.1128/MCB.00210-09

Lee, J., & Bedford, M. T. (2002). PABP1 identified as an arginine methyltransferase substrate using high-density protein arrays. *EMBO Reports*, *3*(3), 268–273. https://doi.org/10.1093/embo-reports/kvf052

Lee, Y. il, Mikesh, M., Smith, I., Rimer, M., & Thompson, W. (2011). Muscles in a mouse model of spinal muscular atrophy show profound defects in neuromuscular development even in the absence of failure in neuromuscular transmission or loss of motor neurons. *Developmental Biology*, *356*(2), 432–444. https://doi.org/10.1016/j.ydbio.2011.05.667

Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., … et al. (1995). Identification and characterization of a spinal muscular atrophy-determining gene. *Cell*, *80*(1), 155–165. Retrieved from http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/referer?http://www.ncbi.nlm.nih.gov/htbin-post/Omim/getmim?field=medline_uid&search=7813012

Lefebvre, S., Burlet, P., Liu, Q., Bertrandy, S., Clermont, O., Munnich, a, … Melki, J.

(1997). Correlation between severity and SMN protein level in spinal muscular atrophy. *Nature Genetics*, *16*(3), 265–269. https://doi.org/10.1038/ng0797-265

Li, H., Park, S., Kilburn, B., Jelinek, M. A., Henschen-Edman, A., Aswad, D. W., … Laird-Offringa, I. A. (2002). Lipopolysaccharide-induced methylation of HuR, an mRNA-stabilizing protein, by CARM1. Coactivator-associated arginine methyltransferase. *J Biol Chem*, *277*(47), 44623–44630. https://doi.org/10.1074/jbc.M206187200

Liu, G. C., Jong, Y. J., Chiang, C. H., & Yang, C. W. (1992). Spinal muscular atrophy: MR evaluation. *Pediatric Radiology*, *22*(8), 584–586. https://doi.org/10.1007/BF02015357

Lorson, C. L., Hahnen, E., Androphy, E. J., & Wirth, B. (1999). A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(11), 6307–11. https://doi.org/10.1073/pnas.96.11.6307

Ma, H., Baumann, C. T., Li, H., Strahl, B. D., Rice, R., Jelinek, M. A., … Stallcup, M. R. (2001). *Hormone-dependent, CARM1-directed, arginine-specific methylation of histone H3 on a steroid-regulated promoter*. *Current Biology* (Vol. 11). https://doi.org/10.1016/S0960-9822(01)00600-5

Ma, W.-J., Chung, S., & Furneaux, H. (1997). The Elav-like proteins bind to AU-rich elements and to the poly(A) tail of mRNA. *Nucleic Acids Research*, *25*(18), 3564–3569. Retrieved from

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC146929/pdf/253564.pdf

Ma, W. J., Cheng, S., Campbell, C., Wright, A., & Furneaux, H. (1996). Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. *The Journal of Biological Chemistry*, *271*(14), 8144–51. https://doi.org/10.1074/JBC.271.14.8144

MacLeod, M. J., Taylor, J. E., Lunt, P. W., Mathew, C. G., & Robb, S. a. (1999). Prenatal onset spinal muscular atrophy. *European Journal of Paediatric Neurology : EJPN : Official Journal of the European Paediatric Neurology Society*, *3*(2), 65–72. https://doi.org/10.1016/S1090-3798(99)80015-4

Martinez, T. L., Kong, L., Wang, X., Osborne, M. A., Crowder, M. E., Van Meerbeke, J. P., … Sumner, C. J. (2012). Survival Motor Neuron Protein in Motor Neurons Determines Synaptic Integrity in Spinal Muscular Atrophy. *Journal of Neuroscience*, *32*(25), 8703–8715. https://doi.org/10.1523/JNEUROSCI.0204-12.2012

Marusich, M. F., Furneaux, H. M., Henion, P. D., & Weston, J. A. (1994). Hu neuronal proteins are expressed in proliferating neurogenic cells. *Journal of Neurobiology*, *25*(2), 143–155. https://doi.org/10.1002/neu.480250206

McAndrew, P. E., Parsons, D. W., Simard, L. R., Rochette, C., Ray, P. N., Mendell, J. R., … Burghes, A. H. (1997). Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNT and SMNC gene copy number. *American Journal of Human Genetics*, *60*(6), 1411–22. https://doi.org/10.1086/515465

Meister, G., Eggert, C., & Fischer, U. (2002). SMN-mediated assembly of RNPs: a

complex story. *Trends Cell Biol*, *12*(10), 472–478. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12441251

Michel, R. N., Vu, C. Q., Tetzlaff, W., & Jasmin, B. J. (1994). Neural regulation of acetylcholinesterase mRNAs at mammalian neuromuscular synapses. *The Journal of Cell Biology*, *127*(4). Retrieved from http://jcb.rupress.org/content/127/4/1061?ijkey=3790afd49f06f890b6369cdf590e5cf4d2508f12&keytype2=tf_ipsecsha

Mobarak, C. D., Anderson, K. D., Morin, M., Beckel-Mitchener, A., Rogers, S. L., Furneaux, H., … Perrone-Bizzozero, N. I. (2000). The RNA-binding protein HuD is required for GAP-43 mRNA stability, GAP-43 gene expression, and PKC-dependent neurite outgrowth in PC12 cells. *Molecular Biology of the Cell*, *11*(9), 3191–3203. https://doi.org/10.1091/mbc.11.9.3191

Monani, U. R. (2005). Spinal muscular atrophy: a deficiency in a ubiquitous protein; a motor neuron-specific disease. *Neuron*, *48*(6), 885–896. https://doi.org/10.1016/j.neuron.2005.12.001

Monani, U. R., Lorson, C. L., Parsons, D. W., Prior, T. W., Androphy, E. J., Burghes, A. H. M., & McPherson, J. D. (1999). A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. *Hum Mol Genet*, *8*(7), 1177–1183. https://doi.org/10.1093/hmg/8.7.1177

Monani, U. R., Sendtner, M., Coovert, D. D., Parsons, D. W., Andreassi, C., Le, T. T., … Burghes, a H. (2000). The human centromeric survival motor neuron gene (SMN2)

rescues embryonic lethality in Smn(-/-) mice and results in a mouse with spinal muscular atrophy. *Human Molecular Genetics*, *9*(3), 333–339. https://doi.org/10.1093/hmg/ddm236

Moosa, A., & Dubowitz, V. (1973). Spinal muscular atrophy in childhood. Two clues to clinical diagnosis. *Arch Dis Child*, *48*(5), 386–388. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4703068

Mourelatos, Z., Abel, L., Yong, J., Kataoka, N., & Dreyfuss, G. (2001). SMN interacts with a novel family of hnRNP and spliceosomal proteins. *EMBO Journal*, *20*(19), 5443–5452. https://doi.org/10.1093/emboj/20.19.5443

Murray, L. M., Beauvais, A., Bhanot, K., & Kothary, R. (2013). Defects in neuromuscular junction remodelling in the Smn2B/− mouse model of spinal muscular atrophy. *Neurobiology of Disease*, *49*, 57–67. https://doi.org/10.1016/j.nbd.2012.08.019

Nölle, A., Zeug, A., Van bergeijk, J., Tönges, L., Gerhard, R., Brinkmann, H., … Claus, P. (2011). The spinal muscular atrophy disease protein SMN is linked to the Rho-kinase pathway via profilin. *Human Molecular Genetics*, *20*(24), 4865–4878. https://doi.org/10.1093/hmg/ddr425

O'Brien, K. B., Alberich-Jorda, M., Yadav, N., Kocher, O., DiRuscio, A., Ebralidze, A., … Kobayashi, S. (2010). CARM1 is required for proper control of proliferation and differentiation of pulmonary epithelial cells. *Development*, *137*(13), 2147–2156. https://doi.org/10.1242/dev.037150

Okano, H. J., & Darnell, R. B. (1997). A Hierarchy of Hu RNA Binding Proteins in Developing and Adult Neurons. *Journal of Neuroscience*, *17*(9). Retrieved from http://www.jneurosci.org/content/17/9/3024.long

Owen, N. (2000). Characterization of the Schizosaccharomyces pombe orthologue of the human survival motor neuron (SMN) protein. *Human Molecular Genetics*, *9*(5), 675–684. https://doi.org/10.1093/hmg/9.5.675

Pang, L., Tian, H., Chang, N., Yi, J., Xue, L., Jiang, B., … Wang, W. (2013). Loss of CARM1 is linked to reduced HuR function in replicative senescence. *BMC Molecular Biology*, *14*, 15. https://doi.org/10.1186/1471-2199-14-15

Paushkin, S., Charroux, B., Abel, L., Perkinson, R. A., Pellizzoni, L., & Dreyfuss, G. (2000). The survival motor neuron protein of Schizosacharomyces pombe: Conservation of survival motor neuron interaction domains in divergent organisms. *Journal of Biological Chemistry*, *275*(31), 23841–23846. https://doi.org/10.1074/jbc.M001441200

Pearn, J. (1980). CLASSIFICATION OF SPINAL MUSCULAR ATROPHIES. *The Lancet*. https://doi.org/10.1016/S0140-6736(80)90847-8

Pellizzoni, L., Charroux, B., & Dreyfuss, G. (1999). SMN mutants of spinal muscular atrophy patients are defective in binding to snRNP proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(20), 11167–72. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10500148

Piazzon, N., Rage, F., Schlotter, F., Moine, H., Branlant, C., & Massenet, S. (2008). In

vitro and in Cellulo evidences for association of the survival of motor neuron complex with the fragile X mental retardation protein. *Journal of Biological Chemistry*, *283*(9), 5598–5610. https://doi.org/10.1074/jbc.M707304200

Piepers, S., van den Berg, L. H., Brugman, F., Scheffer, H., Ruiterkamp-Versteeg, M., van Engelen, B. G., … Wokke, J. H. (2008). A natural history study of late onset spinal muscular atrophy types 3b and 4. *J Neurol*, *255*(9), 1400–1404. https://doi.org/10.1007/s00415-008-0929-0

Ponting, C. P. (1997). Tudor domains in proteins that interact with RNA. *Trends in Biochemical Sciences*. https://doi.org/10.1016/S0968-0004(96)30049-2

Prior, T. W., Swoboda, K. J., Scott, H. D., & Hejmanowski, A. Q. (2004). Homozygous SMN1 deletions in unaffected family members and modification of the phenotype by SMN2. *American Journal of Medical Genetics*, *130 A*(3), 307–310. https://doi.org/10.1002/ajmg.a.30251

Rajendra, T. K., Gonsalvez, G. B., Walker, M. P., Shpargel, K. B., Salz, H. K., & Matera, A. G. (2007). A Drosophila melanogaster model of spinal muscular atrophy reveals a function for SMN in striated muscle. *J Cell Biol*, *176*(6), 831–841. https://doi.org/10.1083/jcb.200610053

Rossoll, W., Jablonka, S., Andreassi, C., Kröning, A. K., Karle, K., Monani, U. R., & Sendtner, M. (2003). Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of β-actin mRNA in growth cones of motoneurons. *Journal of Cell Biology*, *163*(4), 801–812.

https://doi.org/10.1083/jcb.200304128

Rossoll, W., Kröning, A.-K., Ohndorf, U.-M., Steegborn, C., Jablonka, S., & Sendtner, M. (2002). Specific interaction of Smn, the spinal muscular atrophy determining gene product, with hnRNP-R and gry-rbp/hnRNP-Q: a role for Smn in RNA processing in motor axons? *Human Molecular Genetics*, *11*(1), 93–105. https://doi.org/10.1093/hmg/11.1.93

Rudnicki, M. A., & Jaenisch, R. (1995). The MyoD family of transcription factors and skeletal myogenesis. *BioEssays*, *17*(3), 203–209. https://doi.org/10.1002/bies.950170306

Sabra, M., Texier, P., El Maalouf, J., & Lomonte, P. (2013). The Tudor protein survival motor neuron (SMN) is a chromatin-binding protein that interacts with methylated lysine 79 of histone H3. *Journal of Cell Science*, *126*(Pt 16), 3664–77. https://doi.org/10.1242/jcs.126003

Sanchez, G., Dury, A. Y., Murray, L. M., Biondi, O., Tadesse, H., El Fatimy, R., … Cote, J. (2013). A novel function for the survival motoneuron protein as a translational regulator. *Hum Mol Genet*, *22*(4), 668–684. https://doi.org/10.1093/hmg/dds474

Schrank, B., Götz, R., Gunnersen, J. M., Ure, J. M., Toyka, K. V, Smith, A. G., & Sendtner, M. (1997). Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(18), 9920–5. https://doi.org/10.1073/pnas.94.18.9920

Schroth, M. K. (2009). Special considerations in the respiratory management of spinal muscular atrophy. *Pediatrics*, *123 Suppl*, S245-9. https://doi.org/10.1542/peds.2008-2952K

Selenko, P., Sprangers, R., Stier, G., Bühler, D., Fischer, U., & Sattler, M. (2001). SMN tudor domain structure and its interaction with the Sm proteins. *Nature Structural Biology*, *8*(1), 27–31. https://doi.org/10.1038/83014

Setola, V., Terao, M., Locatelli, D., Bassanini, S., Garattini, E., & Battaglia, G. (2007). Axonal-SMN (a-SMN), a protein isoform of the survival motor neuron gene, is specifically involved in axonogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(6), 1959–64. https://doi.org/10.1073/pnas.0610660104

Shafey, D., Côté, P. D., & Kothary, R. (2005). Hypomorphic Smn knockdown C2C12 myoblasts reveal intrinsic defects in myoblast fusion and myotube morphology. *Experimental Cell Research*, *311*(1), 49–61. https://doi.org/10.1016/j.yexcr.2005.08.019

Shafey, D., MacKenzie, A. E., & Kothary, R. (2008). Neurodevelopmental abnormalities in neurosphere-derived neural stem cells from SMN-depleted mice. *Journal of Neuroscience Research*, *86*(13), 2839–2847. https://doi.org/10.1002/jnr.21743

Sharma, A., Lambrechts, A., Le, T. T. H., Le, T. T. H., Sewry, C. A., Ampe, C., … Morris, G. E. (2005). A role for complexes of survival of motor neurons (SMN) protein with gemins and profilin in neurite-like cytoplasmic extensions of cultured

nerve cells. *Experimental Cell Research*, *309*(1), 185–197. https://doi.org/10.1016/j.yexcr.2005.05.014

Simon, A. M., & Burden, S. J. (1993). An E box mediates activation and repression of the acetylcholine receptor delta-subunit gene during myogenesis. *Molecular and Cellular Biology*, *13*(9), 5133–40. https://doi.org/10.1128/MCB.13.9.5133

Sprangers, R., Groves, M. R., Sinning, I., & Sattler, M. (2003). High-resolution X-ray and NMR structures of the SMN Tudor domain: conformational variation in the binding site for symmetrically dimethylated arginine residues. *Journal of Molecular Biology*, *327*(2), 507–520. https://doi.org/10.1016/S0022-2836(03)00148-7

Srikantan, S., & Gorospe, M. (2012). HuR function in disease. *Frontiers in Bioscience (Landmark Edition)*, *17*, 189–205. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22201738

Stallcup, M. R., Chen, D., Koh, S. S., Ma, H., Lee, Y.-H., Li, H., … Aswadt, D. W. (2000). Co-operation between protein-acetylating and protein-methylating co-activators in transcriptional activation. Retrieved from http://www.biochemsoctrans.org/content/ppbiost/28/4/415.full.pdf

Staropoli, J. F., Li, H., Chun, S. J., Allaire, N., Cullen, P., Thai, A., … Carulli, J. P. (2015). Rescue of gene-expression changes in an induced mouse model of spinal muscular atrophy by an antisense oligonucleotide that promotes inclusion of SMN2 exon 7. *Genomics*, *105*(4), 220–228. https://doi.org/10.1016/j.ygeno.2015.01.007

Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M., Wong, E., Henson, J., … Furneaux, H.

M. (1991). HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and sex-lethal. *Cell*, *67*(2), 325–333. https://doi.org/10.1016/0092-8674(91)90184-Z

Tadesse, H., Deschênes-Furry, J., Boisvenue, S., & Côté, J. (2008). KH-type splicing regulatory protein interacts with survival motor neuron protein and is misregulated in spinal muscular atrophy. *Human Molecular Genetics*, *17*(4), 506–524. https://doi.org/10.1093/hmg/ddm327

Terns, M. P., & Terns, R. M. (2001). Macromolecular complexes: SMN--the master assembler. *Current Biology : CB*, *11*(21), R862-4. https://doi.org/S0960-9822(01)00517-6 [pii]

Valsecchi, V., Boido, M., De Amicis, E., Piras, A., & Vercelli, A. (2015). Expression of Muscle-Specific MiRNA 206 in the Progression of Disease in a Murine SMA Model. *PloS One*, *10*(6), e0128560. https://doi.org/10.1371/journal.pone.0128560

van Bergeijk, J., Rydel-Könecke, K., Grothe, C., & Claus, P. (2007). The spinal muscular atrophy gene product regulates neurite outgrowth: importance of the C terminus. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *21*(7), 1492–1502. https://doi.org/10.1096/fj.06-7136com

van der Giessen, K., Di-Marco, S., Clair, E., & Gallouzi, I. E. (2003). RNAi-mediated HuR depletion leads to the inhibition of muscle cell differentiation. *J Biol Chem*, *278*(47), 47119–47128. https://doi.org/10.1074/jbc.M308889200

Vitte, J., Fassier, C., Tiziano, F. D., Dalard, C., Soave, S., Roblot, N., … Melki, J. (2007).

Refined characterization of the expression and stability of the SMN gene products. *The American Journal of Pathology*, *171*(4), 1269–80. https://doi.org/10.2353/ajpath.2007.070399

Vitte, J. M., Davoult, B., Roblot, N., Mayer, M., Joshi, V., Courageot, S., … Melki, J. (2004). Deletion of Murine Smn Exon 7 Directed to Liver Leads to Severe Defect of Liver Development Associated with Iron Overload. *The American Journal of Pathology*, *165*(5), 1731–1741. https://doi.org/10.1016/S0002-9440(10)63428-1

Walker, M. P., Rajendra, T. K., Saieva, L., Fuentes, J. L., Pellizzoni, L., & Matera, A. G. (2008). SMN complex localizes to the sarcomeric Z-disc and is a proteolytic target of calpain. *Hum Mol Genet*, *17*(21), 3399–3410. https://doi.org/10.1093/hmg/ddn234

Wang, I.-F., Reddy, N. M., & Shen, C.-K. J. (2016). Higher order arrangement of the eukaryotic nuclear bodies. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(21), 1–6. https://doi.org/10.1073/pnas.212483099

Wang, J., & Dreyfuss, G. (2001). A cell system with targeted disruption of the SMN gene: Functional conservation of the SMN protein and dependence of Gemin2 on SMN. *Journal of Biological Chemistry*, *276*(13), 9599–9605. https://doi.org/10.1074/jbc.M009162200

Wang, W., Furneaux, H., Cheng, H., Caldwell, M. C., Hutter, D., Liu, Y., … Gorospe, M. (2000). HuR regulates p21 mRNA stabilization by UV light. *Molecular and Cellular Biology*, *20*(3), 760–9. Retrieved from

http://www.ncbi.nlm.nih.gov/pubmed/10629032

Wen, H. L., Lin, Y. T., Ting, C. H., Lin-Chao, S., Li, H., & Hsieh-Li, H. M. (2010). Stathmin, a microtubule-destabilizing protein, is dysregulated in spinal muscular atrophy. *Human Molecular Genetics*, *19*(9), 1766–1778. https://doi.org/10.1093/hmg/ddq058

Wu, J., Cui, N., Wang, R., Li, J., & Wong, J. (2012). A Role for CARM1-Mediated Histone H3 Arginine Methylation in Protecting Histone Acetylation by Releasing Corepressors from Chromatin. *PLoS ONE*, *7*(6), e34692. https://doi.org/10.1371/journal.pone.0034692

Yadav, N., Lee, J., Kim, J., Shen, J., Hu, M. C.-T., Aldaz, C. M., & Bedford, M. T. (2003). Specific protein methylation defects and gene expression perturbations in coactivator-associated arginine methyltransferase 1-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(11), 6464–8. https://doi.org/10.1073/pnas.1232272100

Yang, Y., & Bedford, M. T. (2012). Protein arginine methyltransferases and cancer. *Nature Reviews Cancer*, *13*(1), 37–50. https://doi.org/10.1038/nrc3409

Yano, M., Okano, H. J., & Okano, H. (2005). Involvement of Hu and heterogeneous nuclear ribonucleoprotein K in neuronal differentiation through p21 mRNA post-transcriptional regulation. *The Journal of Biological Chemistry*, *280*(13), 12690–9. https://doi.org/10.1074/jbc.M411119200

Yi, J., Chang, N., Liu, X., Guo, G., Xue, L., Tong, T., … Wang, W. (2009). Reduced

nuclear export of HuR mRNA by HuR is linked to the loss of HuR in replicative senescence. *Nucleic Acids Research*, *38*(5), 1547–1558. https://doi.org/10.1093/nar/gkp1114

Yong, J., Wan, L., & Dreyfuss, G. (2004). Why do cells need an assembly machine for RNA-protein complexes? *Trends in Cell Biology*. https://doi.org/10.1016/j.tcb.2004.03.010

Zerres, K., & Rudnik-Schoneborn, S. (1995). Natural history in proximal spinal muscular atrophy. Clinical analysis of 445 patients and suggestions for a modification of existing classifications. *Arch Neurol*, *52*(5), 518–523. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7733848

Zerres, K., RudnikSchoneborn, S., Forrest, E., Lusakowska, A., Borkowska, J., & HausmanowaPetrusewicz, I. (1997). A collaborative study on the natural history of childhood and juvenile onset proximal spinal muscular atrophy (type II and III SMA): 569 patients. *Journal of the Neurological Sciences*, *146*(1), 67–72.

Zhang, H. L., Pan, F., Hong, D., Shenoy, S. M., Singer, R. H., & Bassell, G. J. (2003). Active transport of the survival motor neuron protein and the role of exon-7 in cytoplasmic localization. *J. Neurosci.*, *23*(16), 6627–6637. https://doi.org/23/16/6627 [pii]

Zhang, H., Xing, L., Rossoll, W., Wichterle, H., Singer, R. H., & Bassell, G. J. (2006). Multiprotein Complexes of the Survival of Motor Neuron Protein SMN with Gemins Traffic to Neuronal Processes and Growth Cones of Motor Neurons.

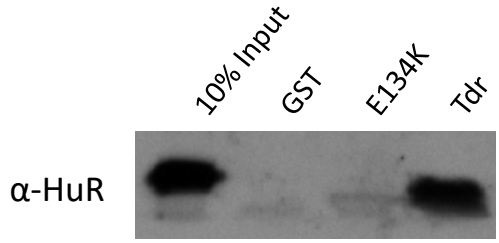
Journal of Neuroscience, *26*(33), 8622–8632. https://doi.org/10.1523/JNEUROSCI.3967-05.2006

Zhang, Z., Lotti, F., Dittmar, K., Younis, I., Wan, L., Kasim, M., … al., et. (2008). SMN Deficiency Causes Tissue-Specific Perturbations in the Repertoire of snRNAs and Widespread Defects in Splicing. *Cell*, *133*(4), 585–600. https://doi.org/10.1016/j.cell.2008.03.031

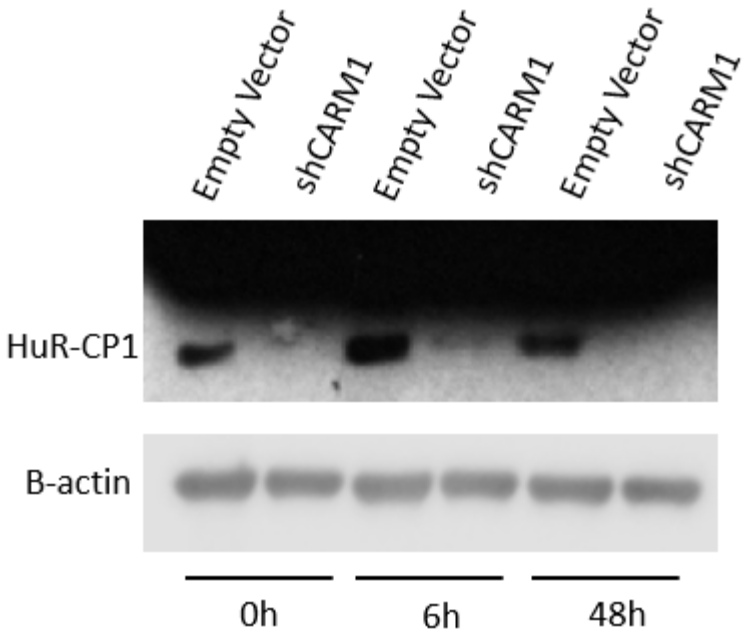
Zhang, Z., Lotti, F., Dittmar, K., Younis, I., Wan, L., Kasim, M., & Dreyfuss, G. (2008). SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. *Cell*, *133*(4), 585–600. https://doi.org/10.1016/j.cell.2008.03.031

Zhao, Z., Chang, F.-C., & Furneaux, H. M. (2000). The identification of an endonuclease that cleaves within an HuR binding site in mRNA. *Nucleic Acids Research*, *28*(14), 2695–2701. https://doi.org/10.1093/nar/28.14.2695

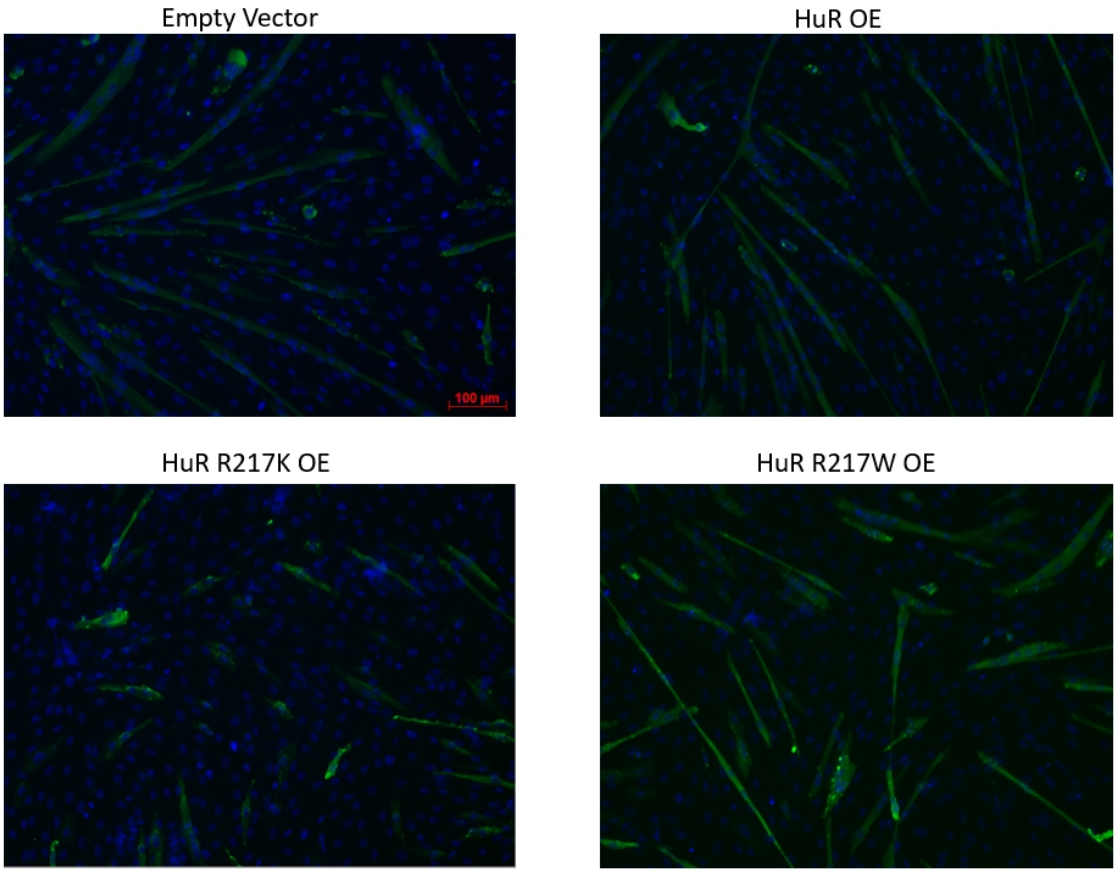
6) APPENDIX



Supplementary Figure 1: HuR interacts with SMN at the Tudor domain. Two variants of a Tudor domain GST-fusion protein were generated and expressed in C2C12. One wildtype variant (Tdr) consisting of the functional Tudor domain, and the other consists of the SMA mutation E134K. GST alone was used as control. GST-pulldowns were performed in proliferating myoblasts and probed for HuR (Genevieve Paris, 2013).



Supplementary Figure 2: Lack of HuR-CP1 in CARM1 deficient cells. Using our stable shCARM1 cell line, alongside our empty vector control, differentiation was induced for 0h, 6h or 48h. Protein extracts were collected and purified from each time point. HuR-CP1 protein levels shown through western blot analysis. (n=1)



Supplementary Figure 3: Methylation of HuR is essential towards myotube formation. C2C12 cells were transfected with our generated methyl-mimic HuR (HuR R217W), our non-methyl mimic (HuR R217K), a wild type HuR as control (HuR WT), and an empty vector control. Cells were then differentiated for 4 days, followed by fixation and immunofluorescent probing for MHC (green). DAPI staining of nuclei is shown in blue. R217K transfected cells revealed patchy areas of myotube formation. Indices not calculated due to the non-random nature of the images. Representative images are shown depicting the localization of HuR relative to its methylation state. Experiment was replicated 4 separate times, each of which 6-7 non-random fields of view were captured.