Characterization of Novel Post-Transcriptional Events Misregulated in Disease: Implications for the Development of Future Therapies

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A thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the
Doctorate in Philosophy degree in Cellular and Molecular Medicine

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Manuscript #1:

Bondy-Chorney, Emma, Tara E. Crawford Parks, Aymeric Ravel-Chapuis, Roscoe Klinck, Lynda Rocheleau, Martin Pelchat, Benoit Chabot, Bernard J. Jasmin, and Jocelyn Côté. "Staufen1 regulates multiple alternative splicing events either positively or negatively in DM1 indicating its role as a disease modifier." *PLoS Genet* 12, no. 1 (2016): e1005827.

Manuscript #2:

Sanchez, Gabriel*, Emma Bondy-Chorney*, Janik Laframboise, Geneviève Paris, Andréanne Didillon, Bernard J. Jasmin, and Jocelyn Côté. "A novel role for CARM1 in promoting nonsense-mediated mRNA decay: potential implications for spinal muscular atrophy." *Nucleic acids research* 44, no. 6 (2015): 2661-2676.

*These authors contributed equally

License number: 3993711428314
License date: Nov 21, 2016
License content publisher: Oxford University Press
License content publication: Nucleic Acids Research

Manuscript #3:

Bondy-Chorney, Emma, R. Mitchell Baldwin, Andréanne Didillon, Benoit Chabot, Bernard J. Jasmin, and Jocelyn Côté. “Identification of Novel Splicing Regulators of Protein Arginine Methyltransferase 1 Exon 2 Alternative Splicing in Breast Cancer”. Manuscript in prep – to be submitted to the International Journal of Biochemistry and Cell Biology.

Abstract

The misregulation of post-transcriptional mechanisms has been linked to the development and progression of numerous human diseases, in particular neurological disorders and cancer. Investigating these misregulated RNA pathways is essential to fully understand the disease mechanisms, identify novel biomarkers, and to develop effective therapies. In this thesis, I present three manuscripts that investigate the mechanisms behind the post-transcriptional misregulation of RNA in human disease, with a focus on pre-mRNA splicing. In the first manuscript (Bondy-Chorney *et al*., 2016a), we investigated the role of Staufen1 (Stau1) in splicing regulation in the neuromuscular disorder Myotonic Dystrophy Type 1 (DM1). Here we report the first insights into the mechanism that Stau1 uses to regulate the alternative splicing of *INSR* exon 11 through an interaction with Alu elements located in intron 10. Moreover, using a high-throughput RT-PCR screen, we uncovered a number of additional Stau1-regulated alternative splicing events in both wild-type and DM1 myoblast cell lines. As Stau1 is known to be aberrantly upregulated in DM1 skeletal muscle, our findings suggest that Stau1 acts as a disease modifier in this disorder. The second manuscript (Sanchez, Bondy-Chorney *et al*., 2015), describes a novel role of the protein methyltransferase Coactivator-Associated Methyltransferase-1 (CARM1), a protein found to be overexpressed in Spinal Muscular Atrophy (SMA). We found that CARM1 can act as a mediator in the nonsense-mediated decay pathway (NMD) and associated UPF1 to promoted its occupancy on PTC-containing transcripts. We identified a subset of natural non-PTC containing NMD targets that were dependent on CARM1, a number of which were misregulated in SMA. This work uncovered a novel role for CARM1 in the NMD pathway and revealed that defective targeting of PTC-containing mRNAs should be included in the complex array of molecular defects associated with SMA. Finally, the third manuscript (Bondy-Chorney *et al*., – in prep) examines the alternative

splicing regulation of the Protein Arginine Methyltransferase *PRMT1* exon 2, an event shown to alter the growth, survival, and invasion of breast cancer cells. Here, we used an RNA interference (RNAi) RT-PCR screen to uncover several splicing proteins that regulate the inclusion of exon 2, several of which we found to be misregulated in a panel of breast cancer cell lines and patient tumours. These findings confirmed that the inclusion of *PRMT1* exon 2 was regulated by alternative splicing via splicing factors that are altered in breast cancer. Moreover, depletion of one of these splicing factors, RALY, resulted in a decrease in the motility and invasive potential of an aggressive breast cancer cell line. These three manuscripts represent a collection of work focused on elucidating the mechanisms involved in post-transcriptional misregulation of RNA in three diverse human diseases. Taken together, the data presented here highlight the broad impact that proteins, such as Stau1 and CARM1, can have in neuromuscular disorders. Moreover, we also uncovered novel misregulation of splicing proteins that alter alternative splicing patterns in breast cancer. Elucidating these mechanisms is of the highest importance in order to identify potential new and effective treatment avenues.

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List of abbreviations

A/G PLUS: Protein A, Protein G

ACCN3: Amiloride-sensitive cation channel 3

ADPGK: ADP-Dependent Glucokinase

AKT2: AKT Serine/Threonine Kinase 2

Alu: Arthrobacter luteus

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMV: Avian Myeloblastosis Virus

ANK1: Ankyrin 1

ANK2: Ankyrin 2

ANOVA: Analysis of variance

APOBEC3C: Apolipoprotein B MRNA Editing Enzyme, Catalytic Polypeptide-Like 3C

ARC: Activity-regulated cytoskeleton-associated protein (also known as Arg3.1)

ARF1: ADP-ribosylation factor 1

ARFRP1: ADP Ribosylation Factor Related Protein 1

ASE: Alternative splicing event

ASF/SF2: Serine/arginine-rich splicing factor 1 (SRSF1), alternative splicing factor 1 (ASF1), pre-mRNA-splicing factor SF2 (SF2)

ASNS: Asparagine Synthetase (Glutamine-Hydrolyzing)

ATCC: American Type Culture Collection

ATF4: Activating Transcription Factor 4

ATM: Ataxia Telangiectasia Mutated

ATPase: Adenylpyrophosphatase

Ay: Lethal Yellow Agouti Mutation

bp: base-pair

BSF: Bicoid stability factor; homolog of human LRPPRC

CACNA1d: Calcium Channel, Voltage-Dependent, L Type, Alpha 1D Subunit

CARM1: Coactivator-Associated Methyltransferase-1

CASC3: Cancer Susceptibility Candidate 3

CCAR1: Cell division cycle and apoptosis regulator protein 1

cDNA: Complementary DNA

CIHR: Canadian Institutes of Health Research

CLC1: Chloride channel 1

CLCN2: Chloride channel 2

CLCN6: Chloride channel 6

CNMD: University of Ottawa Centre for Neuromuscular Disease

CTG: Cytosine-thymine-guanine

CTRL: Control

CUG: Cytosine-uracil-guanine

CUGBP1: CUG-binding Protein 1

DDX5/p68: Asp-Glu-Ala-Asp (DEAD)-Box Helicase 5/Protein 68kDa

DH5α: Doug Hanahan Stain 5

DM1: Myotonic Dystrophy Type I

DMD: Duchenne Muscular Dystrophy

DMEM: Dulbecco's Modified Eagle Medium

DMPK: dystrophia myotonica protein kinase

DNA-PK: DNA-dependent protein kinase

DROSHA: Drosha Ribonuclease III

DTT: Dithiothreitol

E. coli: Escherichia coli

E2+: Exon 2 including transcript

E266Q: Glutamic acid 266 Glutamine

eIF4A3: Eukaryotic Translation Initiation Factor 4A3

EJC: Exon Junction Complex

ELAVL1: Embryonic lethal, abnormal vision - like RNA Binding Protein 1

ENSMUST: Ensembl transcript identifiers mouse transcript

ESE: Exonic Splicing Enhancer

ESS: Exonic Splicing Silencer

FHL3: Four And A Half LIM Domains 3

FMRP: Fragile X mental retardation protein

FN1: Fibronectin 1

FSMA: Families of SMA

FUS: FUS RNA Binding Protein

G6PC3: Glucose 6 Phosphatase Catalytic 3

GADD45a: Growth Arrest And DNA Damage-Inducible Protein Alpha

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GFP: Green fluorescent protein

HA: Human influenza hemagglutinin

HCl: Hydrochloric Acid

HEK293T: Human embryonic kidney cells T antigen of SV40

HeLa: Henrietta Lacks

HepG2: Liver hepatocellular cells

HIF1α: Hypoxia-inducible factor 1-alpha

HITS-CLIP: High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation

hnRNP A2B1: Heterogeneous Nuclear Ribonucleoprotein A2/B1

hnRNP C: Heterogeneous Nuclear Ribonucleoprotein C

hnRNP H: Heterogeneous Nuclear Ribonucleoprotein H

hnRNP R/Q: Heterogeneous Nuclear Ribonucleoprotein R/Q

HPA: Human Protein Atlas

HRP: Horseradish Peroxidase

Hs578T: Human breast tissue the aneuploid mammary epithelial

HuD: Hu Antigen D

IgG: Immunoglobulin G

IHC: Immunohistochemistry

IMP1/ZBP: Insulin-Like Growth Factor 2 MRNA Binding Protein 1/Zipcode-Binding Protein 1

INSR: Insulin Receptor

IP: Immunoprecipitate

IRAlus: Inverted Repeat Alus

ISE: Intronic Splicing Enhancer

ISS: Intronic Splicing Silencer

JAG2: Jagged 2

KCNMA1: Potassium Channel, Calcium-Activated Large Conductance Subfamily M Alpha, Member 1

K-M: Kaplan-Meier

KSRP: KH Type-Splicing Regulatory Protein

LRRC23: Leucine Rich Repeat Containing 23

LTBP4: Latent Transforming Growth Factor-β Binding Protein 4

MAGOH: Mago-Nashi (Drosophila) Homolog

MBNL1: Muscleblind-Like 1

MCF7: Michigan Cancer Foundation-7

MEF: Mouse embryonic fibroblasts

MFE: Minimum free energy

MN-1: Murine motor neuron line

mRNA: Messenger Ribonucleic Acid

MT: Mutant

MyoD: Myogenic Differentiation

NEBuilder: New England Biolabs builder

NFYB: Nuclear Transcription Factor Y Subunit Beta

NF-κB: Nuclear factor-κB

NLS: Nuclear localization signal

NMD: Nonsense-mediated mRNA decay

NRG1: Neuregulin 1

NUTF2: Nuclear Transport Factor 2

OGDH: Oxoglutarate Dehydrogenase

OS: Overall survival

PABP1: Poly-A Binding Protein 1

PBS: Phosphate-buffered saline

pcDH-CMV-MCS-EF1-copGFP:

PDVF: Polyvinylidene fluoride

PMA: Pharbol myristate

PRMT1: Protein Arginine Methyltransferase 1

PRMT3: Protein Arginine Methyltransferase 3

PRPF8: Pre-mRNA Processing Factor 8

PSI: Percent Splicing Index

PSI: Percent splicing index

PTC: Premature terminating codon

PTM: Post-translation modification

RABL5: Intraflagellar Transport 22; RAB, Member RAS Oncogene Family-Like 5

RALY: RNA-binding protein RALY

RAN: Repeat-associated non-ATG translation

RBDs: RNA-binding domains

RBFOX1: RNA Binding Protein, Fox-1 Homolog 1

RBM8a: RNA Binding Motif Protein 8A

RBPs: RNA-binding proteins

RIP: RNA Immunoprecipitate

RIPA: Radioimmunoprecipitation

RNA: Ribonucleic Acid

RNAi: RNA Interference

RPS2: Ribosomal Protein S2

RRM: RNA recognition motif

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

RT-qPCR: Reverse Transcriptase Quantitative Polymerase Chain Reaction

SBSs: Stau1-binding-sites

SDS-PAGE: Sodium dodecyl polyacrylamide gel electrophoresis

SEM: Standard Error of the Mean

SF3B1: Splicing Factor 3b, Subunit 1

SF3B2: Splicing Factor 3b, Subunit 2

SFRS1: Serine/arginine-rich splicing factor 1

SFRS10: Splicing Factor, Arginine/Serine-Rich 10, aka. TRA2β

SHKBP1: SHC Binding And Spindle Associated 1

shRNA: Short Hairpin RNA

SMA: Spinal Muscular Atrophy

SMA: Spinal muscular atrophy

SMG-1: Serine/Threonine-Protein Kinase SMG1

SMN: Survival of Motor Neurons

snRNP: Small nuclear ribonucleoprotein particle

SNW1: SNW domain containing protein 1

SOC: Super Optimal broth with Catabolite repression

STaR: Scholarships in Translational Research

Stau1: Staufen1

Stau2: Staufen2

SUMO: Small ubiquitin-like modifiers

TBC1D12/13: TBC1 Domain Family, Member 12

TBPH: TAR DNA-binding protein-43 homolog or TDP-43

TDD: Translation-dependent decay

TGFβ: Transforming Growth Factor Beta

THRA: Thyroid Hormone Receptor, Alpha

TNFα: Tumor necrosis factor, Alpha

TNNT2: cardiac Troponin T

U2AF65: U2 snRNP auxiliary factor 65-kDa subunit

UCSC: University of California, Santa Cruz

uOBMRI: University of Ottawa Brain and Mind Research Institute

uORF: Upstream open reading frame

UPF1: Up-Frameshift Suppressor 1 Homolog

UPF2: Up-Frameshift Suppressor 2 Homolog

UPF3: Up-Frameshift Suppressor 3 Homolog

USPL1: Ubiquitin-specific protease-like 1

UTR: Untranslated region

WT: Wild Type

Acknowledgments

As always, I would like to first thank science. I am forever grateful that I live in a time and a place where any individual can strive to understand the universe around them through science. To paraphrase Elon Musk, why be afraid of the dark when the dark is just the absence of photons in the visible wavelength between 400-700 nanometers.

I would like to sincerely thank my co-supervisors, Dr. Jocelyn Côté and Dr. Bernard J. Jasmin. Thanks to their continued support I have been able to spend the last four years perusing my passion of investigating the mechanisms of pre-mRNA splicing. The hard work and dedication of both Jocelyn and Bernard gave to my research will always be appreciated and never forgotten. I would also like to thank the members of my TAC, Dr. F. Jeffrey Dilworth, Dr. Martin Holcik, and Dr. Katey Rayner for the help and discussions during my PhD. Moreover, I would like to thank all of the collaborators I had the opportunity to work with over these years, in particular Dr. Benoit Chabot.

The friends and labmates from the Côté and Jasmin labs I have worked with at the University of Ottawa are amazing and I will forever be inspired by the level of dedication and commitment to research I have seen from them. I would like to extend a special thanks to Dr. Tara Crawford-Parks, Andréanne Didillon, Christine Peladeau, Dr. Alan Morettin and Dr. Mitch Baldwin for the immense amount of support and patience they have given to me during the good times and the bad, we know there was more than enough of both!

To my family, you are the reason I can do this. I was raised with the freedom to choose my path in life and I can never be more thankful to my mom Jan, and dad Richard, for supporting my decisions along the way. I honestly believe not many people have been raised with such love and acceptance for who they are. I have also been lucky enough to have an amazing mother-, father- and sister-in-law, Fabie, Dave and Carol-Ann, who have given much support and who always listened to my endless work stories around the dinner table. Finally, my love Stacy. You are an incredible man whose kindness and love for me, my research and our families (including the all of the weasels, chickens, puppies, and spiders!), never ceases to amaze me. I am so lucky to have you in my life.

Chapter 1 – Introduction

In eukaryotic cells, genetic information is encoded by DNA, which is transcribed to form RNA, before it is converted into polypeptides by translation, the process that produces proteins, the workhorses of the cell. This idea is referred to as the central dogma and describes the major steps generally taken in the flow of genetic information (Crick, 1970). Although, the central dogma portrays RNA as simply an intermediate step required to go from DNA to protein, the importance of RNA should not be underestimated. In fact, the 'RNA world' hypothesis, the idea that RNA molecules are the precursors to all of life on Earth, is a widely accepted model that highlights the diversity, complexity, and necessity of RNA (Joyce, 2002). Indeed, as described in this RNA world, RNA served to play all the genetic, catalytic and regulatory roles required for life, prior to the emergence of DNA and proteins (Copley *et al.*, 2007). Eventually, some of the diverse roles RNA played in this world were replaced by DNA and proteins, however, the importance of successful RNA regulation remains crucial. In fact, as will be discussed in this thesis, the disruption of many steps in the processes of RNA regulation can cause or contribute to numerous human diseases.

The processes involved in the post-transcriptional regulation of RNA require various molecular machines and numerous layers of regulatory mechanisms. Here, we focus on several regulatory pathways involved in the post-transcriptional regulation of gene expression. Post-transcriptional regulation occurs immediately following transcription initiation (although many RNA processing steps do occur co-transcriptionally) and continues until translation termination. The following brief introduction describes the important steps involved in the post-transcriptional regulation of precursor messenger mRNA (pre-mRNA) and its mRNA product (Fig 1).

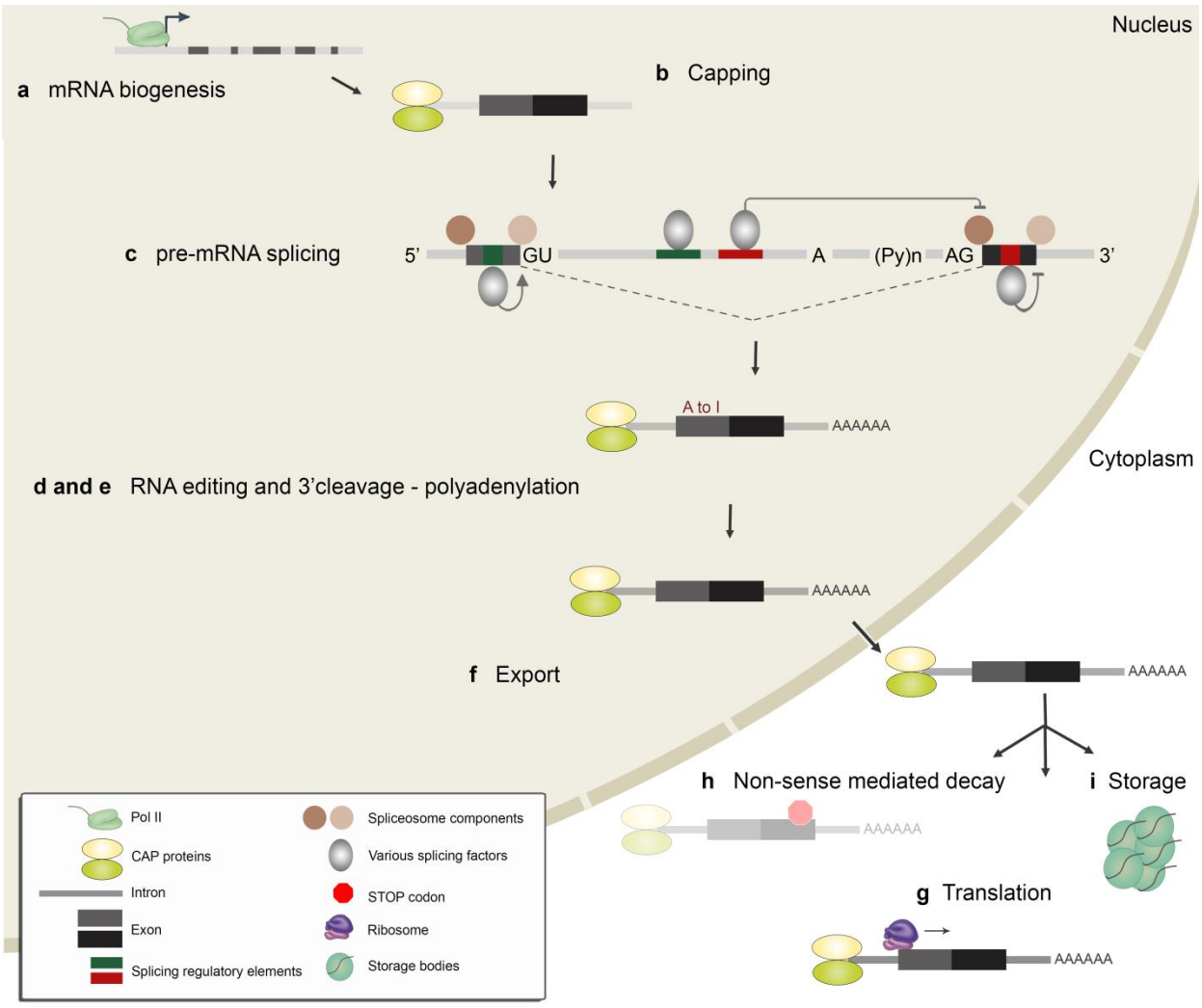

Figure 1. The major processes that occur during post-transcriptional regulation of RNA.

(A) mRNA biogenesis is represented here with binding and initiation of transcription by RNA Polymerase II (Green structure). (B) Shown here is the addition of a 7-methylguanosine cap structure to the 5' end (yellow and green circles) of a RNA following the synthesis of 20-30 nucleotides of the transcript. (C) pre-mRNA splicing and alternative splicing by the spliceosome (grey circles) and other splicing factors (dark and light brown circles) – coupled to transcription (D-E) The additional modification steps of the RNA including

RNA editing, 3’end cleavage and polyadenylation. (F) The mRNA is exported from the nucleus to the cytoplasm. (H) The decay of

(B) mRNA units that habours a pre-termination stop codon (red hexagon) during the pioneer round of translation located in the cytoplasm. (G-I) mRNA location, mRNA storage and translation of mRNA into a polypeptide chain by the formation of the ribosome (pink and purple structure) in the cytoplasm.

1.1 Post-transcriptional regulation of gene expression

Post-transcriptional regulatory steps begin quickly following the start of transcription of pre-mRNA by the DNA-directed RNA polymerase II (Pol II) (Fig 1a). Considered the first step in the processing of pre-mRNA, the addition of a 7-methylguanosine cap structure to the 5' end of a RNA, occurs after the synthesis of 20-30 nucleotides (nts) of the transcript and has been shown to be essential for cell viability (Cowling, 2010; Ghosh and Lima, 2010; Martinez-Rucobo *et al.*, 2015; Shuman, 2000) (Fig 1b). Similar to capping, pre-mRNA splicing, the process of removing intervening sequences of genes called introns and the joining together of the coding regions called exons, occurs on actively transcribing RNA molecules (Beyer and Osheim, 1988) (Fig 1c). This highly regulated phenomenon is considered one of the most complex cellular process and requires the formation of a dynamic multi-megadalton ribonucleoprotein (RNP) complex called the spliceosome. The pre-mRNA is subjected to several additional modification steps while still in the nucleus including RNA editing, 3'end cleavage and polyadenylation (Fig 1d and e). Once these processing steps are complete, the pre-mRNA, now referred to as a mature mRNA transcript, is surrounded by and interacts with a specific array of specialized proteins that regulate and assist in the mRNA's nuclear export into the cytoplasm, and subsequently, its fate there (Fig 1f) (Glisovic *et al.*, 2008; Wickramasinghe and Laskey, 2015).

Cytoplasmic mRNA transcripts are then subject to several key regulatory mechanisms such as localization, translation, storage and/or degradation (Fig 1g-i). mRNA localization provides both temporal and spatial control over transcript expression through the movement of translationally repressed mRNAs to distinct regions in the cytoplasm (Martin and Ephrussi, 2009). The benefits of mRNA localization can easily be recognized in the regulation of several mRNAs in *Drosophila* required to establish the morphogen gradients required for spatial patterning of the developing

embryo (Duchaîne *et al.*, 2002; Johnstone and Lasko, 2001; Mallardo *et al.*, 2003). In mammals, mRNA localization is mostly seen in highly polarized cells such as neurons, however, this regulatory mechanism is also important in fibroblasts during development and has been observed in invading cancer cells (Orr-Urtreger *et al.*, 1993; Wang *et al.*, 2005). Several post-transcriptional processes, such as mRNA decapping, mRNA storage, and mRNA silencing, are thought to occur within cytoplasmic RNA processing bodies, termed P-bodies, which are discrete aggregates containing both protein and RNA factors (Fig 1i) (Eulalio *et al.*, 2007). Nonsense-mediated mRNA decay (NMD) is a type of surveillance mechanism employed by the cell to allow for quality control of specific mRNA transcripts (Hentze and Kulozik, 1999; Hug *et al.*, 2016; Yepiskoposyan *et al.*, 2011). NMD ensures that transcripts, which could produce truncated proteins harbouring potential deleterious effects, are degraded prior to translation (Wagner and Lykke-Andersen, 2002). Furthermore, the NMD process is now recognized as an important pathway that regulates the overall gene expression patterns of diverse classes of transcripts (Mendell *et al.*, 2004; Pan *et al.*, 2006).

Finally, mRNAs undergo translation, a process by which the mRNA is read by the ribosome, in groups of three base pairs called codons that correspond to specific amino acids, to form the chain of amino acids that make a protein (Chekulaeva and Landthaler, 2016) (Fig 1j). Taken together, these processes represent the major steps in post-transcriptional regulation of mRNA transcripts. Virtually every step in the life of an RNA transcript requires the formation RNP complexes, dynamic structures formed through interactions between RNA and RNA-binding proteins (RBPs).

1.2 RBPs

RBPs are a diverse group of >500 proteins, each possessing unique binding characteristics towards both RNA transcripts and other proteins (Anantharaman *et al.*, 2002). To date, eleven classical RNA-binding domains (RBDs) have been described and recently, several unorthodox RBPs which lack the classical RBDs were identified (Castello *et al.*, 2016). It is thought that through the diverse arrangement and number of these domains, RBPs can achieve differing degrees of specificity and affinity. These domains include the RNA-recognition motif (RRM), K-homology (KH) domain (type I and II), double-stranded RBD (dsRBD), Arg-Gly-Gly (RGG) box, Sm domain, DEAD/DEAH box, Zinc fingers, S1 domain, Piwi/Argonaut/Zwille (PAZ) domains, cold-shock domain, trp RNA-binding Attenuation Protein domain (TRAP), Pumilio/FBF (PUF), and the Sterile alpha motif (SAM) domain. Some RBPs, such as the most common RBP the RRM, typically recognize several nts of single-stranded RNA of a specific sequence (Burd and Dreyfuss, 1994). Alternatively, some RBDs, for instance the dsRBDs found in the Drosophila Staufen RBP (St Johnston *et al.*, 1992), identify their dsRNA target through shape-specific recognition of the minor-major-minor groove pattern of dsRNA and are likely acting independent of sequence content (Li *et al.*, 2014). Taken together, RBPs are a diverse group of proteins made up of hundreds of members that participate in virtually every step of RNA processing. It has been suggested that one of the major drivers of the RBP expansion in evolution emerged from the development of pre-mRNA splicing, a highly complex process that requires a large number of RBPs (Anantharaman *et al.*, 2002; Glisovic *et al.*, 2008).

1.3 Pre-mRNA splicing

Pre-mRNA splicing is a critical regulatory step in the regulation of gene expression. Although pre-mRNA splicing is evolutionary conserved, the extent to which an organism uses this process

varies. For example, there are only a handful of instances of pre-mRNA splicing in the genome of S. cerevisiae, as only 3% of this organism's annotated genes even contain introns, accounting for <1% of the entire genome (Spingola *et al.*, 1999). In contrast, human introns are reported to make up 25.9% of the genome with the typical human gene containing 7.8 introns which on average, are 10-100 times longer than exons (Lander *et al.*, 2001). These introns retain many essential functions, such as contributing to regulating the transcriptional expression of a gene, however they must be spliced out in order to form the mature mRNA transcript required for functional protein production.

1.3.1 Small-nuclear ribonucleoproteins (snRNPs) and SMN

Pre-mRNA splicing requires the assembly of complex ribonucleoparticles, called snRNPs. snRNPs are made up of specialized snRNAs and a collection of highly-bound associated proteins. The snRNAs, namely U1, U2, U4, U5, and U6, belong to the Sm and Sm-like classes of RNA, respectively (Matera *et al.*, 2007). The cytoplasmic assembly of the core snRNPs is mediated by the survival of motor neuron (SMN) protein complex. This large, stable ~50S macromolecular complex is made up of SMN and at least eight tightly associated components including GEMINS 2-8 and unr-interacting protein (Unrip) (Carissimi *et al.*, 2005; Pellizzoni *et al.*, 2002). SMN is transported into the nucleus, quickly dissociates from the snRNPs, and has been shown to accumulate in distinct nuclear structures called Gemini of Cajal bodies (Gems) (Gubitz *et al.*, 2004). snRNPs can then be observed, along with numerous other pre-mRNA splicing factors, enriched in highly dynamic nuclear structures called nuclear speckles. These structures are localized in interchromatin regions and are thought to serve as storage, assembly, and/or modification compartments (Spector and Lamond, 2011). It is between these nuclear speckles and the chromatin domains where the majority of splicing activity is thought to occur, although the

exact mechanisms regarding the transport of splicing proteins from the nuclear speckles to the actively transcribing sites are still poorly understood (Matera and Wang, 2014).

1.3.2 The spliceosome and the splicing cycle

Humans have two types of spliceosomes, the major and minor spliceosome, which catalyze the removal of U2-type and the less abundant U12-type introns, respectively. The major spliceosome, which processes ~95.5% of all introns (Turunen *et al.*, 2013), is comprised of 5 U snRNPs (U1, U2, U4, U5, and U6) and >300 additional protein cofactors (Jurica and Moore, 2003; Will and Lührmann, 2001). The initiation of pre-mRNA splicing begins with the assembly of the spliceosome on the pre-mRNA in order to carry out the splicing reaction (Fig 2). This complex process involves the recognition of junctions that will allow for the definition between the introns and the exons. This recognition is driven by the ability of the spliceosomal snRNPs to recognize core elements comprised of conserved short nucleotide motifs found in the pre-mRNA, called splicing signals. These signals include the 5' splice site (5'SS) at the beginning of an intron, the branch point (BP) adenosine, and the 3' splice site (3'SS) AG found at the intron end (Burge, 1999; Cartegni *et al.*, 2002).

The four phases of the splicing cycle can be described as: 1) spliceosome assembly, 2) spliceosome activation, 3) catalytic reactions, and 4) spliceosome disassembly (Liu and Cheng, 2015). Splicing occurs by two sequential chemical transesterification steps: 1) the 2'-hydroxyl (OH) of the branch point adenosine acts as a nucleophile that attacks the 5'SS to form 2'-5' branched lariat intermediate. This step liberates the 5' exon, 2) the 3'OH of the 5'exon attacks the phosphate at the 3'SS to join the exons forming the mature mRNA product. This step liberates the lariat intron. These reactions are catalyzed by the spliceosome and in fact, are the same steps used by self-splicing group II catalytic introns (Sharp and Eisenberg, 1987). Indeed, it is now widely

accepted that the spliceosome is an RNA-catalyzed machine, whereby its catalytic center, made up of the stem between the snRNAs U2 and U6, can catalyze a splicing-related reaction by themselves (Fica *et al.*, 2013; Valadkhan *et al.*, 2007). The spliceosome assembly is a complex and dynamic, but highly ordered pathway that has been extensively studied *in vitro* and is known to be largely conserved between yeast and humans (Du and Rosbash, 2002; Fica *et al.*, 2013; Will and Lührmann, 2011). The assembly of the major spliceosome can also occur through alternative assembly pathways, called exon or intron definition, that is dependent on whether the intron size is greater than or less than 200-250 bps in length, respectively (Will and Lührmann, 2011).

1.4 Alternative pre-mRNA splicing

Alternative splicing is a phenomenon that allows for the generation of multiple mRNA alternative isoforms from a single pre-mRNA transcription unit. Recent deep sequencing results estimate that alternative splicing occurs in 95-100% of all multi-exon human genes (Gerstein *et al.*, 2014; Nilsen and Graveley, 2010; Pan *et al.*, 2008; Wang *et al.*, 2008). Alternative splicing is considered to be one of the major pathways which allows for such high degrees of diversity and complexity seen in both the eukaryotic transcriptome and proteome (Barbosa-Morais *et al.*, 2012; Merkin *et al.*, 2012; Nilsen and Graveley, 2010; Pan *et al.*, 2008; Wang *et al.*, 2008). One of the most striking examples of the complexity of alternative splicing is seen in the *Drosophila melanogaster* gene Down syndrome cell adhesion molecule (*Dscam*), which can generate 38,016 distinct mRNA isoforms, a number which exceeds the total number of genes (~14,500) in the organism (Graveley, 2005).

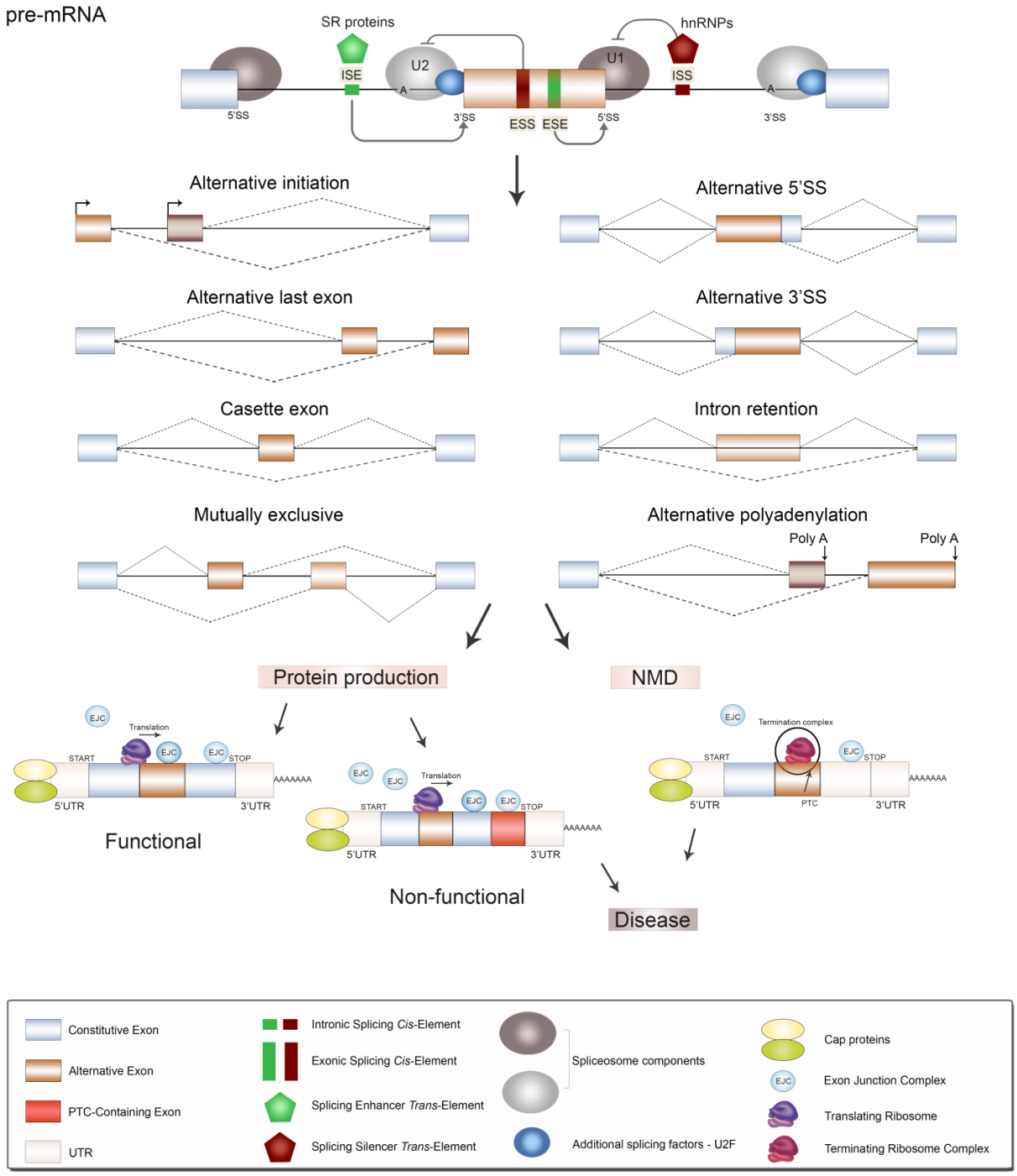

Figure 2. Pre-mRNA constitutive and alternative splicing.

Formation of the spliceosome components over the splice sites and exonic/intronic splicing enhancers and silencers on the pre-mRNA. Alternative exon is represented as an orange box. The several common types of alternative splicing in eukaryotes. The alternative exon affected in the

alternative splicing event is indicated in orange. Following splicing the mRNA is exported from the nucleus and several events can occur including translation into a functional protein (left), translation into a non-functional protein isoform (middle), or the mRNA triggers a decay pathway (eg. NMD) and the transcript is decayed (right).

Several types of alternative splicing are observed in the eukaryotic genome. These types are classified into several major subgroups including: exon skipping, alternative 5' and 3'SS selection, mutually exclusive exons, alternative initiation, alternative polyadenylation, and intron retention (Fig 2) (Keren *et al.*, 2010). Interestingly, many alternative splicing events are evolutionarily conserved among species, both in the conservation of the sequence of the alternatively spliced exon and the occurrence of the event (Keren *et al.*, 2010).

1.4.1 Alternative splicing regulation

Alternative splicing is regulated by both *cis*- and *trans*-regulatory elements, comprised of RNA and protein elements, respectively. *Cis*-regulatory elements serve to either enhance or repress splicing and based on their location in the pre-mRNA, are classified as exonic and intronic splicing enhancers (ESE and ISE) or silencers (ESS and ISS) (Fig 2) (Blencowe, 2000; Cartegni *et al.*, 2002; Graveley, 2000). Additionally, sites located within introns can act as "decoy" splice sites to regulate alternative splicing (Côté *et al.*, 2001). Traditionally, focus has been on regulatory elements located 200-300 nts adjacent to observed splice sites, however recent studies demonstrate that remote regulatory sequences in distal intronic regions are just as important (Lovci *et al.*, 2013). It is through the recruitment of *trans*-acting regulatory factors, such as splicing proteins, to these *cis*-regulatory elements that result in differential splice site selection and thus alternative splicing.

Proteins that can influence splicing pathways are diverse and their contributions to splicing regulation vary. Classic examples of *trans*-acting splicing regulators include serine/arginine-rich (SR) proteins and heterogeneous nuclear (hn)RNPs, which traditionally act to activate or repress splicing activity, respectively. SR proteins usually promote exon inclusion by binding exonic sequences and recruiting spliceosomal components, such as the U1snRNP and the U2 auxiliary factor (U2AF) to the 5'SS and the 3'SS, respectively. Alternatively, hnRNPs most often act as

negative regulators of exon inclusion through less well-defined mechanisms, although, several hnRNPs are known to antagonize the function of SR proteins and mediate downstream splicing events (Fu and Ares Jr, 2014). Moreover, splicing pathways can be context-dependent, for example the neuronal RBP NOVA1 either inhibits or enhances the inclusion of an alternative exon depending on the location of YCAY motifs in the upstream or downstream intron, respectively (Licatalosi *et al.*, 2008). Numerous splicing proteins also rely on features such as cell and/or tissue type, protein concentration and cellular localization to mediate splicing regulatory mechanisms.

1.5 pre-mRNA splicing and disease

In humans, a large number of diseases are associated with mutations or misregulation of various splicing processes (Cooper *et al.*, 2009; Singh and Cooper, 2012). Splicing dysfunction/misregulation can occur by alterations of both *cis*-regulatory elements, such as mutations in core signals (5'SS, 3'SS, etc) or regulatory elements (ESE, ESS, ISE, ISS), and in *trans*-regulatory elements, for instance altered activity/expression levels of splicing proteins (Appendix A). Recent analysis using the SpliceDisease database estimate that >200 human diseases are caused by mutations in splice sites that result in altered splicing patterns of RNA (Wang *et al.*, 2012a). Some of the most striking examples of altered splicing in human disease are seen in neuromuscular disorders, such as Myotonic dystrophy type I (DM1) and Spinal Muscular Atrophy (SMA), as well as in cancer. The three manuscripts presented in this thesis focus on post-transcriptional misregulation, with a focus on pre-mRNA splicing, in DM1, SMA and breast cancer.

1.6 DM1

One of the most studied human disorder associated with splicing defects is DM1. DM1 is an autosomal dominant neuromuscular disorder subdivided into four general phenotype classifications, with each group displaying a varied degree in the severity of their symptoms. The four groups are congenital, childhood onset, adult onset "classic DM1", and late onset/asymptomatic, with the age of onset starting at birth, 1-10 years, 10-30 years, and 20-70 years, respectively. It is estimated that the prevalence of DM1 is 1 in 8000 individuals although the incidence of DM1 varies greatly within certain populations. For example, DM1 is reported to be rarer in Taiwan (Hsiao *et al.*, 2003), whereas the prevalence is much higher in certain populations, where the frequency can reach 1 in 550 in North-eastern Quebec regions (Yotova *et al.*, 2005).

In 1992, 83 years after the first recorded case of DM1, it was discovered that the genetic defect responsible for DM1 was an expansion of CTG repeats in the 3'UTR of the Dystrophia Myotonica Protein Kinase (*DMPK*) gene (Fig 3) (Brook *et al.*, 1992; Fu and Pizzuti, 1992; Mahadevan *et al.*, 1992). In 2001, another muscle dystrophy called DM2 was described and found to also be caused by the expansion of a repeat motif, in this case a CCTG expansion in intron 1 of the Zinc finger domain protein 9 (*ZNF9*) gene (Liquori *et al.*, 2001). In DM1, the number of CTG repeats, can vary from 50 – 4,000 and are thought to be one of the major underlying reasons for the diverse spectrum of DM1 severity seen in patients (Thornton, 2014). CTG repeats have been found to be highly unstable with different repeat sizes being frequently generated with a bias towards an increase expansion in new alleles, on average an increase of >200 repeats from one generation to the next. This increase in repeat number leads to the presence of symptoms at earlier ages in future generations, a phenomenon called anticipation

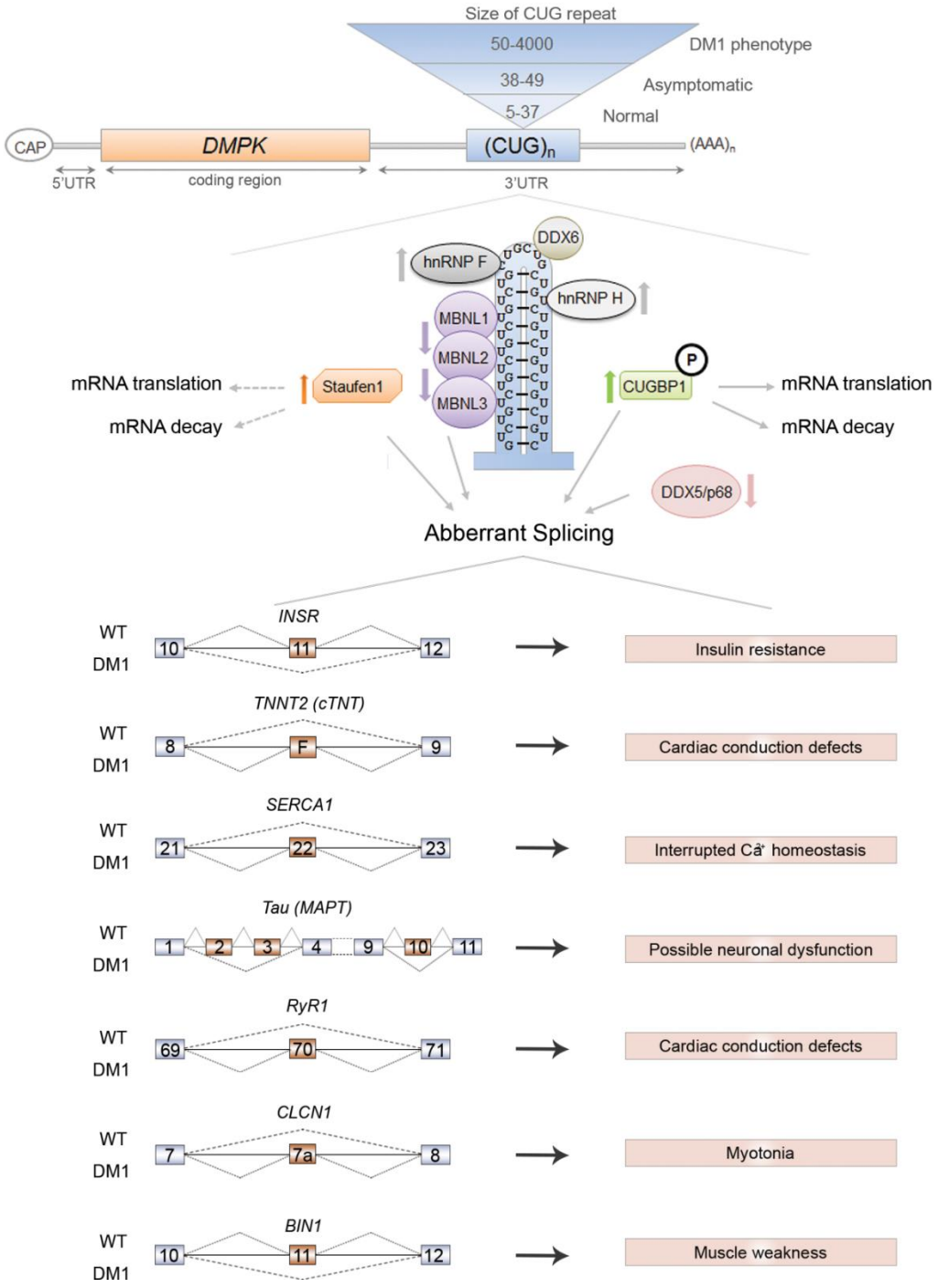

Figure 3. The molecular mechanisms underlying the RNA toxic splicing model of Myotonic Dystrophy Type 1.

Diagram of the RNA-toxic gain of function model of DM1 which shows the expansion of CUG-repeats in the 3'UTR of the DMPK gene. Different sizes of the CUG expansion are shown in the

3’UTR in the blue triangle. Aggerates of the CUG-repeats are found in the nucleus resulting misregulation of RBPs (coloured circles). Arrows located beside the RBPs represent the decrease/increase in either protein levels and/or activities previous found of these protein in DM1. The misregulation of these RBPs is suggested to lead to numerous misregulated alternative splicing events, several common ones of which are represented here (alternative exon shown as an orange box). Each misregulated alternative splicing events is matched with the symptoms seen in DM1 models/patients (detailed in text).

(De Temmerman *et al.*, 2004). This instability of the CTG repeats is also found throughout a person's life in their somatic cells and can vary depending on cell type. In skeletal muscle the CTG repeats have been shown to grow to >2000 and >4000 repeats in patients by 20 and 40 years of age, respectively (Nakamori *et al.*, 2013; Zatz *et al.*, 1995). The increase in CTG repeat number has also been shown to correlate with disease severity in DM1 (Mahadevan *et al.*, 1992; Mahadevan, 2012). Alternatively, in DM2 there does not appear to be a strong correlation between repeat expansion size and disease severity (Day *et al.*, 2003).

Although DM1 is a complex, multisystemic disorder affecting numerous tissues, the skeletal muscle is severely affected with muscle weakness, reduced muscle tone, and myotonia (Bird, 2015). In addition to muscle defects, other symptoms include heart conduction defects (e.g. arrhythmia), cognitive impairment, gastrointestinal tract problems, endocrine abnormalities (e.g. diabetes), and a high prevalence of obesity in adults patients (Bassez *et al.*, 2004; Håkansson *et al.*, 2015; Osborne *et al.*, 2009). Many phenotypic similarities have been reported between DM1 and DM2. For instance as seen in DM1, muscle weakness, myotonia, cataracts, cardiac defects, primary gonadal failure, and insulin insensitivity are several of the common symptoms in DM2 patients (Dalton *et al.*, 2013). Interestingly, one important distinction between these two disorders that unlike DM1, DM2 has a lack of developmental abnormalities and does not present with severe childhood symptoms (Dalton *et al.*, 2013; Day *et al.*, 2003).

1.6.1 Molecular mechanisms of DM1

Several molecular mechanisms have been proposed to explain why the CTG repeat expansion in the 3'UTR of the *DMPK* gene causes DM1. Several early studies suggested that the CTG expansion could interfere with the processing and/or transport of the *DMPK* mRNA leading to a decreased DMPK expression levels. As the DMPK protein is a Ser/Thr protein kinase mainly

expressed in skeletal and cardiac muscles its loss could potentially contribute to the DM1 pathology somehow (Lam *et al.*, 2000). Although several reports found decreases in both DMPK mRNA and protein levels in DM1 patient tissues it was demonstrated that knockout mice for the *DMPK* gene only share some of the pathological features of DM1 (Pettersson *et al.*, 2015; Reddy *et al.*, 1996). Alternatively, or potentially in conjunction with the loss of DMPK, there was the idea that the CTG repeat expansion may affect the transcription of other genes located around the *DMPK* gene, such as the flanking gene SIX Homeobox 5 (*SIX5/DMAHP*) (Sarkar *et al.*, 2000). Again, although reports did show decreased *DMAHP* expression levels in DM1 patient cells (Inukai *et al.*, 2000; Klesert *et al.*, 1997) mouse models with Six5 knockout could not account for the full DM1 disease severity, as only some characteristic features similar to those seen in DM1, such as ocular cataracts, were present (Jansen G, 1996). Lastly, it has been proposed that the CUG expanded RNA itself causes a toxic effect in the cell. This hypothesis is supported by several lines of evidence. For example, once transcribed, the CUG repeats of the *DMPK* mRNA form hairpin-like secondary structures, causing the mRNA to aggregate and become retained within the nucleus forming RNA foci (Davis *et al.*, 1997; Taneja *et al.*, 1995). Experimental evidence showed that these RNA foci were toxic, having a gain-of-function effect on the cell caused by the misregulation and/or sequestration of several RBPs and transcription factors. The misregulation of these factors were found to disrupt the expression, metabolism and/or splicing of target mRNAs (Gauthier *et al.*, 2013; Lee *et al.*, 2013; Miller *et al.*, 2000; Osborne *et al.*, 2009; Philips *et al.*, 1998; Savkur *et al.*, 2001; Timchenko *et al.*, 1996). These findings lead to the formation of the favoured "RNA toxic" model for the DM1 pathology, in which the major focus has been on the effects that the toxic RNA expansion has on RBPs that regulate pre-mRNA splicing (Fig 3). The RNA toxic model hypothesis is further supported by the fact that in DM2, a disorder also caused by a microsatellite

expansion of RNA repeats but in a totally different gene, presents with similar pathogenesis and a strong clinical similarity to the multisystemic features observed in DM1 (Liquori *et al.*, 2001).

1.6.2 The aberrant pre-mRNA splicing mechanisms in DM1

Initial investigation into pre-mRNA splicing in DM1 revealed several aberrant splicing events, many of which can be linked to the specific symptoms seen in the disease (Mankodi *et al.*, 2002; Philips *et al.*, 1998; Savkur *et al.*, 2001) (Fig 3) (Table 1). For example, an isoform of the insulin receptor *INSR* (IR-A), generated when the alternative exon 11 is skipped, is predominantly produced resulting in insulin resistance in DM1 patients (Savkur *et al.*, 2001). Altered splicing of the Troponin T2, Cardiac Type (*TNNT2*) mRNA at exon 5 has also been observed in DM1 and overexpression of the fetal isoform is suggested to contribute to the reduced myocardial function and conduction abnormalities observed in patients (Philips *et al.*, 1998). Missplicing of the main chloride channel in muscle, *CLCN1* pre-mRNA, an event which involves the inclusion of the fetal exon 7a, is also observed in DM1 and has been linked to the reduced expression of the CLC-1 protein and myotonia (Mankodi *et al.*, 2002). The expression of *MAPT* isoforms with decreased inclusion of exon 2 and exon 10 are observed in DM1 and may contribute to neuronal dysfunction (Jiang *et al.*, 2004). More recent reports have also linked missplicing of the Dystrophin (*DMD*) (Rau *et al.*, 2015), Amphiphysin2 (*BIN1*) (Fugier *et al.*, 2011), and the sodium voltage-gated channel alpha subunit 5 (*SCN5A*) (Freyermuth *et al.*, 2016) pre-mRNAs to compromised muscle fibre maintenance, T tubule alterations and muscle weakness, and the cardiac-conduction delay and heart arrhythmia, respectively, that are seen in DM1 patients. Klinck *et al*., using a high-throughput RT-PCR screening and validation platform in human embryonic DM1 cell cultures, recently identified several new missplicing events

Table 1. Examples of DM1 associated splicing events.

Pre-mRNA	Alternaitve splicing event affected in DM1	Related symptom in DM1 patient	Selected reference
INSR	Exon 11	Insulin resistance	Savkur *et al.*, 2001a
TNNT2	Exon 5	Reduced myocardial function and conduction abnormalities	Philips *et al.*, 1998
CLCN1	Exon 7a	Reduced expression of the CLC-1 protein and myotonia	Mankodi *et al.*, 2002
MAPT	Exon 2 and Exon 10	Neuronal dysfunction	Jiang *et al.*, 2004
DMD	Exon 78	Compromised muscle fibre maintenance	Rau *et al.*, 2015
BIN1	Exon 11	T tubule alterations and muscle weakness	Fugier *et al.*, 2011a
SCN5A	Exon 6a	Cardiac-conduction delay and heart arrhythmia	Freyermuth *et al.*, 2016
SYNE1	N/A	Potential defects in muscle sarcomere and actin cytoskeleton	Klinck *et al.*, 2014
RyR1	Exon 70	Excitation-contraction coupling	Kimura et al., 2009

including in the Spectrin Repeat Containing Nuclear Envelope Protein 1 (*SYNE1*) pre-mRNA, a gene that is involved in a network that links subcellular structures in the muscle sarcomere to those in actin cytoskeleton (Klinck *et al.*, 2014). The well-studied splicing patterns of these pre-mRNAs in wild-type and DM1 conditions are shown in Fig 3 (Table 1).

1.6.3 Misregulation of RBPs in DM1

There are several examples of the missplicing events occurring in DM1 being linked to the misregulation of specific RBPs. The members of the muscle-blind protein family, specifically Muscleblind-Like Splicing Regulator (MBNL)1-3 and a prominent member of the CUG-BP, Elav-like family CELF protein family, CUGBP1 are misregulated in DM1. MBNL1 and CUGBP1 are among the group of proteins predominantly thought to cause the majority of missplicing in DM1 (Ho *et al.*, 2005; Miller *et al.*, 2000; Timchenko *et al.*, 1996). The major isoform of MBNL in skeletal muscle is MBNL1 and in addition to binding directly to the CUG-repeats, MBNL1 is also sequestered by the RNA foci in the nucleus (Warf *et al.*, 2009). This sequestration is thought to cause MBNL1 loss-of-function and subsequent missplicing events, supported by the DM1-like symptoms observed in a Mbnl1 knockout mouse model (Kanadia *et al.*, 2003). In 2007, Wheeler *et al.*, uncovered that MBNL1 was also sequestered in the motor neurons in DM1, and although the CUG repeat expansion was poorly expressed there, authors suggest that the function or stability of the neuromuscular junction may still be affected in DM1 due to the loss-of-function of MBNL1 (Wheeler *et al.*, 2007). A recent study furthermore demonstrated the sequestration of MBNL proteins to C(C)UG repeat expansions in the DM1 brain (Goodwin *et al.*, 2015). Upon further examination, authors uncovered that the depletion of MBNL in the DM1 brain led to the dysregulation of normal MBNL protein function in this tissue, similar to what is seen in DM1 skeletal muscle (Goodwin *et al.*, 2015). In contrast, CUGBP1 is upregulated in DM1 myoblasts,

heart, and skeletal muscle as a result of protein stabilization via PKC phosphorylation (Dansithong *et al.*, 2005; Kuyumcu-Martinez *et al.*, 2007; Savkur *et al.*, 2001). Transgenic mouse models overexpressing CUGBP1 recapitulate several DM1 phenotypes suggesting that it too is a key player in DM1 pathogenesis (Ho *et al.*, 2004; Timchenko *et al.*, 2004; Ward and Cooper, 2010). One example of the effect of the misregulation of MBNL1 and CUGBP1 is the *INSR*, which has been found to harbour multiple MBNL1 and CUGBP1 binding sites and is misspliced in DM1 due to misregulation of these two splicing factors (Savkur *et al.*, 2001; Sen *et al.*, 2010). Although MBNL knockout mice and CUGBP1 transgenic mice recapitulate several DM1 features, they do not account for all DM1 symptoms (Gomes-Pereira *et al.*, 2011; Ward *et al.*, 2010). This suggests that numerous factors contribute to the DM1 pathology and more studies are required that expand the repertoire to include other RBPs that are misregulated in DM1.

Indeed, the desire to uncover more factors that contribute to the DM1 pathology has revealed that numerous other RBPs, in addition to MBNL1 and CUGBP1, are misregulated in the disease (Fig 3). For example, Paul *et al*. reported increased steady-state levels of hnRNP H in DM1 myoblasts which regulated the splicing of the *INSR* exon 11 in a manner similar to that observed in DM1 (Paul *et al.*, 2006). Recently, it was shown that the protein levels of RNA helicase p68/DDX5 were significantly decreased in skeletal muscles of a DM1 mouse model (Jones *et al.*, 2015). DDX5/p68 has also been shown to modify MBNL1 activity by influencing its binding to CUG repeats, which, in turn, can regulate the missplicing of the *TNNT2* pre-mRNA (Laurent *et al.*, 2012). Another DEAD-box helicase protein, DDX6, has also been shown to interact with the CUG mRNA-foci in fibroblasts isolated from DM1 patients (Pettersson *et al.*, 2014). In 2012, our group uncovered that the dsRBP Stau1 was naturally upregulated in DM1 skeletal muscles obtained from three different DM1 mouse models, as well as human DM1 muscle biopsies (Ravel-

Chapuis *et al.*, 2012). A striking finding from this report was the discovery that Stau1 acted as a novel splicing regulator of two pre-mRNAs that are misspliced in DM1. Specifically, the overexpression of Stau1 in DM1 conditions caused an increase of exon 11 inclusion for the *INSR* pre-mRNA and the intronic retention event in the *CLC1* pre-mRNA (Ravel-Chapuis *et al.*, 2012). Following our publication, several other groups reported results that were consistent with Stau1's involvement in pre-mRNA splicing regulation (Laver *et al.*, 2013; Milev *et al.*, 2012). Taken together these studies suggest that the missplicing events seen in DM1 are due to the misregulation of numerous RBPs that regulate pre-mRNA splicing.

1.6.3 Additional molecular mechanisms in DM1

Importantly, although emphasis has been placed on the effects of aberrant pre-mRNA splicing in DM1, both in this thesis and the DM1 field, there are numerous additional mechanisms that are misregulated in DM1 that contribute to the complex pathology, perhaps completely independent of splicing.

In 2004, it was shown that CUG repeat expansions also cause sequestration of transcription factors resulting in the reduced expression of several mRNAs, including those implicated in DM1, such as *CLC-1* (Ebralidze *et al.*, 2004). Recent reports have now demonstrated that repeat-associated non-ATG translation (RAN translation) occurs in DM1 CAG repeat expansion transcripts. RAN translation has been suggested to produce toxic homopolymeric (polyglutamine: Poly(Q)) peptides that contribute to the DM1 pathogenesis (Sicot *et al.*, 2011; Zu *et al.*, 2011). Multiple stress-related pathways are also affected in cells containing CUG repeat expansions. In 2010, Huichalaf et al., generated stable cells that overexpressed mutant CUG repeats and found the overexpression induced the formation of stress granules (SG), which resulted in the trapping of certain mRNAs that coded for DNA repair factors, such as *MORF4L1* (Huichalaf *et al.*, 2010).

Interesting, in 2016 our lab demonstrated that SG formation was actually deficient in DM1 myoblasts, an effect that was partially due to Stau1 overexpression, as we further demonstrated that Stau1 depletion via RNA interference rescued normal SG formation (Ravel-Chapuis *et al.*, 2016).

Several groups have now reported that numerous microRNAs (miRNAs) are also differentially expressed in DM1 tissues. In 2014, Kalsotra *et al*. used a high-throughput screen to examine the expression of >500 miRNAs in heart tissue of a DM1 mouse model and identified 54 miRNAs whose expression was altered when compared to wild-type levels (Kalsotra *et al.*, 2014). A hypothesis regarding the cause of the progressive DM1 muscular features has also been put forth by Thornell *et al*., which is independent of pre-mRNA splicing defects (Thornell *et al.*, 2009). This group proposed that an increase in DM1 satellite cells, the precursor to skeletal muscle cells, is one of the first responses to the muscle dysfunction caused by the CUG repeat expansion (Thornell *et al.*, 2009). Furthermore Thornell *et al*. suggest that these DM1 satellite cells are defective and do not have the inability to ensure proper muscle fibre maintenance, an effect that could lead to the progressive muscle atrophy seen in the disease (Thornell *et al.*, 2009). It was recently reported that in DM1 there is misregulation of alternative polyadenylation, a wide-spread, tissue-specific, post-transcriptional regulatory mechanism that generates distinct 3'termini of mRNA (Batra *et al.*, 2014; Tian and Manley, 2016). Lastly, Buckley *et al*., investigated potential epigenetic features at the DMPK gene and its flanking genes in multiple DM1 tissues and non-muscle samples and reported the presence of DNA hyper- and hypomethylation and enhancer chromatin modifications in this gene neighbourhood, effects that may contribute to regulation of these genes in DM1 (Buckley *et al.*, 2016). These studies, and others highlight the importance of alternate mechanisms

in addition to the spliceopathic features observed that also have important roles in the DM1 pathology.

1.6.4 Current DM1 therapeutic approaches

There is no cure for DM1, and currently the only options following diagnosis are focused on management of the symptoms. These measures are taken to improve a patient's quality of life and include both medical interventions, such as treatment of pain, and preventive measures (Moxley, 1992). However, in recent years several groups have reported promising results from potential therapeutic avenues. These approaches are diverse and can include, but are not limited to, steroid treatments, small molecule targeting, modulation of misregulated RBP expression, and RNA-based therapies.

Preliminary studies using a precursor of human sex steroid biosynthesis, called dehydroepiandrosterone sulfate (DHEA-S), showed improvements in both muscle strength and myotonia upon treatment, however a later multicentre, double-blind, placebo-controlled trial with 75 DM1 patients showed no effect (Penisson-Besnier *et al.*, 2008). The modulation of misregulated RBPs in order to restore the aberrant pre-mRNA splicing patterns observed in DM1 is also a highly-investigated idea in the treatment of DM1. Indeed, promising results were shown in 2006 when the overexpression of Mbnl1 protein in the widely-used HSA^{LR} DM1 mouse model, a transgenic mouse with a 250 CUG-repeat expansion in the 3'UTR of the human skeletal actin gene, rescued both myotonia and several pre-mRNA splicing events that are defective in DM1 (Kanadia *et al.*, 2006). Our work in 2012 with the overexpression of Stau1 provided another important example demonstrating the potential benefits that altering the expression levels of RBPs in DM1 can have, such as correction of pre-mRNA splicing (Ravel-Chapuis *et al.*, 2012). In two studies published this year, the design, synthesis and testing of several small molecules to treat

DM1 was described (Angelbello *et al.*, 2016; Li *et al.*, 2016). In one of these studies, Angelbello *et al*., demonstrated the effectiveness of several small molecules to disrupt the CUG repeat expansion interaction with MBNL1, an action that resulted in improvement of DM1-assiocated defects including the aberrant pre-mRNA splicing of *cTNT* exon 5 (Angelbello *et al.*, 2016). Finally, in a recent study Witherspoon *et al*., investigated the effect of using several sodium channel blockers, including mexiletine, prilocaine, procainamide, and sparteine, to suppress the expression of the *DMPK* mRNA as a therapeutic avenue in DM1 based on the observation that this transcript is central in the pathology (Witherspoon *et al.*, 2015).

RNA-based therapeutics in the DM1 field are mainly focused on using antisense oligonucleotides (ASOs) to target the CUG repeat expansion and/or correct aberrant pre-mRNA splicing that occurs in DM1. For example, Wheeler *et al*., demonstrated that DM1-assiocated CUG repeats were sensitive to antisense silencing upon the systemic administration of specifically designed ASOs that target the toxic RNA (Wheeler *et al.*, 2012). Their ASO treatment (subcutaneous injections; 25mg/kg - twice weekly) caused the significant and rapid knockdown of the CUG repeats (>80%) in the hindlimb muscle of the HSA^{LR} DM1 mouse model, which resulted in a correction of many features of the disease with the effect being sustained for up to one year (Wheeler *et al.*, 2012). Another group designed a miRNA-based RNA-interference expression cassette that targeted the CUG repeat expansion (Bisset *et al.*, 2015). Delivery of this miRNA-based system via a recombinant adeno-associated viral (rAAV) vector into the muscles of HSA^{LR} mice reduced the CUG repeat mRNA, positively affected MBNL1 localization patterns, and corrected several aberrant pre-mRNA events (Bisset *et al.*, 2015). These studies are just a few examples of the current avenues that show promising results in the treatment of this complex,

neuromuscular disorder. However, despite these significant findings there is still no efficient treatment for DM1, thus, more work is needed.

1.7 SMA

SMA is an autosomal recessive neuromuscular disorder with an international incidence of 1/10,000 live births and is considered one of the leading genetic causes of infant mortality (Kirwin *et al.*, 2013). SMA has historically been characterized by the selective degeneration of lower alpha motor neurons in the spinal cord. Interestingly although researchers have observed that motor neurons are the primarily affected cell type in this disorder for many years, the reason behind this selective vulnerability remains largely unknown, as discussed below. Moreover, in addition to motor neurons, there is now an accumulation of data showing that other tissues are also affected in the SMA pathology (Fig 4).

A great deal of data has now been collected by numerous groups demonstrating a direct effect of SMN depletion on the neuromuscular junction (NMJ), as described in detail in several extensive reviews (B Goulet *et al.*, 2013; Bottai and Adami, 2013; Murray *et al.*, 2008). Indeed, SMN is found localized at the NMJ (Fan and Simard, 2002) and developmental abnormalities of the NMJ are observed in SMA mice (Kariya *et al.*, 2008; Kong *et al.*, 2009). For instance, in *Smn-/-*;SMN2 mice, a commonly used, severe SMA mouse model, McGovern et al., found a lack of innervation of the intercostal muscles in early development which has been suggested to occur from both a lack of axon outgrowth and NMJ formation (B Goulet *et al.*, 2013; McGovern *et al.*, 2008). Some of the specific defects that have been found in the NMJ of various SMA mouse models include, but are not limited to: impairment of NMJ maturation (Kariya *et al.*, 2008), massive accumulation of neurofilaments in terminal axons of the NMJ (when NMJs are present) (Cifuentes-Diaz *et al.*, 2002), defective Ca^{2+} channel clustering (Jablonka *et al.*, 2007), impaired synaptic vesicle release

(Kong *et al.*, 2009), a loss of terminal Schwann cells (Murray *et al.*, 2013), and abnormal acetylcholine receptors (AChRs), a membrane protein the responds through binding of the neurotransmitter acetylcholine, clustering. In cultured muscle cells from SMA patients a failure of AChR clustering was also observed (Arnold *et al.*, 2004), a defect that was later associated with prenatal defects in SMA fetuses (Martínez-Hernández *et al.*, 2013). Muscle cells from SMA mouse models show altered expression of key muscle developmental factors (Bricceno *et al.*, 2014), increase and cultured muscle cells from SMA patients have been reported to be significantly smaller than wild-type (Boyer *et al.*, 2014). Mustsaers *et al*., showed the presence of disruptions of the molecular composition of skeletal muscle in *Smn-/-*;SMN2 mice (Mutsaers *et al.*, 2011). Furthermore, these authors found an increased activity of cell death pathways, an observation that is supported by the previously reported presence of apoptotic cell death in SMA muscle (Fidzianska *et al.*, 1990). The differentiation of muscle satellite cells of the *Smn-/-*;SMN2 mouse model was also found to be dysfunctional and was associated with the premature expression of muscle differentiation makers and a reduced efficiency in myotube formation (Hayhurst *et al.*, 2012). Astrocytes, the specialized glial cells that serve as support cells in the mammalian central nervous system, derived from SMA human induced pluripotent stem cells (iPSCs) are reported to have a reduced Ca^{2+} response to ATP, an effect that is suggested to precede the motor neuron loss in SMA (McGivern *et al.*, 2013). Recently, it also was reported that there is an impairment of proper spleen development in SMA mice and patients (Thomson *et al.*, 2016). Lastly, recent reviews have highlighted work from numerous studies showing defects a multitude of additional systems affected in SMA, including the endocrine, lymphatic, bone and reproductive systems (Nash *et al.*, 2016). These findings emphasize the newly-recognized multi-systemic nature of the SMA pathology.

SMA patients present with skeletal muscle weakness and atrophy of the proximal muscles, hypotonia and eventual paralysis (Hamilton and Gillingwater, 2013; Lunn and Wang, 2008). A broad range of SMA phenotypes are recognized; Type I (Werdnig (Werdnig, 1891) –Hoffmann (Hoffmann, 1893)) is the most severe, with death occurring before 6 months of age, and accounts for >50% of SMA cases, Type II (Intermediate), Type III (Kugelberg-Welander, Juvenile), and Type IV (Adult). Although palliative care based on symptoms is given to maintain the quality of life and health of SMA patients, there is currently no cure for this debilitating disease.

1.7.1 Genetics of SMA

The genetic cause of SMA is the homozygous disruption, by deletion, conversion or mutation, of the *SMN1* gene. First described in 1990, linkage analysis identified the locus of the SMA gene to chromosome 5q13 (Hoffmann, 1893), which was later narrowed down to the disease-causing *SMN1* gene in 1995 (Lefebvre *et al.*, 1995). The human *SMN1* gene is composed of 8 exons (Lefebvre *et al.*, 1995), with the two exons making up exon 2 referred to as exons 2a and 2b (Bürglen *et al.*, 1996) (Fig 4). As described above, the *SMN* gene encodes for the 294-amino acid, 38 kDa SMN1 protein. Humans have two *SMN* genes that share >99% nucleotide identity, the telomeric *SMN1*, and its centromeric homolog *SMN2*, which exists in multiple copies. *SMN2* has a C to T substitution at base pair position 840 that has been shown to attenuate the activity of an ESE bound by the splicing factor SRSF1 and results in the production of an SMN protein product lacking exon 7 (Cartegni and Krainer, 2002; Lorson *et al.*, 1999). Interestingly, the activity of SRSF1 associated with the new ESE has been shown to be antagonized by hnRNP A/B, although it was shown that this activity is independent of the C to T substitution (Cartegni *et al.*, 2006). Other splicing proteins that have been shown to regulate the pre-mRNA splicing of exon 7 include Tra2β (Hofmann *et al.*, 2000), hnRNPG (Hofmann and Wirth, 2002), and SRp30c (Young *et al.*,

2002). Alternatively, it has been proposed that this C to T substitution also creates a negative splicing element, an ESS, that can be recognized by hnRNP A1, leading to an inhibition of exon 7 inclusion (Kashima and Manley, 2003). This SMNΔ7 transcript is produced nearly 90% of the time from *SMN2* and the translated protein is truncated, unstable and rapidly degraded (Le *et al.*, 2005) (Fig 4). Of note, early studies also indicated a role of the neighbouring genes to *SMN*, such as the Neuronal Apoptosis Inhibitory protein (*NAIP*) and *p44t* (Akutsu et al., 2002; Chen et al., 1998).

1.7.2 The molecular mechanisms of SMA

The precise molecular mechanism of the SMA pathology are still not fully understood and considered somewhat controversial (Lorson *et al.*, 2010). Over the past several decades, two major hypotheses have emerged to explain the SMA phenotype, both of which highlight specific consequences of SMN loss: 1) splicing defects 2) dysfunctional neuronal RNP transport granules. These two hypotheses are particularly important when investigating the selective vulnerability of motor neurons that is observed in SMA. Indeed, it has been suggested that motor neurons may be more sensitive to loss of SMN than other cell types, however whether this is caused through an increased susceptibility to splicing defects or dysfunctional RNP transport, or more likely a combination of both, is still unknown. These two central mechanisms, with their consequences to motor neuron degeneration, as well as several additional pathways misregulated in SMA, are discussed in detail below.

1.7.3 Pre-mRNA splicing misregulation in SMA

The best-described cellular function for the SMN complex is the critical role it plays in snRNP biogenesis (Battle *et al.*, 2006; Meister *et al.*, 2001; Neuenkirchen *et al.*, 2008; Nilsen and Graveley, 2010). Indeed, reduced snRNP assembly activity has been reported in extracts from

SMA patient cells (Wan *et al.*, 2005) and spinal cord of a severe SMA mouse model (Gabanella *et al.*, 2007). Winkler *et al*., showed impaired snRNP assembly upon SMN depletion in HeLa cells (Winkler *et al.*, 2005). Moreover, they found that the silencing of SMN expression in zebrafish led to SMA-like motor axon degeneration, a defect rescued by the injection of purified snRNPs (Winkler *et al.*, 2005). The "snRNP-centric model" of the SMA pathology hypothesizes that the depletion of SMN will cause mis-expression of U snRNPs, which in turn will result in wide-spread splicing defects and lead to the disease phenotype (Pellizzoni *et al.*, 1998). Indeed, there are several lines of evidence that show the existence of splicing defects in SMA.

In a thorough study done by Zhang *et al*., multiple tissues from a 6-day-old mouse model of type II SMA phenotype (Le *et al.*, 2005) were examined to look for snRNP expression levels. Using exon arrays, Zhang *et al*. demonstrated the presence of widespread, cell-type specific splicing defects in these tissues (Zhang *et al.*, 2008). These splicing defects appeared more predominantly in pre-mRNAs that harboured a large number of introns. Moreover, there was also a reduction in the expression levels of snRNAs, specifically a 30-60% reduction in minor spliceosomal snRNAs U11, U12, and U4atac, and interestingly, increased levels were observed for several major snRNAs U2 and U6, in several tissue types (Zhang *et al.*, 2008). This defect in the minor spliceosomal snRNAs and subsequent splicing changes associated with the minor spliceosome in some minor introns was also reported other groups (Boulisfane *et al.*, 2011; Gabanella *et al.*, 2007; Lotti *et al.*, 2012). For instance, Lotti *et al*., uncovered expression and splicing defects of a subset of U12 intron-containing genes upon SMN depletion, including the gene for the Stasimon protein, a protein required for proper motoneuron function (Lotti *et al.*, 2012). A more recent study by Huo *et al*., showed numerous splicing changes associated with the

major U2-dependent spliceosome, in the motor neurons isolated from a severe SMA mouse model (*mSmn* -/-; *hSMN2* +/+) (Huo *et al.*, 2014). Another study, that used exon-array analysis of RNA from pre-, early-, and late-symptomatic SMN deficient mouse spinal cords, found that although widespread splicing disturbances were present in SMA, they appeared as a late feature of the disease (Baumer *et al.*, 2009). The authors hypothesized that these late splicing features are not actually a primary feature of SMA, but instead a secondary effect as a result of cell injury that appears in late phases of the disease (Baumer *et al.*, 2009). Taken together, these studies and others, show the presence of splicing defects associated with decreased SMN levels and in several SMA models.

Although these large-scale studies have shown widespread defects in snRNP assembly and splicing the examination of several SMA animals models suggest that these defects are not enough to fully cause the SMA pathogenesis, prompting researchers to look further into the molecular mechanisms of SMA (Hamilton and Gillingwater, 2013).

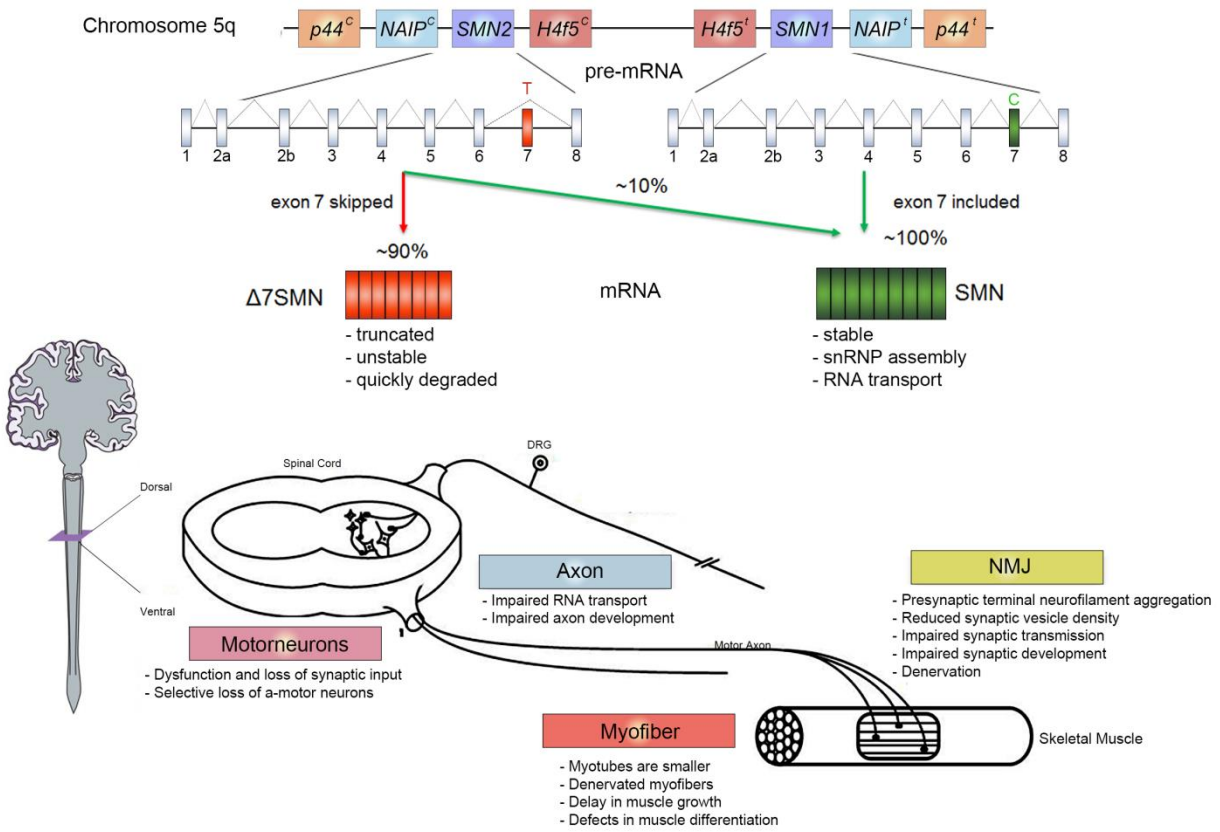

Figure 4. The molecular mechanisms of the Spinal Muscular Atrophy pathology.

(A) Genetic map of the SMA locus and SMN protein synthesis. The genes located within the centromeric and telomeric regions of chromosome 5q13. Pre-mRNA splicing and resulting translated SMN proteins from the SMN genes. (B) Schematic representation of the different areas affected in SMA.

1.7.4 The role of SMN in neuronal RNP complexes

In addition to the historically favoured "snRNP-centric model" as the cause of the SMA phenotype, there have emerged numerous SMA-associated molecular mechanisms that are focused on the non-splicing functions of SMN in motor neurons. It should be of particular importance to consider these other hypotheses, in addition to the "snRNP-centric model", when investigating the SMA pathology.

For instance, the underlying reasons as to why motor neurons are primarily affected in SMA when SMN is ubiquitously expressed and decreased SMN levels are present in all cells in SMA, is still unknown. One hypothesis put forth that would agree with the "snRNP-centric model" is that motor neurons are somehow more sensitive to defects in pre-mRNA splicing than other cell types, although this is still unknown at this time (Vuong *et al.*, 2016). Recent evidence however suggests that the answer to this lies within the additional roles for SMN, such as its involvement in multiple signalling pathways, the neuromuscular junction maturation, and importantly, in a crucial role as a chaperone of RNP complexes involved in axonal RNA metabolism, and neuronal transport granules (Fallini *et al.*, 2014; Hubers *et al.*, 2011; Kariya *et al.*, 2008; Le *et al.*, 2005; Zhang *et al.*, 2003b). Consistent with SMN's role in these pathways, it has been shown to interact with numerous RBPs with known roles in axonal RNA handling, such as FUS/TLS, IMP1/ZBP, TDP-43, hnRNP R/Q, FMRP, KSRP and HuD (Akten *et al.*, 2011; Fallini *et al.*, 2014; Fallini *et al.*, 2011; Hubers *et al.*, 2011; Piazzon *et al.*, 2008; Rossoll *et al.*, 2002; Tadesse *et al.*, 2008). It is the dysfunction of these interactions of SMN and these proteins in SMN-associated RNP complexes in SMA that is considered to be one of the leading causes, in addition to defective splicing pathways, of the SMA pathology. Neuronal RNP complexes are required for mRNA processing, transport, localization and local translation, all functions that are particularly important

to maintain normal motor neuron physiology (Thomas *et al.*, 2011). Several studies have now shown that defects associated with misregulation of RNP complexes in motor neurons, such as defects in axonal growth, maintenance, and local protein synthesis, have been observed in SMA models (Fallini *et al.*, 2012).

Although the mechanism underlying how exactly low levels of SMN results in misregulation of neuronal RNP complexes is still poorly understood, it has been suggested that the answer lies in the protein-protein interactions between SMN and RNA-binding proteins and in SMN's ability to regulate the localization and/or expression of these proteins. For example, our lab and others demonstrated that the RNA-binding protein HuD interacts with SMN and this interaction was found to be required for proper recruitment of HuD mRNA targets to neuronal RNA granules, a defect seen in SMA-like conditions (Akten *et al.*, 2011; Hubers *et al.*, 2011). The proper transport of these RNA transcripts is essential and the misregulation of this pathway may be critical to understanding the selective vulnerability of motor neurons in SMA. For example, an important study by Rossoll *et al*., revealed through work in SMA mice and zebrafish models, that motor neurons deficiency of SMN showed reduced axon growth that correlated with reduced β-actin protein and mRNA transport in distal axons and to growth cones (Rossoll *et al.*, 2003). Importantly, these results suggest that the misregulation of mRNA transport induced by low SMN levels in highly polarized cells such as motor neurons, could contribute to the selective degeneration of motor neurons in SMA.

1.7.5 Additional roles of SMN that may play roles in SMA

Several groups have also examined the potential role of SMN in the RhoA/ROCK signaling pathway and proposed that this relationship may play an important role in SMA (Ahmad *et al.*,

2016). Indeed, this idea is supported by the observation that RhoA/ROCK signaling is important for the regulation of cytoskeleton dynamics involving pathways essential for proper neuron function including growth, differentiation and degeneration (Govek *et al.*, 2005). Low levels of SMN have been shown to result in the activation of RhoA/ROCK complexes, through altered expression patterns of the profilin II mRNA, and subsequent phosphorylation of several downstream targets, an effect that results in SMA-like characteristics such as defects in neuritogenesis (Bowerman *et al.*, 2007). These findings have since been confirmed by other groups, and furthermore, additional alterations in the phosphorylation of downstream targets of ROCK have been identified, including cofilin, and profilin IIa (Hensel *et al.*, 2015; Nölle *et al.*, 2011).

An additional novel role for SMN was uncovered by our lab in 2012, when we determined that SMN cofractionates with polyribosomes and acts as a translational regulator of the Coactivator-Associated aRginine Methyltransferase 1 (CARM1) (Sanchez *et al.*, 2012). CARM1 is recognized as a positive transcriptional regulator (Di Lorenzo and Bedford, 2011; Kawabe *et al.*, 2012; Lee and Stallcup, 2009; Wu and Xu, 2012), a factor that plays a role in pre-mRNA splicing and also stability through the methylation of splicing factors and other RBPs (Cheng *et al.*, 2007; Yang and Bedford, 2013). In Sanchez *et al*., we further demonstrated that in tissues depleted of SMN, such as the spinal cord tissue from SMA mice and cells from severe Type I SMA patients, CARM1 was abnormally upregulated (Sanchez *et al.*, 2012). In this recent work, our lab used genome-wide array technology to uncover that this aberrant upregulation of CARM1 in SMA led to a number of misregulated transcriptional and alternative splicing events (Sanchez *et al.*, 2012). This data strongly suggested that CARM1 upregulation might contribute to altered gene expression profiles that are observed in the SMA pathology. Indeed, CARM1 can play diverse roles in many RNA-

related pathways, for example in pre-mRNA splicing, and we wonder how this could contribute to the SMA pathology. This is essential, as we know that altered gene expression and/or splicing profiles observed in many diseases have been shown to contribute to the pathology.

These studies and others demonstrate the important roles that SMN plays in RNP complex pathways such as transport, that go beyond SMN's best-characterized role in snRNP assembly and splicing. It is important to consider SMN's involvement in these diverse pathways in order to fully understand the complex SMA pathology.

1.7.6 Current SMA therapies

To date, only palliative care based on symptoms is given to maintain the quality of life and health of SMA patients, as there is currently no cure for this debilitating disease. However, several promising therapeutic approaches have recently emerged and excitingly, the National Institutes of Health listed SMA as the neurological disorder that is closest to a cure.

Similar to DM1, SMA research has uncovered therapeutic strategies that focus on using drugs treatments, small molecules, and RNA-based approaches. In 2007, Avila *et al*., investigated the potential beneficial effects of the highly specific and potent histone deacetyltransferase (HDAC) inhibitor trichostatin A (TSA) on the SMA phenotype (Avila *et al.*, 2007). Upon a single intraperitoneal dose of TSA (10 mg/kg) in SMA mice, an increased level of histone acetylation (H3 and H4) was observed followed by a slight increase in the expression of *SMN* mRNA. Multiple doses of TSA resulted in the increase of both the *SMN2* transcript levels and importantly, SMN protein levels in multiple tissues (Avila *et al.*, 2007). More recently, Abera *et al*., identified several small molecules, highlighting one called ML372, that were able to increase the protein levels of SMN in cells derived from SMA patients, and in tissues from SMA mice through the inhibition of SMN protein ubiquitination and degradation pathways (Abera *et al.*, 2016). Another recent study

examined the effects of administrating a butyrate-based compound called pivaloyloxymethyl butyrate in SMA mice (Edwards and Butchbach, 2016). The oral administration of this compound almost doubled the average lifespan and significantly increased the growth rate of the SMA mice, as compared to vehicle-treated SMA mice (Edwards and Butchbach, 2016). In 2011, Faroog *et al*., demonstrated that prolactin, a luteotropic hormone, upregulated the mRNA and protein levels of SMN in neuronal cells from both human and mouse, through STAT5 pathway activation (Farooq *et al.*, 2011). Examining this results further, Faroog *et al*., showed that treatment of prolactin increased the SMN levels and improved both motor function and survival in a SMAΔ7 mouse model (Farooq *et al.*, 2011). The results from these studies highlight the potential of several small molecules that can increase SMN levels and, thus, could be highly beneficial in SMA therapeutics.

The most widely-used RNA-based therapeutic approaches in SMA treatments is the use of ASOs, also known as splice-switching oligonucleotides (SSOs), which can be used to switch a disease altered splicing event towards wild-type splicing patterns (Lin *et al.*, 2016; Osman *et al.*, 2016). When ASOs are used to alter splicing patterns, this particular approach requires the identification and careful mapping of specific *cis*-regulatory elements that influence the affected splicing event. Much work has been done to identify the numerous *cis*- and *trans*- regulatory elements that regulate the pre-mRNA splicing of the *SMN2* mRNA (Cartegni *et al.*, 2002; Cartegni *et al.*, 2006; Cartegni and Krainer, 2002; Hofmann *et al.*, 2000; Kashima and Manley, 2003). The data gathered from this crucial work, as the major goal of many RNA-based approaches is to design ASOs that modulate the pre-mRNA splicing of *SMN2* exon 7 by targeting specific *cis*-regulatory elements in the pre-mRNA, in order to restore full-length, function SMN protein expression (Lorson *et al.*, 2010).

Hua *et al*., showed that through the identification and subsequent blocking of ISS elements in intron 7 of the *hSMN2* gene by a 2'-O-methoxyethyl (MOE) ASO (ASO-10-27), they were able to promote the inclusion of exon 7, from 21% to 91%, in tissues of mice harbouring a human *SMN2* transgene (Hua *et al.*, 2008). This ASO is currently being tested in Phase III clinical trials (Faravelli *et al.*, 2015). A similar strategy was used by Singh *et al*., although in this case, they developed a short (8 nt) ASO that binds to a GC-rich sequence in intron 7 of the *SMN2* transcript to modulate the pre-mRNA splicing of exon 7 (Singh *et al.*, 2009). More recent studies have also used ASOs in iPSCs and iPSC-differentiated motor neurons that not only modulate exon 7 splicing but are also designed to target the *SMN2* promoter and improve pre-mRNA processing, in order to increase SMN protein levels (Nizzardo *et al.*, 2015). It was reported that all three of these approaches indeed increased the levels of SMN protein and a significant improvement in phenotype was observed in SMA motor neurons, *in vitro* (Nizzardo *et al.*, 2015). These results, and many others, demonstrate the immense potential to exploit RNA-based therapies that modulate pre-mRNA splicing patterns in neuromuscular disorders. Finally, as the majority of these studies only demonstrate the potential of their therapy with regards to motor neurons, it is important to remember that SMA is a multi-systemic disorder, and these promising treatments will have to consider the many other tissues affected in this disease, as described above.

1.8 Hallmarks of cancer

The immense diversity of the characteristics underlying neoplastic diseases have been organized into several essential "hallmarks of cancer", best outlined in the detailed reviews by Hanahan and Weinberg (Hanahan and Weinberg, 2000, 2011). Originally presented as the six hallmarks of cancer: 1) proliferative signalling, 2) evading growth suppressors, 3) resisting cell death, 4) enabling replicative immortality, 5) inducing angiogenesis, and 6) activating invasion and metastasis, additional emerging characteristics such as deregulating cellular energetics and avoiding immune destruction, are now also considered crucial hallmarks (Hanahan and Weinberg, 2011). Moreover, two enabling characteristics that are now regarded as essential for the acquisition of these hallmarks, have been described as genome instability and mutation, and tumour-promoting inflammation (Hanahan and Weinberg, 2011). These hallmarks outline the numerous characteristics that enable the formation and progression of tumours, and importantly the adaption of features that are important for the prognosis of cancer, such as metastasis.

1.8.1 Metastasis in breast cancer

In order for cancer cells to metastasize, several changes need to occur. Two major changes that are required include an enhancement of their motility or migration, coupled with an increased ability to invade, through an extracellular matrix (ECM) of a surrounding tissue. Understanding the mechanisms that drive specific hallmarks of cancer such as invasive potential and metastasis are essential to understanding the disease and develop effective treatments. For example, breast cancer starts as a local disease with the ability to metastasize to the lymph nodes and other organs usually manifesting later in disease progression and being associated with poor prognosis. Currently, breast cancer is the most common malignant disease affecting Western women with recent reports estimating that breast cancer accounts for 29% of all new cancer cases in women (Siegel *et al.*,

2015). It is essential to elucidate the mechanisms the underlie hallmarks, such as metastasis, as it is not the primary tumour, but the metastasis into distant sites, that is the main cause of death in breast cancer (Weigelt *et al.*, 2005). There are several established breast cancer metastasis prognostic markers such as tumour size (i.e >5cm very high risk of metastasis), axillary lymph node status, histological grade (i.e grade 1 (low risk of metastasis), 2 (intermediate), 3 (high), and over 70-gene expression profiles that can be used to predict the probably of distant metastasis (Weigelt *et al.*, 2005). Identification of novel genes that contribute to the invasive potential of a breast cancer cell will allow for better prediction tools for earlier diagnosis and the development of tailored treatments for individual patients.

1.8.3 The role of pre-mRNA splicing misregulation in cancer

The molecular mechanisms that can be deregulated in order for a cell to exhibit the essential hallmarks of cancer are highly diverse and vary among cancer types. The misregulation of post-transcriptional mechanisms is common in cancer and can involve disruptions in virtually all steps of RNA processing, including pre-mRNA splicing.

As discussed above the role of pre-mRNA splicing in neuromuscular disorders, like DM1 and SMA has long been studied and more recently, the emerging role of pre-mRNA splicing, particularly in cancer, have been recognized. Defects in pre-mRNA splicing, stemming from alterations in both *cis*- and *trans*-acting factor alterations can cause and/or contribute to many human cancers. For example, genomic mutations in splice sites are considered the most common form of splicing defects found in cancer (Venables, 2004). Importantly, alterations in both the expression and activity of many splicing factors has been shown in numerous cancer types. These alterations can have widespread effects on the splicing patterns of the associated pre-mRNA targets. In many cases, the altered expression of splicing factors can result in the production of

"pro-cancer" isoforms, as described below. Splicing changes associated with altered expression of splicing factors have been reported in every step of the transformation process and are observed affecting every major hallmark of cancer, such as resisting cell death, enabling replicative immortality, activating invasion and metastasis (Anczuków and Krainer, 2016) (Fig 5).

Among the numerous splicing factors altered in cancer are both essential components of the spliceosome, involved in constitutive splicing, and those that play important roles in alternative splicing pathways (Fig 5) (Appendix A). For example, the most frequently mutated spliceosome component in cancer is SF3B1, an essential spliceosome component that assists in the recognition of the branch point during 3'SS selection (Anczuków and Krainer, 2016). Cancer-associated mutations found in hot spots of the *SF3B1* gene have recently been shown to cause changes in the splicing of a subset of alternative or cryptic 3'SS (DeBoever *et al.*, 2015).

Other altered splicing factors in cancer include the SRSFs 1,2,3,5,6, and 10, hnRNPs A1, A2/B1, H, K, M, and other RBPs such as PTB, RBFOX2, Sam68, HuR, RBM5 and RBM10 (Appendix A) (Anczuków and Krainer, 2016; Grosso *et al.*, 2008; Silipo *et al.*, 2015). Splicing factors can be up- or -downregulated, as compared to wild-type levels (Fig 5). For example, the overexpression of SRSF1 has been shown to change the splicing patterns of multiple pre-

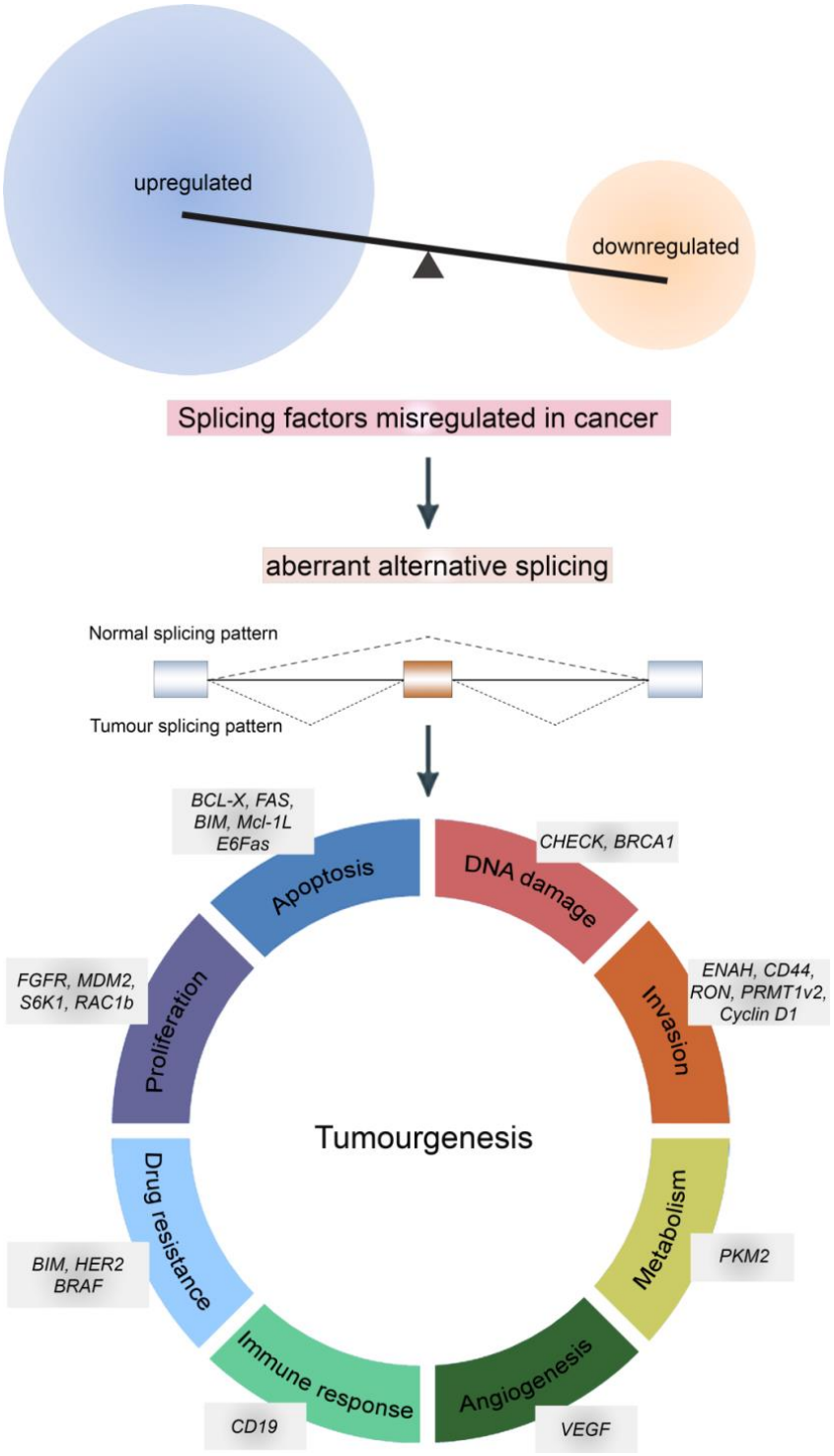

Figure 5. Splicing alterations in cancer.

Splicing factors that are misregulated in cancer (either up- or -down-regulated) are represented here proportional to the size of the colour circles. This misregulation leads to altered alternative

splicing (orange box) events in breast cancer which can lead to a “pro-cancer” of tumour-specific spliced isoform. These types of events have been reported in all of the major hallmarks of cancer (hallmarks of cancer adapted from Hanahan and Weinberg, 2000 and 2011).

mRNAs to produce pro-cancer biological effects and phenotypes. Karni *et al*., showed that the overexpression of SRSF1 altered the splicing of the tumour suppressor *BIN1,* to promote the inclusion of exon 12A, an event that resulted in the loss of the suppressor activity of *BIN1* (Karni *et al.*, 2007). Recently, SRSF1 overexpression was demonstrated to alter the splicing pattern of *BIM* to produce two novel *BIM* isoforms, γ1 and γ2, which lack the normal BIM pro-apoptotic functions (Anczuków *et al.*, 2012).

The overexpression of several protein arginine methyltransferases (PRMTs), proteins that can play roles in pre-mRNA splicing, have also been observed in several cancer types (Yang and Bedford, 2013). For example, a recent study showed that MYC, a transcription factor found to be overexpressed/amplified in the majority of human cancers, directly upregulated the transcription of multiple snRNP particle genes, such as PRMT5, a PRMT responsible for methylating Sm proteins and promoting snRNP assembly (Koh *et al.*, 2015). In 2012, our lab uncovered that the relative balance of the *PRMT1* spliced isoforms was altered in breast cancer, specifically that the isoform *PRMT1v2*, was increased in a number of breast cancer cell lines and tumours (Goulet *et al.*, 2007). Our lab pursued this research and in 2012, we reported that the depletion of *PRMT1v2* mRNA resulted in a significant decrease in cancer cell survival and invasion (Baldwin *et al.*, 2012). This work not only investigated the influence of splicing of *PRMT1* in breast cancer but also showed that the isoforms have distinct functional differences (Baldwin *et al.*, 2012). Taken together, these splicing proteins with altered expression have been shown to contribute to important hallmarks of cancer.

1.8.4 Current therapies for cancer treatment

Several treatment options are currently being used in cancer therapy including, surgery, radiation therapy, chemotherapy, immunotherapy, hormone therapy (which is used to both treat the disease

and its symptoms), stem cell transplants, and importantly, the recently developing options related to precision or personalized medicine.

Personalized medicine is a long-standing concept in cancer treatment and recent advances in cancer genomics technologies are allowing for the development of novel ways to implement this idea (Chin *et al.*, 2011). For example, the identification of specific cancer markers can vary among individuals, a situation that presents opportunities for personalized targeted approaches. Furthermore, some tumours types have been reported to be specifically "addicted" to certain pro-cancer isoforms produced by alternative splicing (Salton and Misteli, 2016), which could be targeted with the ASO approaches mentioned above. Indeed, although it not yet at the stage of personalized medicine, the concept of targeting pre-mRNA splicing in cancer treatment has been heavily studied (Dutertre *et al.*, 2010; Salton and Misteli, 2016).

Targeting pre-mRNA splicing for cancer treatment has previously described using small-molecules/splicing modulators or ASO/SSO technologies. To date, there are >20 small molecule modulators of pre-mRNA splicing that have been discovered (Salton and Misteli, 2016). These include several natural compounds extracted from different bacteria stains, such as *Pseudomonas* and *Streptomyces*, that were shown to modulate pre-mRNA splicing and inhibit tumour growth, through the targeted inhibition of the Splicing Factor 3b Subunit 1 (SF3B1), a component of the U2 snRNP complex (Albert *et al.*, 2009; Fan *et al.*, 2011; Nakajima *et al.*, 1996; Roybal and Jurica, 2010). Although these small molecules, and others, modulate cancer-specific splicing effects and in some cases inhibit tumour growth, much work remains to be done to evaluate their therapeutic potential. For instance, several of these modulators report with high toxicity in phase I clinical trials, thus, the specific concentrations required to yield beneficial outcomes are still unknown (Eskens *et al.*, 2013).

Similar to DM1 and SMA, results for RNA-based cancer therapeutic avenues using ASOs appear promising. In 1999, Taylor *et al*., investigated the effects of an ASO that targeted the pre-mRNA splicing regulation of the *bcl-x* gene, an mRNA that when alternatively spliced gives rise to either the *Bcl-xL* isoform (anti-apoptotic "pro-cancer") or the *Bcl-xS* (pro-apoptotic "anti-cancer") isoforms (Taylor *et al.*, 1999). This ASO successfully altered the ratio of these isoforms from the anti- to the pro-apoptotic form, resulting in an increased sensitivity of cancer cells to undergo apoptosis when exposed to radiation or chemotherapeutic treatments (Taylor *et al.*, 1999). A similar approach was used to inhibit the production of the oncogenic alternatively spliced isoforms of the Fibroblast Growth Factor Receptor 1 (*FGFR1*) gene (Bruno *et al.*, 2004). A more recent example was carried out by Dewaele *et al*., this year where they not only identified that the upregulation of *MDM4* expression, an event often seen in cancer cells, is largely dependent on a switch in the alternative splicing of *MDM4* isoforms, but also used an ASO to target this specific ASE (Dewaele *et al.*, 2016). The administration of this MDM4-targeting ASO decreased MDM4 expression levels and increased the sensitivity of the treated cells to MAPK-targeted therapeutics (Dewaele *et al.*, 2016). Taken together, the results from these studies highlight the potential that modulating pre-mRNA splicing, through multiple approaches, can have in cancer treatments.

1.9 Outstanding questions

The disease mechanisms described in this thesis for DM1, SMA and breast cancer, are highly complex and there are many questions that still need to be answered in order to understand these disease pathologies. For example, the tendency to classify DM1 as a spliceopathy is common in DM1 studies. This raises the question of how widespread and disruptive is the misregulation of pre-mRNA splicing in DM1? There is a growing list of RBPs that are misregulated in DM1, many of which are multi-functional, suggesting the misregulation of these RBPs can affect numerous

other pathways that may contribute to the DM1 pathology. Indeed, as described above it is now known that additional post-transcriptional regulatory pathways other than pre-mRNA splicing, are disrupted in DM1. To this end, it is important to investigate the extent of the pre-mRNA splicing defects caused by RBPs such as Stau1, in DM1, as is addressed in Chapter 2, while keeping in mind the multi-functional nature of the protein.

In SMA, one of the major outstanding questions is why there is a selective degeneration of motor neurons in the pathology? Moreover, will the restoration of SMN levels, which has been achieved through several RNA-based therapeutic approaches, be required in every cell or just the motor neurons? As outlined above, there are several lines of evidence suggesting multiple pathways may be responsible for the motor neuron specific degeneration, with a major focus on the dysregulation of neuronal mRNA transport in SMN-associated RNP complexes. The discovery of the multiple roles of SMN also raises the question, what other SMN-associated post-transcriptional regulatory pathways are misregulated in SMA? Could these other pathways contribute to the SMA pathology? In the work presented here in Chapter 3, we work towards understanding the additional roles of SMN and their effect on the SMA pathology.

The role of pre-mRNA splicing in cancer is emerging as an important characteristic of many of the hallmarks of cancer, such as metastasis. Although many splicing proteins have now been shown to be misregulated in cancer we still do not know the extent of these altered protein networks. Which splicing proteins are upregulated or downregulated in cancer? What triggers the altered expression levels of certain splicing proteins in the cancer pathology? Moreover, what effect does the altered expression, both up and downregulation of these splicing factors have on their downstream targets? For example, we uncovered that the overexpression of the PRMT1v2 protein was due to an increase in the inclusion of the alternative exon2 in the *PRMT1* mRNA.

However, it is unknown what splicing factors regulate the inclusion of exon 2, and whether these factors themselves are misregulated in breast cancer models. In Chapter 4 of this thesis, we present data examining altered splicing proteins and specific downstream target pre-mRNAs that can contribute to the invasive potential of breast cancer cells.

From this brief list of outstanding questions, it is evident that more work is required to fully understand the mechanisms behind these diseases. It is the overarching theme of the three manuscripts presented in this thesis to investigate and elucidate novel post-transcriptional mechanisms in human disease in order to address important aspects of these outstanding questions.

1.10 Description of rationales and hypotheses

Taken together, these studies highlight the potential to treat multiple human diseases by targeting misregulated post-transcriptional mechanisms. In order to develop effective therapies, it is essential to better understand the complex mechanisms behind these misregulated pathways. In this thesis, I present three distinct projects, which focus on elucidating these pathways, with a focus on pre-mRNA splicing, in DM1, SMA and breast cancer.

1.10.1 The role of Stau1 splicing regulation in DM1

1.10.1.1 Rationale

We found that Stau1 was significantly increased in muscle samples from adult DM1 patients and DM1 mouse models. Furthermore, overexpression of Stau1 in DM1 conditions rescued key hallmarks of the pathology, such as the missplicing of the *INSR* and *CLCN1*. These data lead us to speculate that upregulation of Stau1 may represent a potential therapeutic avenue for DM1. In order to explore this idea, we needed to investigate the mechanism by which Stau1 regulates pre-mRNA alternative splicing and the broader impact of Stau1 as a splicing regulator in the context of DM1.

1.10.1.2 Hypotheses

My hypotheses are that:1) Stau1 regulates the splicing patterns of numerous pre-mRNAs 2) The splicing regulatory activity of Stau1 may contribute to the pathology of DM1.

1.10.2 The role of CARM1 in SMA

1.10.2.1 Rationale

We found that SMN acts as a translational regulator of CARM1 and that this mechanism leads to the abnormal upregulation of CARM1 in SMA. Furthermore, using a genome-wide exon array, we

identified CARM1-regulated transcription and splicing targets in motoneuron-derived cells, and validated a few that were also misregulated in an SMA cell culture model.

1.10.2.2 Hypothesis

My hypothesis is that the abnormal upregulation of CARM1 contributes to the altered gene expression profiles that are observed in the SMA pathology.

1.10.3 *PRMT1v2* splicing in breast cancer

1.10.3.1 Rationale

We found that the relative ratio of the *PRMT1* spliced isoforms is altered in breast cancer, specifically that *PRMT1v2*, was increased in a number of breast cancer cell lines and tumours. Furthermore, we reported that the depletion of *PRMT1v2* resulted in a significant decrease in cancer cell survival and invasion. These data lead us to speculate that if we could target the PRMT1v2 splicing mechanism it would result in decreased invasive potential of breast cancer cells and represent a potential therapeutic avenue for this disease. In order to explore this idea, we need to investigate how the *PRMT1v2* splicing pattern is regulated in breast cancer cells by identifying the regulatory elements that control this pathway.

1.10.3.2 Hypothesis

My hypotheses are that: 1) The altered PRMT1 isoforms relative expression profile observed in breast cancer is a result of alternative splicing misregulation.

Chapter 2 - Manuscript #1:

Staufen1 regulates multiple alternative splicing events either positively or negatively in DM1 indicating its role as a disease modifier

Publication information:
Bondy-Chorney, Emma, Tara E. Crawford Parks, Aymeric Ravel-Chapuis, Roscoe Klinck, Lynda Rocheleau, Martin Pelchat, Benoit Chabot, Bernard J. Jasmin, and Jocelyn Côté. "Staufen1 regulates multiple alternative splicing events either positively or negatively in DM1 indicating its role as a disease modifier." *PLoS Genet* 12, no. 1 (2016): e1005827.

Author's contribution:
Conceived and designed the experiments: EBC, TECP, ARC, RK, BC, BJJ, JC. Performed the experiments: EBC, TECP, RK. Analyzed the data: EBC, TECP, ARC, RK, BC, BJJ, JC. Contributed reagents/materials/analysis tools: RK, BC, LR, MP. Wrote the paper: EBC, TECP, BC, BJJ, JC.

Contributions by figure: Fig 6 (EBC), Fig 7 (EBC), Fig 8 (EBC), Fig 9 (EBC, TECP), Fig 10 (EBC TECP ARC RK BC BJJ JC), Fig 12 (EBC), Fig 13 (EBC), Fig 14 (EBC, MP, LR), Fig 15 (EBC), Fig 16 (EBC, JC)

Staufen1 regulates multiple alternative splicing events either positively or negatively in DM1 indicating its role as a disease modifier

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2.1 Abstract

Myotonic dystrophy type 1 (DM1) is a neuromuscular disorder caused by an expansion of CUG repeats in the 3' UTR of the *DMPK* gene. The CUG repeats form aggregates of mutant mRNA which cause misregulation and/or sequestration of RNA-binding proteins causing aberrant alternative splicing in cells. Previously, we showed that the multi-functional RNA-binding protein Staufen1 (Stau1) was increased in skeletal muscle of DM1 mouse models and patients. We also showed that Stau1 rescues the alternative splicing profile of pre-mRNAs, e.g. the *INSR* and *CLC1*, known to be aberrantly spliced in DM1. In order to explore further the potential of Stau1 as a therapeutic target for DM1, we first investigated the mechanism by which Stau1 regulates pre-mRNA alternative splicing. We report here that Stau1 regulates the alternative splicing of exon 11 of the human *INSR* via binding to Alu elements located in intron 10. Additionally, using a high-throughput RT-PCR screen, we have identified numerous Stau1-regulated alternative splicing events in both WT and DM1 myoblasts. A number of these aberrant ASEs in DM1, including *INSR* exon 11, are rescued by overexpression of Stau1. However, we find other ASEs in DM1 cells, where overexpression of Stau1 shifts the splicing patterns away from WT conditions. Moreover, we uncovered that Stau1-regulated ASEs harbour Alu elements in intronic regions flanking the alternative exon more than non-Stau1 targets. Taken together, these data highlight the broad impact of Stau1 as a splicing regulator and suggest that Stau1 may act as a disease modifier in DM1.

2.2 Author Summary

Myotonic Dystrophy Type 1 (DM1) is an inherited disorder affecting many systems, including skeletal muscle, heart, eyes and endocrine system. DM1 is known as a 'trinucleotide repeat disorder' because it is caused by an abnormal expansion of a highly repeated motif within the *DMPK* locus. Such an expansion results in the expression of a 'toxic RNA' which causes misregulation of proteins involved in many essential cellular pathways. Research efforts have largely focused on misregulation of a very few splicing regulators that can be linked with many defects observed in the pathology. We have recently uncovered that the multifunctional RNA-binding protein Staufen1 is increased in DM1 and that it is capable of rescuing selected defects in DM1 cells, including alternative splicing of the *INSR* pre-mRNA, which is linked with insulin resistance. Given the potential impact of this novel function for Staufen1, we investigated the mechanism by which it regulates splicing and determined that it mediates its effects through binding to conserved genomic repetitive sequences called Alu elements. We also uncovered that Staufen1 influences the splicing of numerous genes in DM1 patient cells, predictive to either improve or worsen the pathology, thus identifying Staufen1 as a novel disease modifier in DM1.

2.3 Introduction

Alternative splicing of pre-mRNAs is a phenomenon allowing multiple mRNA transcripts to be produced from a single pre-mRNA. Recent reports estimate that 95-100% of human multi-exon genes produce two or more mRNA splice variants, with a majority yielding an average of eight variants (Barbosa-Morais *et al.*, 2012; Djebali *et al.*, 2012; Merkin *et al.*, 2012; Pan *et al.*, 2008; Wang *et al.*, 2008). Generation of these variants by alternative splicing is a major mechanism responsible for the complexity of the transcriptome and proteome observed in eukaryotes (Nilsen and Graveley, 2010). Constitutive RNA splicing occurs through the recognition of the core splicing signals: the 5' splice site, branch point, polypyrimidine tract, and the 3' splice site AG by the spliceosome components. Additional cis-regulatory elements including exonic splicing silencers (ESS) and enhancers (ESE), and intronic splicing silencers (ISS) and enhancers (ISE) can influence the usage of core splicing signals. Moreover, intronic elements resembling splice sites can act as "decoy" splice sites to influence alternative splicing (Côté *et al.*, 2001). There is also a variety of conserved RNA secondary structures that interfere with the recognition of splicing signals and influence splice site selection (Raker *et al.*, 2009; Shepard and Hertel, 2008). The binding of these regulatory elements by RNA-binding proteins can inhibit or enhance the use of core splice sites and results in alternative splicing. Regulation of alternative splicing is thus mediated through the intricate interplay between these cis-acting and trans-acting regulatory elements.

Deciphering the mechanisms that regulate alternative splicing is essential for understanding how cellular diversity and specialization are generated but, importantly, it is also critical to develop novel therapeutic approaches for a growing number of diseases caused by misregulation of pre-mRNA splicing ((Kornblihtt *et al.*, 2013) and refs. therein). For example, in the neuromuscular

disorder Myotonic Dystrophy Type 1 (DM1), an expansion of CTG repeats in the 3'UTR of the Dystrophia Myotonica Protein Kinase (*DMPK*) gene results in retention of CUG-containing DMPK mRNAs within specific RNA foci in the nucleus. The length of CTG repeats varies in DM1 patients and correlates with disease severity (Mahadevan *et al.*, 1992). The mutant CUG-expanded mRNA causes a large misregulation of many splicing factor proteins, such as MBNL1, CUGBP1, hnRNP H, ASF/SF2 and RBFOX1 (for review see (Du *et al.*, 2010; Sen *et al.*, 2009; Shin *et al.*, 2009)). The misregulation of these splicing factors is reflected in the splicing defects observed in the DM1 pathology. Previously, it has been reported that 13 aberrant splicing events could be linked with the complex DM1 phenotype (Gatchel and Zoghbi, 2005; Ranum and Cooper, 2006), but more recent studies suggest the existence of numerous additional splicing defects in DM1 tissues (Dhaenens *et al.*, 2011; Fugier *et al.*, 2011; Klinck *et al.*, 2014). One important example of mis-splicing in DM1 is the increase in exon 11 exclusion of the insulin receptor (*INSR),* which results in the overproduction of the IR-A splice variant, thereby contributing to insulin resistance in DM1 patients (Savkur *et al.*, 2001). A variety of splicing factors acting on multiple cis-regulatory elements contribute to the splicing control of *INSR* and of other alternative splicing events. Indeed, a recent report suggests that CUGBP1 and MBNL1 antagonistically regulate hundreds of alternative exons and compete for binding to specific pre-mRNAs (Wang *et al.*, 2015).

Stau1 is a highly conserved multi-functional double-stranded RNA-binding protein involved in key aspects of RNA metabolism. These include mRNA transport and localization, translation efficiency, stability, regulation of stress granule assembly, and both nuclear and unconventional cytoplasmic mRNA alternative splicing (Bélanger *et al.*, 2003; Ephrussi *et al.*, 1991; Kim Y K, 2007; Martel *et al.*, 2010; Martel *et al.*, 2006; Micklem *et al.*, 2000; Ravel-Chapuis *et al.*, 2012; St Johnston and Nüsslein-Volhard, 1992; Sugimoto *et al.*, 2015; Thomas *et al.*, 2009; Wickham *et*

al., 1999). In mammals, Stau1 pre-mRNA is alternatively spliced to produce two major forms Stau1^{55}, Stau1^{63} and one variant reported to not bind RNA, Stau1^{i} (Duchaîne *et al.*, 2002; Furic *et al.*, 2008; Wickham *et al.*, 1999). Recently, several high-profile studies have focused on elucidating Stau1 binding sites (SBS), which are crucial for understanding Stau1's ability to regulate mRNA metabolism (de Lucas *et al.*, 2014; Laver *et al.*, 2013; Ricci *et al.*, 2014; Sugimoto *et al.*, 2015). Extensive work by numerous groups has utilized various immunoprecipitation techniques to investigate SBS, which appear to be represented by a highly diverse group of RNA secondary structures. These include double-stranded RNA structures containing stems and motifs ranging in size from 5-22 base pairs (bps) to hundreds of bps long which, in turn, can contain multiple short binding sites with varying degree of perfect base pairing, displaying little to no sequence specificity (de Lucas *et al.*, 2014; Laver *et al.*, 2013; Ricci *et al.*, 2014; Sugimoto *et al.*, 2015). Notably, in all large-scale studies performed to date, Stau1 has been reported to bind preferentially to the primate-specific, mobile element called Alu elements. SBS, comprised from both Alu and non-Alu element containing sequences, have been found everywhere in the genome including 3'UTRs, 5'UTRs, intronic regions, coding sequences and intergenic regions (Sugimoto *et al.*, 2015). This diversity of SBS location highlights the potential complexity surrounding events regulated by Stau1.

Recently, our group identified Stau1 as being significantly increased in muscle samples from adult DM1 patients and DM1 mouse models (Ravel-Chapuis *et al.*, 2012). Additionally, we saw that further overexpression of Stau1 in DM1 was able to rescue key hallmarks of the pathology, such as increased export and translation of CUG-expanded mRNAs and a significant increase in *INSR* exon 11 inclusion (Ravel-Chapuis *et al.*, 2012). Interestingly, our study revealed for the first time the ability of Stau1 to regulate alternative pre-mRNA splicing suggesting a novel role for

Stau1 as a splicing regulator (Ravel-Chapuis *et al.*, 2012). These data lead us to speculate that the upregulation of Stau1 represents a positive and protective adaptation in the DM1 pathology.

Here, we first set out to determine the mechanism by which Stau1 regulates pre-mRNA alternative splicing. Second, we examined the broader impact of Stau1 as a splicing regulator in the context of DM1. We report that Stau1 regulates the alternative splicing of human *INSR* exon 11 via binding to a region harbouring Alu elements within intron 10. Additionally, using a high-throughput RT-PCR screen, we identified numerous Stau1-regulated Alternative Splicing Events (ASEs) in both WT and DM1 myoblasts. These Stau1-induced changes in ASEs are expected to be beneficial or detrimental for the DM1 pathology. Importantly, a higher number of Stau1-regulated ASEs harbour Alu elements in intronic regions flanking the alternative exon when compared to non-Stau1 ASE targets. We thus propose that Stau1 uses Alu elements to regulate a large set of ASEs and that it acts as a disease modifier impacting on the severity of DM1.

2.4 Results

Human Insulin Receptor (*INSR*) alternative exon 11 inclusion is regulated by Stau1

We recently reported that Stau1 overexpression rescues specific alternative splicing defects associated with DM1, including that of exon 11 in the *INSR* pre-mRNA (Ravel-Chapuis *et al.*, 2012). Moreover, our observation that Stau1 also promotes exon 11 inclusion in muscle cells in the absence of pathological RNA repeats suggests that Stau1 may be a bona fide splicing regulator. In order to explore this idea further, we first assessed whether Stau1 could affect *INSR* exon 11 alternative splicing in non-muscle cells. First, HeLa cells were transiently transfected with a Stau1-HA expression construct and the relative level of endogenous *INSR* exon 11 inclusion was determined using semi-quantitative RT-PCR. A high level of exon 11 inclusion was observed in

these cells which agrees with previous findings (Mykowska *et al.*, 2011). Similar to our previous work in C2C12 myoblasts, the overexpression of Stau1-HA, as confirmed by Western blotting with HA-antibodies, resulted in a small, but reproducible ~5% increase in exon 11 inclusion (Fig 6A). To address whether Stau1 is required to maintain normal levels of *INSR* exon 11 inclusion, we also assessed *INSR* splicing event when levels of Stau1 are reduced. This would also mitigate the possibility that our observed effects on splicing were due to spurious, non-specific RNA binding of overexpressed Stau1-HA. HeLa cells were thus transiently transfected with a Stau1-targeting shRNA mix (described in materials and methods) and Western blotting was performed to assess Stau1 protein levels. This analysis demonstrated a 40% reduction of Stau1 protein level compared to CTRL (Fig 6B). This reduction of Stau1 levels caused a significant ~10% decrease in the relative inclusion of *INSR* exon 11 (Fig 6B). To confirm further the role of Stau1 as a splicing regulator in non-muscle cells, we extended our work to include an additional cell line, namely, HEK293Ts. In agreement with our findings in HeLa cells, the overexpression and reduction of Stau1 resulted in a significant ~5% increase and ~10% decrease, respectively, of exon 11 inclusion (Fig 7).

The splicing of exon 11 is known to be regulated by a number of splicing factors, including, but not limited to MBNL1, CUGBP1, and hnRNP H (Dansithong *et al.*, 2005; Sen *et al.*, 2010; Sen *et al.*, 2009). These splicing proteins, similar to Stau1, are misregulated in DM1 (Kanadia *et al.*, 2003; Kim *et al.*, 2005; Philips *et al.*, 1998; Savkur *et al.*, 2001). Thus, it was important to determine if our data are indicative of a direct effect of Stau1 on exon 11 splicing regulation or an indirect effect mediated through modified expression of other splicing regulators. In both

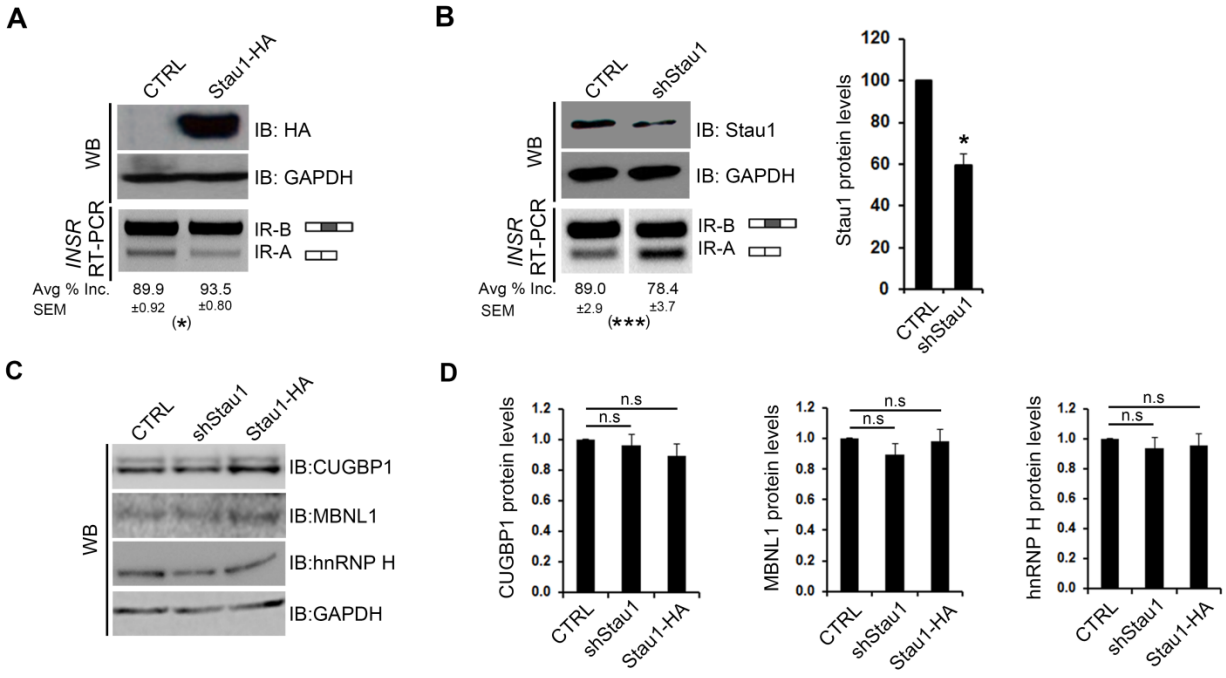

Figure 6. Stau1 levels regulate the pre-mRNA splicing of the human *INSR* in HeLa cells.

(A) pGIPZ (CTRL) or Stau1-HA (Stau1-HA) plasmids were transiently transfected into HeLa cell lines, and total RNA and protein lysate was collected after 48 hours. RT-PCR using primers specific to the human endogenous *INSR* were used on cDNA synthesized from total RNA to amplify the two isoforms (IR-A and IR-B) of the *INSR*. Stau1-HA protein levels were assessed by Western blot using HA-specific antibodies, and GAPDH was used as a loading control. (B) shCTRL or shStau1 were transiently transfected into HeLa cell lines and total RNA and protein lysate was collected after 48 hours. RT-PCR was performed to amplify the *INSR* isoforms. Stau1 protein levels were assessed by Western blot and quantified using GAPDH as a loading control. (C) Representative Western blots showing protein levels of CUGBP1, MBNL1 and hnRNP H in HeLa cells transfected with CTRL, shRNA or Stau1-HA plasmids. GAPDH was used as a loading control. (D) Quantification of Western blot analysis of splicing factors upon Stau1 level

modulation. In all cases, bar graphs show an average of $\geq$3 independent experiments. Error bars represent SEM * = $p < 0.05$, ** = $p < 0.01$.

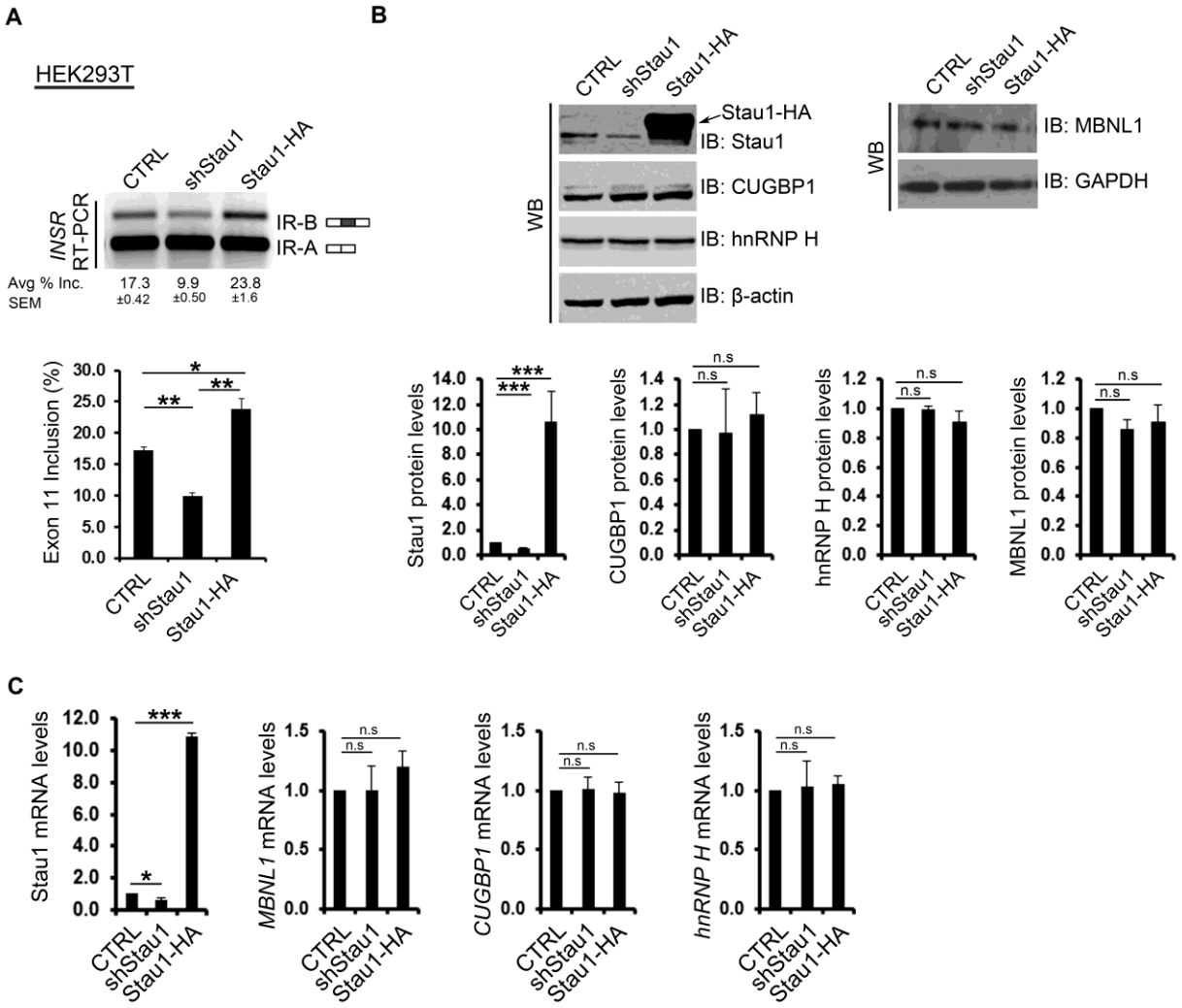

Figure 7. Stau1 levels regulate the pre-mRNA splicing of the human *INSR* in HEK293Ts cells.

(A) pGIPZ (CTRL), shStau1 or Stau1-HA (Stau1-HA) plasmids were transiently transfected into HEK293T cell lines and total RNA and protein lysate was collected after 48 hours. RT-PCR using primers specific to the human endogenous *INSR* were used on cDNA synthesized from total RNA to amplify the two isoforms (IR-A and IR-B) of the *INSR*. (B) Stau1, CUGBP1, MBNL1 and hnRNP H protein levels were assessed by Western blot using β-actin or GAPDH as a loading control. (C) Semi-quantitative RT-PCR using primers specific to the human Stau1 mRNA

demonstrates the increase and decrease of Stau1 mRNA in HeLa cell lines. RT-qPCR using primers specific to the human *MBNL1*, *CUGBP1*, and *hnRNP H* mRNA transcripts in HeLa cell lines with decreased or overexpressed Stau1 levels. 18S was used as for normalization in PCR experiments. In all cases, bar graphs show an average of ≥3 independent experiments. Error bars represent SEM * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

HeLa and HEK293T cells, no significant changes were observed in the mRNA or protein levels of MBNL1, CUGBP1 and hnRNP H, upon modulation of Stau1 levels (Fig 6C and D and Fig 7B and C). Finally, as we have previously confirmed, Stau1 over-expression did not differentially affect the mRNA half-lives of *INSR* alternatively spliced variants (Ravel-Chapuis *et al.*, 2012). Thus, altogether, our results strongly suggest that Stau1 is a bona fide splicing regulator, participating in the maintenance of human *INSR* exon 11 alternative splicing profile.

Stau1 regulates splicing of *INSR* exon 11 through an interaction with Alu elements in intron 10

In order to gain insights into the mechanism by which Stau1 regulates *INSR* exon 11 alternative splicing, we first searched for possible SBSs, which could represent cis-regulatory elements within the *INSR* pre-mRNA. Recently, numerous reports have emerged describing SBSs and although no single SBS has been described, one of the most highly recurring SBS reported is composed of Alu Repeat elements (de Lucas *et al.*, 2014; Elbarbary *et al.*, 2013; Kelley *et al.*, 2014; Laver *et al.*, 2013; Ricci *et al.*, 2014; Sugimoto *et al.*, 2015). Interestingly, an earlier report described the presence of an Alu Repeat element located in intron 10 of the *INSR* (Kosaki *et al.*, 1998). Closer inspection of this region via a bioinformatic analysis revealed that, in fact, there are three Alu elements located upstream of the intron 10-exon 11 boundary (Fig 8A). The fact that Alu elements are preferred SBS (Elbarbary *et al.*, 2013), together with the presence of Alu elements in intron 10 of the *INSR*, led us to propose that Stau1 may bind to these Alu elements to regulate alternative splicing of exon 11. To test this hypothesis, we selected two IR-minigene constructs (Kosaki *et al.*, 1998; Sen *et al.*, 2010; Sen *et al.*, 2009): WT and ΔAlus (in which all three Alu elements are deleted) (Fig 8A). HeLa cells were co-transfected with Stau1-HA and

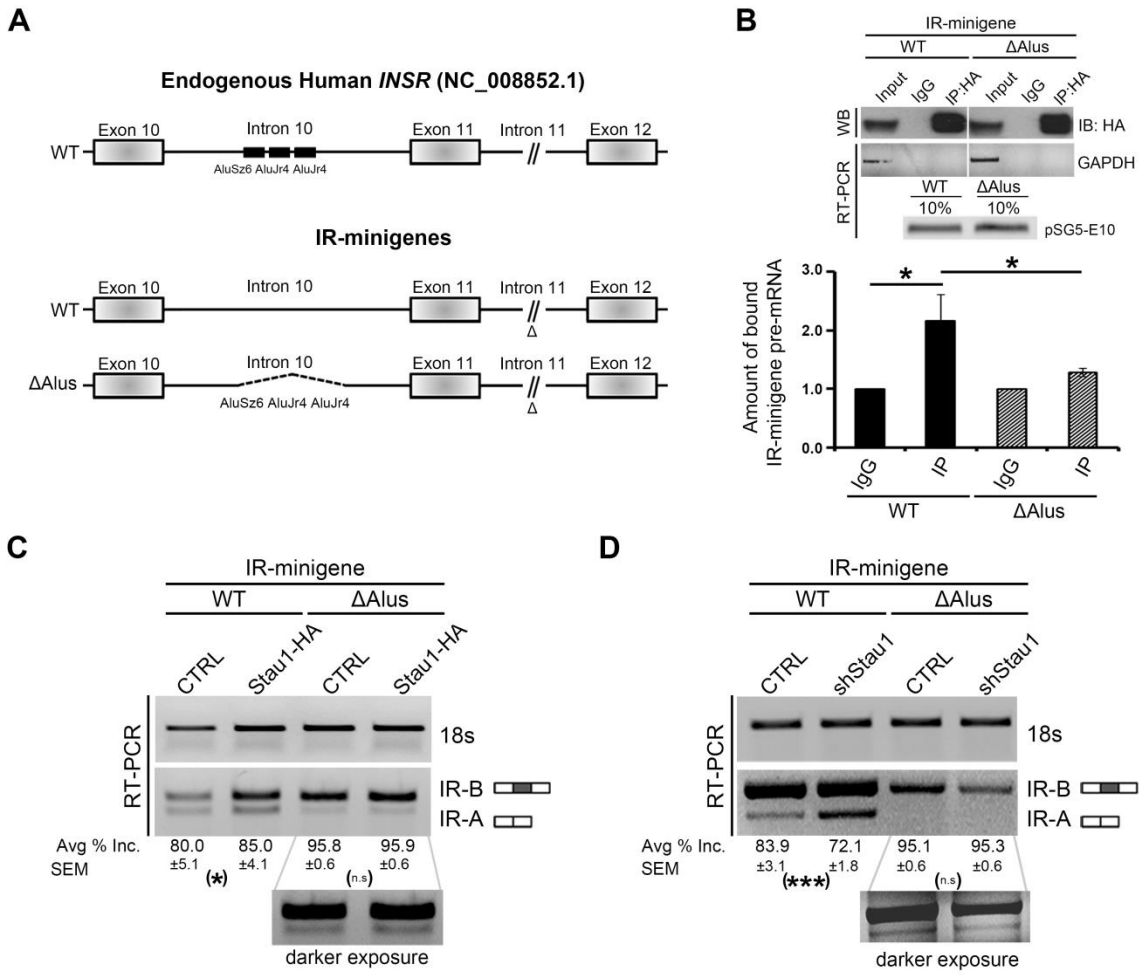

Figure 8. Stau1 regulates splicing of *INSR* exon 11 through an interaction with Alu elements in intron 10.

(A) The genomic DNA sequence of the human INSR (NG_008852.1) was used to assess the Alu elements located in intron 10. Introns are not to scale, and this is indicated in intron 11 (//). The IR-minigene constructs used in this study are shown here. Previously deleted segments of genomic DNA determined not to influence exon 11 splicing are indicated in intron 11 (Δ symbol), and black dotted lines represent the deleted segment containing the three Alu elements. (B) HeLa cells were transiently transfected with Stau1-HA plasmid and either the WT or ΔAlus IR-minigene. Immunoprecipitation (IP) of Stau1-HA protein was carried out using HA-specific antibodies under

RNase-free conditions. Western blot using HA-antibodies show equal amounts of Stau1-HA protein was immunoprecipitated in each condition. RNA was collected and DNase-treated prior to cDNA synthesis. RT-PCR was performed using *GAPDH* specific primers to demonstrate a lack of non-specific binding of RNA to the beads used for immunoprecipitation. Equal amounts of transfected minigenes were confirmed by performing RT-PCR on the cDNA synthesized from the 10% inputs lysates with primers specific to amplify a portion of the plasmid vector (pSG5) and the IR-minigene (Exon 10), corresponds to the pSG5-E10 labelled band. RT-qPCR was carried out using primers specific to an 115 bp region of intron 10 of the IR-minigene to determine the amount of IR-minigene RNA bound to immunoprecipitated Stau1-HA. Bar graphs show an average of four independent RIP experiments. (C-D) HeLa cells were transiently transfected with a CTRL, Stau1-HA plasmid or shStau1 and either the WT or ΔAlus IR-minigene. IR-minigene splicing was determined by RT-PCR. The average of $\geq$3 independent experiments was used. Error bars represent SEM * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

either the WT or the ΔAlus IR-minigene. RIP experiments were performed on cell lysates and RT-qPCR using primers specific for the intronic sequence of intron 10 was performed to identify the amount of IR-minigene pre-mRNA bound to Stau1-HA (Fig 8B). The amount of Stau1-HA bound to WT IR-minigene demonstrated a significant ~2-fold enrichment over the IgG control (Fig 8B; black bars). This degree of association was greatly reduced with the ΔAlus IR-minigene (Fig 8B; crosshatch bars), consistent with the hypothesis that Stau1 binds to the pre-mRNA of the *INSR* via the Alu elements located in intron 10. Next, we investigated whether the Alu elements were necessary for Stau1-regulated splicing of exon 11 by carrying out Stau1 overexpression and knockdown experiments. First, HeLa cells were co-transfected with a Stau1-HA expression or shStau1 construct and one of the IR-minigenes; WT or ΔAlus. Overexpression of Stau1-HA induced an ~5% increase in exon 11 inclusion in the WT IR-minigene (Fig 8C), as determined by RT-PCR as above. The reduction in Stau1 led to an ~12% decrease in exon 11 inclusion in the WT IR-minigene (Fig 8D). Importantly, in the absence of the Alu elements in the IR-minigene, neither overexpression nor reduction of Stau1 resulted in a significant change in exon 11 inclusion (Fig 8C and D, respectively). Thus, our data demonstrate that the Alu elements located in intron 10 are essential for Stau1 splicing regulation of exon 11 of the IR-minigene.

High-throughput RT-PCR reveals that Stau1 regulates the alternative splicing of numerous mRNAs in both WT and DM1 conditions

The observation that Stau1 regulates the splicing of the *INSR* led us to examine whether Stau1 regulates the splicing of additional pre-mRNAs. To address this central question, we carried out a screen using a high-throughput RT-PCR screen that measured the changes in the splicing ratios of 487 selected events (as described in (Klinck *et al.*, 2014)) in MyoD-converted WT and DM1 myoblasts either overexpressing GFP or Stau1-HA. The 487 ASEs comprising the RT-PCR

alternative splicing screen were chosen based on their association with the specific key terms: "muscle", "glucose metabolism", "wasting", and "ion-channel". WT (GM03377) or DM1 fibroblasts harbouring 1700 CTG (GM03132) repeats in the 3'UTR of the *DMPK* gene, were converted to myoblasts. Briefly, the conversion was done via two rounds of infection over 48 hours with a retrovirus engineered to express MyoD, followed by selection with Puromycin (1 μg/mL) for 5 days. Cultures were then infected with either GFP- or human Stau1-HA lentiviral particles. GFP expression was used to confirm infection efficiency 48 hours post-infection (Fig 9A). Semi-quantitative RT-PCR and Western blot analysis using *MyoD* specific primers and MyoD antibodies confirmed the overexpression of *MyoD* mRNA and protein (Fig 9B and C). Moreover, Western blot analysis with anti-HA tag antibodies confirmed Stau1-HA overexpression (Fig 9D). Total RNA from the MyoD-converted WT and DM1 cells was then isolated and used to carry out the high-throughput RT-PCR splicing screen.

Initial heat map data from the RT-PCR screen revealed that the overexpression of Stau1 in both WT and DM1 conditions had a broad effect on the splicing profile of numerous ASEs through the observed alteration of the Percent Splicing Index (PSI) for each ASE (Fig 10A and B). Although several ASEs were not affected by Stau1-HA overexpression (e.g. *ITGA7a*; Fig 10B), a number of ASEs showed important changes in both WT and DM1 conditions (e.g. *INSR*; Fig 10B). To determine quantitatively whether Stau1 increase regulates the PSI of an ASE, Stau1-HA overexpression was compared to GFP CTRL from WT and DM1 cell lines, yielding a value referred to as the change in PSI (ΔPSI). A threshold of a ΔPSI ≥10% was established to denote relevant changes in splicing regulated by Stau1 (Fig 10C and D, Table 2). Altogether, data from the high-throughput RT-PCR screen demonstrate that overexpression of Stau1-HA affects the splicing of 75 and 88 ASEs in WT and DM1 cell lines, respectively, with 27 ASEs

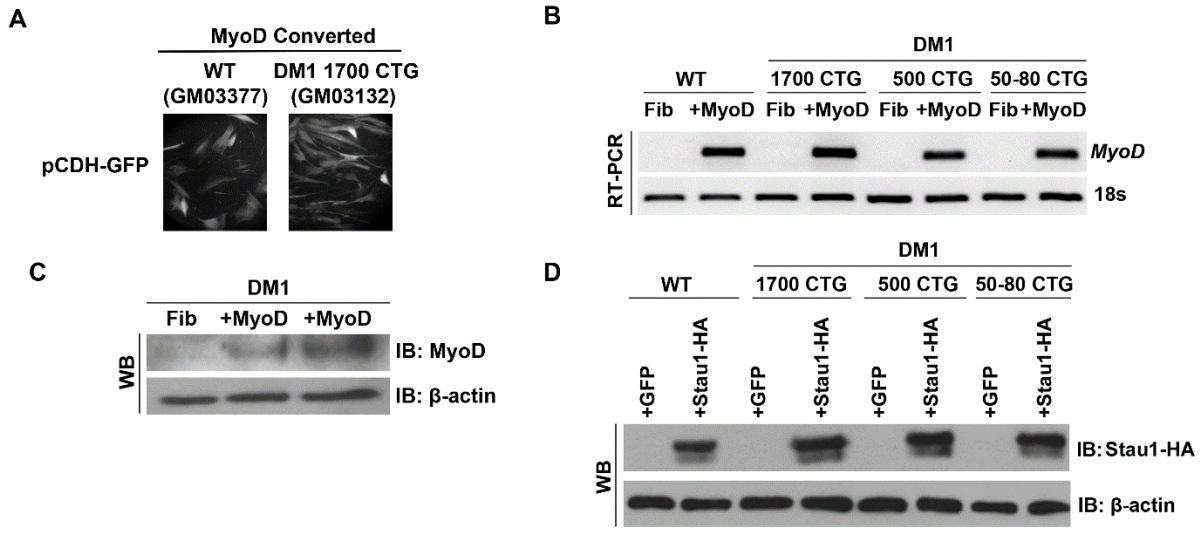

Figure 9. Confirmation of MyoD expression and overexpression of Stau1-HA protein in GM0 cells.

(A) Representative image of GFP positive MyoD converted WT and DM1 cell lines. (B) One WT (GM03377) and three DM1 (GM03132, GM03987, GM03991) primary fibroblast cell lines were converted to myoblasts using MyoD retrovirus. Semi-quantitative RT-PCR using primers specific to amplify *MyoD* plasmid demonstrates plasmid expression in all MyoD converted myoblast cell lines as compared to uninfected fibroblast cell lines. 18S was used as a loading control. (C) Protein was collected from GM03132 cell lines and western blot was used to analyze the levels of MyoD protein from virus infected MyoD converted myoblasts compared to uninfected fibroblast cell lines. β-actin was used as a loading control. (D) Representative Western blot showing levels of Stau1-HA in MyoD converted myoblast GM0 cell lines as compared to GFP infected MyoD converted cell lines. β-actin was used as a loading control.

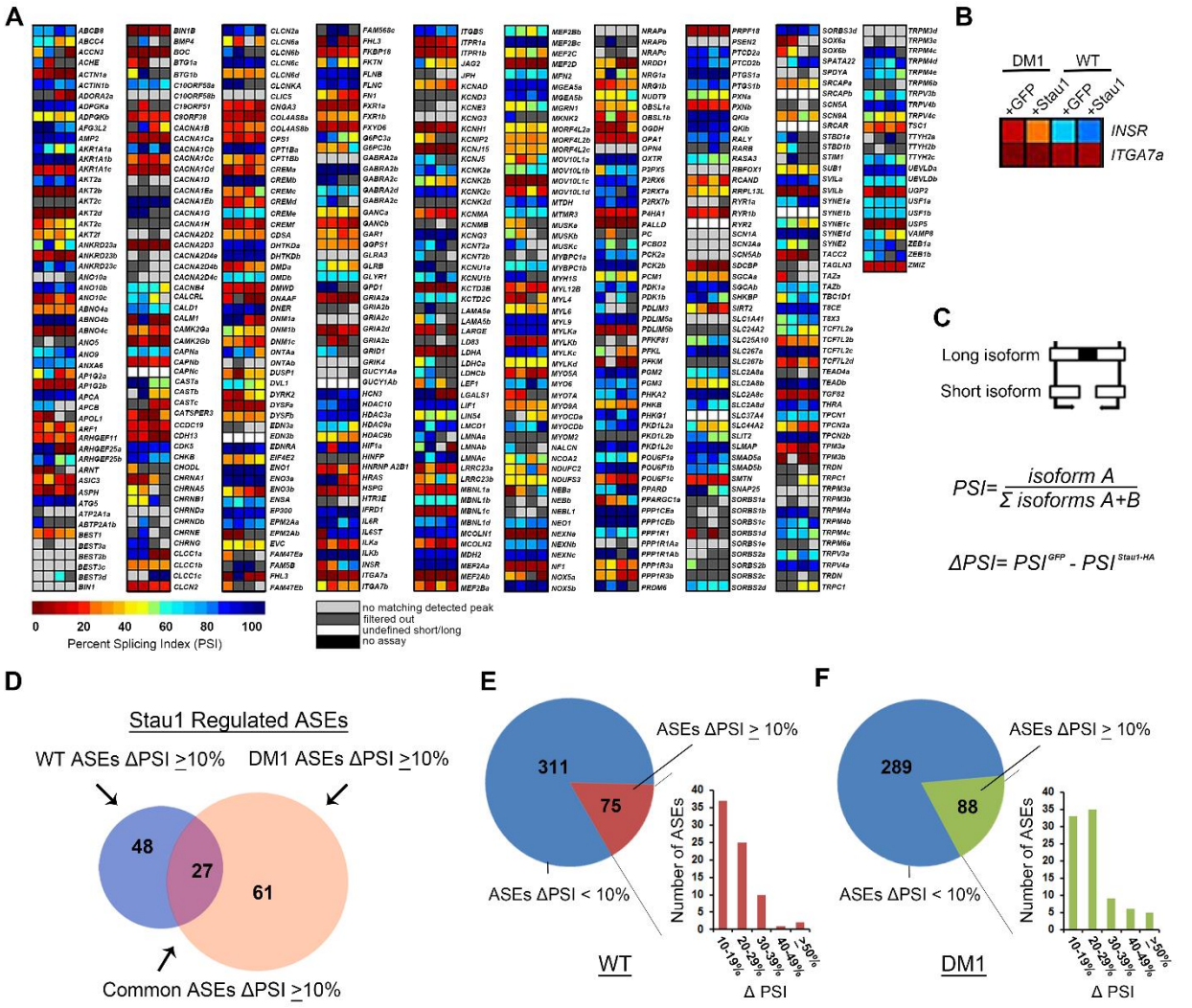

Figure 10. Analysis of high-throughput RT-PCR splicing screen.

(A) Raw data output displayed as heatmap from RT-PCR splicing screen. A total of 489 ASEs were analysed in the high-throughput RT-PCR Screen. Any ASEs that showed a blank reading for Percent Splicing Index (PSI) in any of the four conditions was excluded. (B) Enlarged image of two ASEs in DM1 and WT conditions. (C) PSI represented here as a ratio between the long isoform and the short isoform. The changed in PSI (ΔPSI) was calculated for each ASE in both conditions. (D) Total number of ASEs that showed a PSI change ≥10% with Stau1-HA overexpression in WT

and DM1. (E-F) Stau1 and non-Stau1 (<10% ΔPSI) regulated ASEs. Bar graphs display the ΔPSI groups (according to percent change) of Stau1-regulated ASEs in both WT and DM1.

Table 2. RT-PCR splicing screen information.

This excel file contains: Tab 1: The raw data PSI values from all four conditions performed in this study, Tab 2: The ASEs PSI values from DM1 conditions compared to CTRL from Klinck *et al*., 2014, Tab 3: The comparison between ASEs in DM1 between Klinck *et al*., 2014 and the splicing screen from the current study, Tabs 5 and 6: Comparison between ASEs regulated by Stau1 to MBNL1 and/or RBFOX1. All PSI≥5% were considered for any analysis comparing our data with that our Klinck *et al*., 2014., Tab 6: The subfamilies of Alu elements in Stau1-regulated targets.

*Due to the size of this table please refer to Appendix B

common in both conditions (Fig 10D). Similar trends were seen in both WT and DM1 conditions where the majority of Stau1-regulated ASEs showed a ΔPSI between 10-30% upon Stau1-HA overexpression. Accordingly, only a few ASEs showed large (≥50%) ΔPSI when Stau1-HA was overexpressed, compared to GFP CTRL, such as *INSR and NRG1* (Fig 10E and F). These data suggest that Stau1 does not dramatically alter the PSI of the majority of Stau1-regulated ASEs, but instead appears to fine-tune the alternative splicing of many ASEs. These results confirm our hypothesis that Stau1 is a splicing regulator and further show that Stau1 levels can alter the splicing profile of numerous pre-mRNAs both in WT and DM1 conditions.

ASEs regulated by Stau1-HA overexpression are both potentially beneficial and detrimental for the DM1 pathology

We recently demonstrated that overexpression of Stau1 in DM1 conditions induced an increased inclusion of the *INSR* exon 11 (Ravel-Chapuis *et al.*, 2012). This splicing modulation should be beneficial for DM1 as it reverts the *INSR* aberrant splicing towards WT conditions, an event that would also be predicted to reduce insulin resistance in patients. This prompted us to investigate whether the splicing events regulated by Stau1 overexpression were all beneficial for the DM1 pathology. For this analysis, only ASEs which showed a change in splicing pattern ≥10% from WT to DM1 conditions were considered. ASEs that shifted back towards WT splicing patterns when Stau1-HA was overexpressed were considered beneficial. Conversely, an ASE was considered detrimental if the overexpression of Stau1-HA in DM1 conditions exacerbated the splicing pattern observed in the pathology, i.e. opposite direction of WT. Using these criteria, 25 ASEs were classified as beneficial, whereas 8 ASEs would be potentially detrimental upon Stau1-HA overexpression in DM1 patient cells (Fig 11A and B). This suggests that promoting

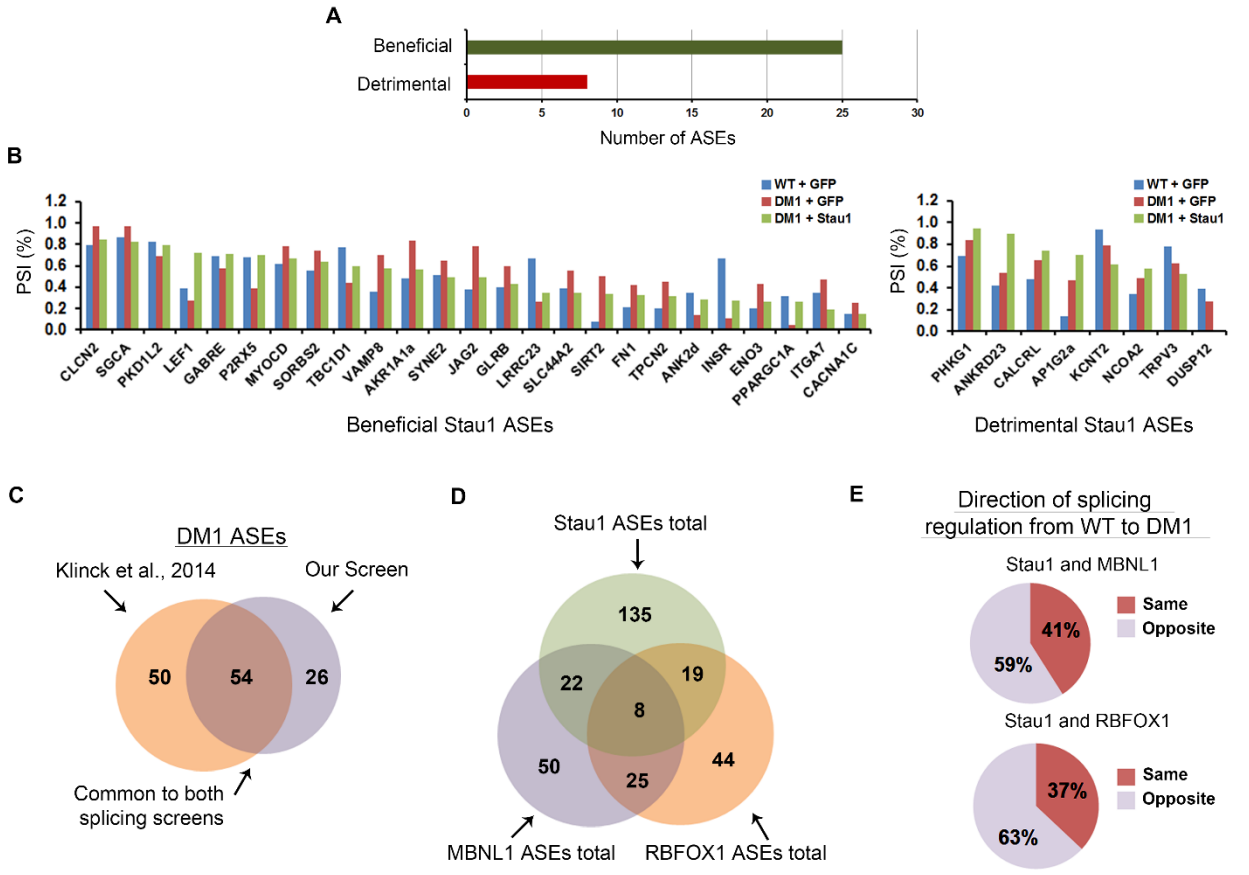

Figure 11. Stau1 overexpression regulates the alternative splicing of numerous ASEs which are both beneficial and detrimental in DM1 conditions.

(A) A total of 33 ASEs were considered for this analysis based on the following criteria. First, ASEs that showed a ≥10% ΔPSI from WT to DM1 conditions were selected. From this, ASEs that showed a ≥10% ΔPSI with Stau1 overexpression were selected and identified as either beneficial or detrimental for the DM1 pathology (refer to results). (B) Bar graphs showing the specific ASEs that the overexpression of Stau1-HA under DM1 conditions either returned towards the WT splicing pattern (left bar graph) or continued towards a detrimental effect (right bar graph). (C-E) Comparison of the data obtained from the RT-PCR splicing screen presented here to that of Klinck *et al*., 2014. The threshold used by Klinck *et al*., was applied (≥5% PSI) to compare the 163 ASEs

used in both screens (Table 2). (E) The direction of splicing was determined by identifying whether modification of the splicing proteins (i.e. Stau1 and/or MBNL1/RBFOX1) shifted the splicing pattern of an ASE in similar directions, for example, modification of both splicing factors resulted in an increased splicing of the long isoform.

Stau1-regulated splicing in DM1 could potentially have both beneficial and detrimental effects depending on the specific alternative splicing event considered. Taken together, these results demonstrate that Stau1 is a splicing factor that regulates a broad range of splicing events and highlights the importance of Stau1 as a potential disease modifier for DM1.

We extended the results collected from our RT-PCR splicing screen by generating a comparison between ASEs altered in DM1 identified in the current study relative to those documented in a recent report by Klinck and colleagues (Klinck *et al.*, 2014). Using the same thresholds set by Klinck *et al*., we identified 54 ASEs that appeared altered in the DM1 conditions that were common to both screens, including previously described events such as the *INSR*, *ANK2* and various chloride channels (Fig 11C and Table 2). The ASEs that were distinct between the two screens may be due to the different cell lines used in each independent study. More specifically, normal adult satellite muscle cells were used in the study by Klinck *et al*., and MyoD-converted myoblast cell cultures were used in our study. These differences in cell culture models may account for the variability in the DM1 associated ASEs identified. We also compared the ASEs regulated by Stau1-HA, to ASEs regulated by MBNL1 or RBFOX1 described by Klinck and colleagues. A total of 22 and 19 ASEs were identified that appeared to be co-regulated by either Stau1 and MBNL1 or RBFOX1, respectively (Fig 11D and Table 2). A total of eight ASEs were identified as being co-regulated by all three splicing proteins, including *INSR*. A comparison of the direction of splicing regulation of Stau1 to either MBNL1 or RBFOX1 revealed that 51% and 63% of the ASEs co-regulated by Stau1 and MBNL1 or RBFOX1, respectively, proceeded in the same direction (Fig 11E). The fact that Stau1 regulates the same splicing events as MBNL1 and/or RBFOX1 suggests that Stau1 may act as both an agonist and antagonist to other splicing factors.

Validation of Stau1-regulated splicing targets

Of the ASEs affected by Stau1 over-expression in the high-throughput screen, we validated by semi-quantitative RT-PCR 13 of 19 (68%). Among the 13 ASEs that were identified as Stau1-regulated events by our screen and independently shown to be regulated by Stau1 by semi-quantitative RT-PCR validation, we found *INSR*, *hnRNP A2B1*, *LRRC23*, *HIF1α*, *NRG1*, *FN1*, *ACCN3*, *FHL3*, *G6PC3*, *CLCN2* and *CLCN6* (Fig 12A-D and Fig 13A-G). Splicing analysis of four ASEs were extended to include two additional DM1 myogenic cell lines with varying numbers of CTG repeats; 500 CTGs and 50-80 CTGs (Fig 12A-D and Fig 13B and D). These additional DM1 cell lines were included to investigate the influence of Stau1-HA, which was overexpressed at relatively equal amounts (Fig 13D), on splicing regulation in varying degrees of the DM1 pathology. As expected, exon 11 inclusion of the *INSR* decreased with an increase of CTG repeats, and in all cases, Stau1-HA overexpression increased exon 11 inclusion by ≥15%, independent of the number of CTG repeats (Fig 12A). Taken together, the data suggest that Stau1 regulates the splicing of numerous ASEs even in cases of varying degrees of severity of the DM1 pathology.

Stau1-regulated ASEs contain Alu elements in their flanking introns

Given that Alu elements in the upstream intron flanking the alternative exon 11 of the *INSR* is required for both binding and splicing regulation by Stau1 (Fig 7), we examined how many other Stau1-regulated ASEs contained one or more Alu elements as compared to non-Stau1 regulated ASEs. Briefly, we first mapped out the genomic regions corresponding with each primer pairs used in the RT-PCR splicing screen using UCSC Genome Browser (human genomic data v.37).

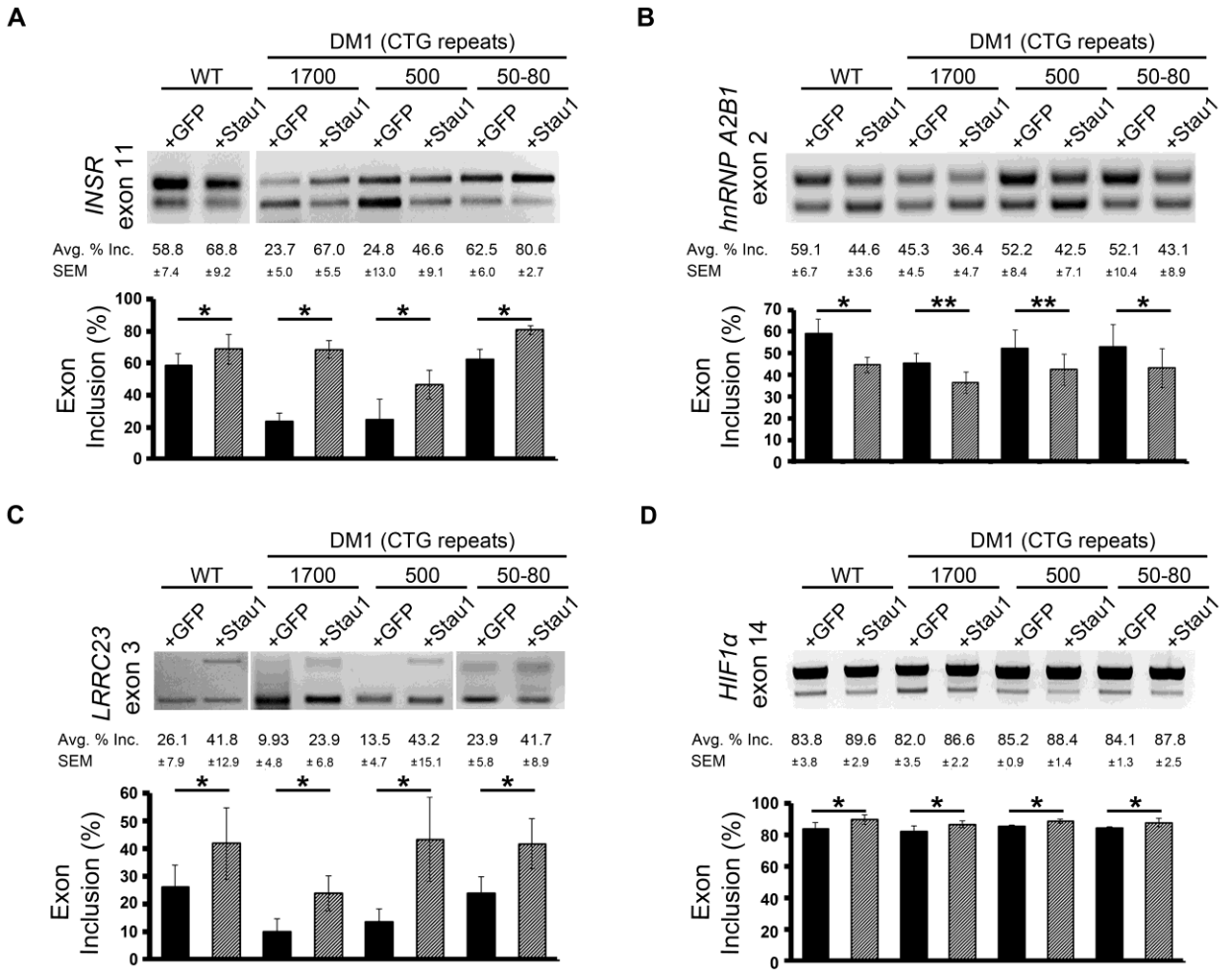

Figure 12. Validation of Stau1-regulated ASEs from RT-PCR splicing screen, in WT and DM1 cell lines.

Total RNA was collected from four GM0 cell lines; WT (GM01653) and DM1 cell lines GM03132 (1700 CTG), GM03987 (500 CTG), and GM03991 (50-80 CTG). RT-PCR was performed to determine splicing ratios of ASE in the (A) *INSR* (B) *hnRNPA2B1*, (C) *LRRC23*, (D) *HIF1α* mRNA isoforms. ASE is indicated by exon number for each event. Bar graphs show an average of three independent experiments. Error bars represent SEM * = $p < 0.5$, ** = $p < 0.01$.

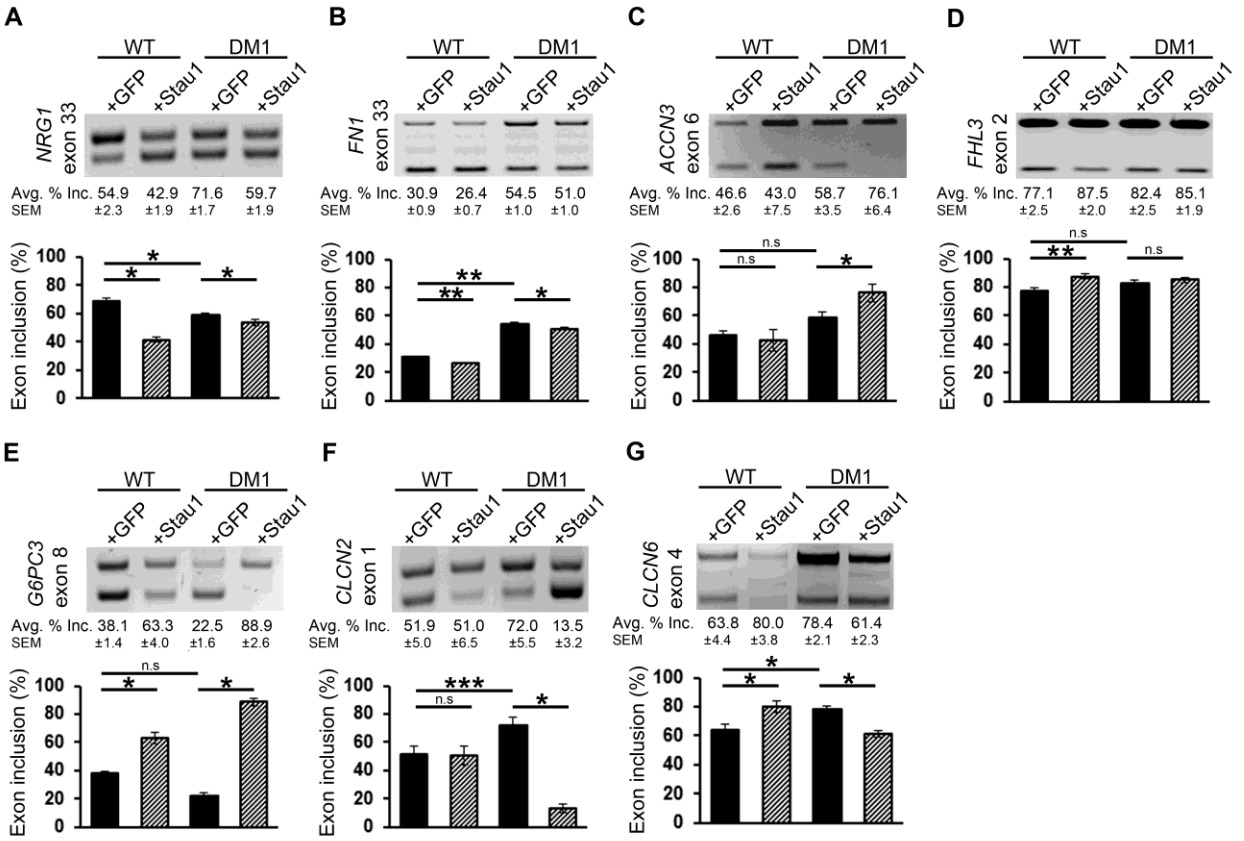

Figure 13. Additional validation of high-throughput RT-PCR splicing screen.

(A-G) Total RNA was collected from WT and DM1 (1700 CTG) cell lines. Semi-quantitative RT-PCR was performed to determine splicing ratios of (A) *NRG1*, (B) *FN1*, (C) *ACCN3*, (D) *FHL3*, (E) *G6PC3*, (F) *CLCN2* and (G) *CLCN6* mRNA long and short isoforms. ASE is indicated by exon number for each event. Bar graphs show an average of three independent experiments. Error bars represent SEM * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Next, utilizing RepeatMasker (release v.4.0.6), the number of Alu elements that were present within each genomic region of the ASEs was determined. We then compared the number of Stau1-regulated ASEs that harboured Alu elements to non-Stau1-regulated ASE targets. In total, 80.5% of Stau1-regulated targets (ΔPSI ≥15%) contained Alu elements. In contrast, only 65.7% of non-Stau1 regulated targets contained Alu elements (Fig 14A). A similar value of 68.3% was obtained when considering the whole dataset. Focusing on the Stau1 ASEs that contained Alu elements, we again used RepeatMasker to identify the subfamilies of the Alu elements in order to see whether any particular Alu subfamilies were prevalent in Stau1 ASE targets. This analysis revealed no obvious preference toward a specific subfamily of Alu element in the introns flanking Stau1 ASEs (Fig 14B). Further analysis comparing the proportions of the major subfamilies, i.e. AluY, AluS, and AluJ, identified in our study revealed a similar distribution of Alu family proportions to those reported in primate genomes (Deininger, 2011; Kapitonov and Jurkal, 1996). It has been observed for a number of splicing factors that their recruitment either upstream or downstream of the alternatively spliced cassette-exon correlated with whether they were promoting inclusion or skipping of that exon (Wang *et al.*, 2015; Wang *et al.*, 2012b). To determine if this seemed to be the case for Stau1, we examined the distribution of Alu elements relative to the alternative cassette-exon amongst top Stau1-regulated ASEs. This analysis revealed that the 35% of Alu elements were found within the upstream introns, relative to 17% in downstream introns, while the remaining 48% were found in both flanking introns (Fig 14B). However, this distribution did not seem to correlate strongly with whether Stau1 induced exon inclusion or skipping for those ASEs. Since Stau1 has also been proposed to bind to various non-Alu RNA secondary structures (Laver *et al.*, 2013; Ricci *et al.*, 2014), the predicted secondary

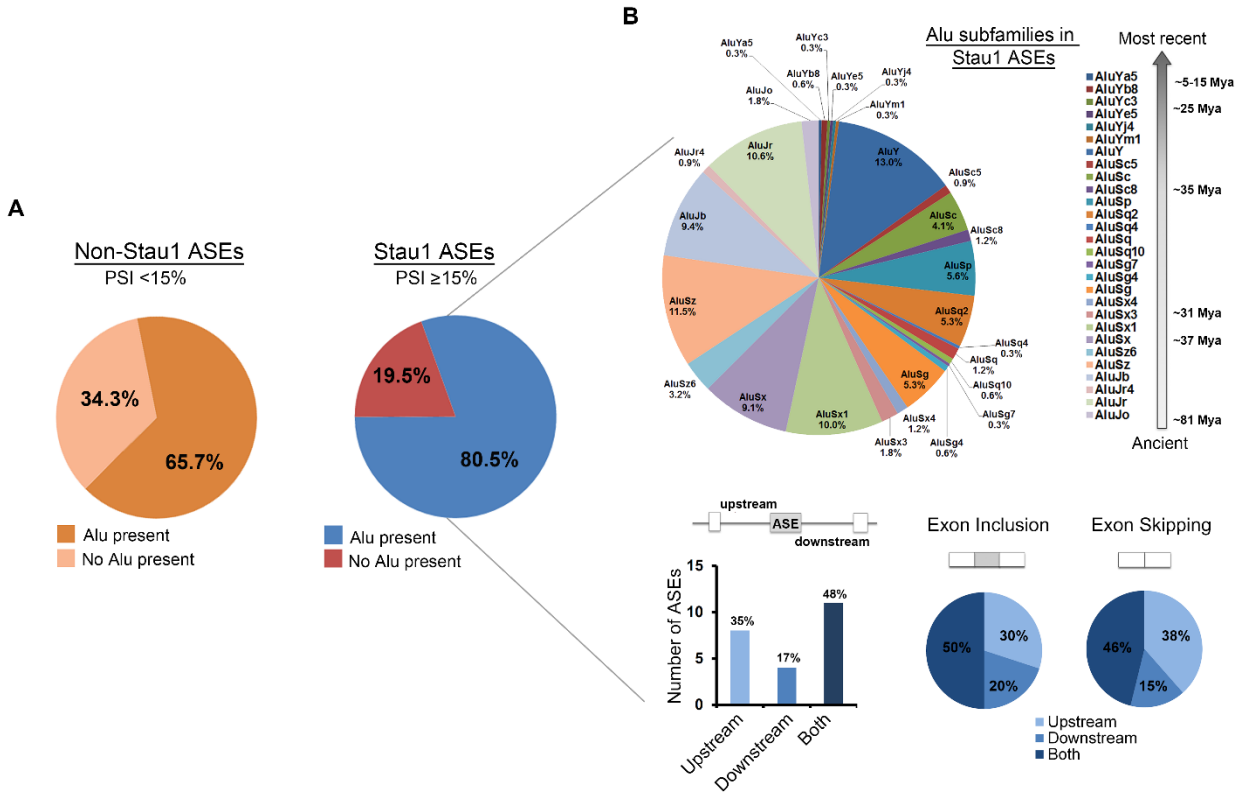

Figure 14. Alu elements in Stau1 and non-Stau1 regulated ASEs.

(A) All ASEs were considered for this analysis with the exception of any that had a blank PSI, as described above. Stau1 (≥15% PSI) and non-Stau1 regulated ASEs targets were searched using RepeatMasker software v 4.0.5, to identify the presence of Alu elements in introns flanking the ASE. (B) The subfamilies of Alu elements in Stau1-regulated targets are presented. The locations of Alu elements in Stau1-regulated cassette-type ASE containing targets are represented in a bar graph as the number of Alu elements found in the upstream, downstream or in both introns surrounding the ASE. The locations of these intronic Alu elements were then correlated to the type of splicing event induced by Stau1 overexpression, i.e. exon inclusion or exon skipping.

structures of the flanking introns of three non-Alu containing Stau1-regulated ASEs, were scanned for RNA secondary structures that resembled possible SBS. This additional analysis revealed the

presence of secondary structures resembling known or predicted SBS within the proximity of the ASEs of *hnRNP A2B1*, *LRRC23*, and *NRG1* (Fig 15A-C). These may serve as SBS allowing Stau1 to regulate the splicing of these pre-mRNAs when no Alu elements are present.

2.5 Discussion

We report here that Stau1 regulates the alternative splicing of *INSR* exon 11 through its interaction with Alu elements located in intron 10. Using a high-throughput alternative splicing screen, we also demonstrate that Stau1 regulates a broad range of pre-mRNAs, many of which also harbour Alu elements within introns flanking the ASE. Importantly, although Stau1 overexpression in DM1 myoblasts did rescue splicing patterns of many pre-mRNAs towards WT, as previously observed for *INSR* exon 11, a number of Stau1-induced splicing changes were also found shifting away from WT patterns, and thus would be predicted to exacerbate the DM1 pathology. Taken together, these findings are consistent with the notion that Stau1 may act as a disease modifier in DM1.

Stau1 regulates alternative splicing of *INSR* exon 11 through binding to Alu elements

In the present study, we demonstrated that modulation of Stau1 levels regulates *INSR* exon 11 splicing. This regulation was demonstrated in two non-muscle (HeLa and HEK293Ts) cell lines and complements our previous findings in DM1 muscle cell lines (Ravel-Chapuis *et al.*, 2012). Modulating Stau1 levels did not result in major changes in the expression of other splicing factors known to regulate exon 11 inclusion, such as MBNL1, CUGBP1 or hnRNP H.

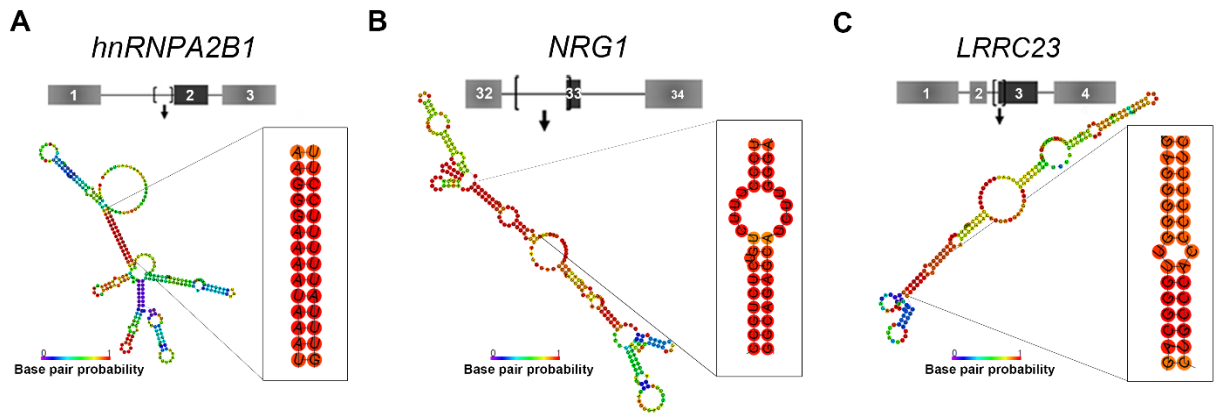

Figure 15. Proposed SBS in validated Stau1-regulated ASEs.

The genomic DNA sequence of the human (A) *hnRNPA2B1* (NG_000007.14) (B) *NRG1* (NG_000008.11) and (C) *LRRC23* (NG_000012.12) was used to assess the possible non-Alu SBS. RNA secondary structure of indicated introns was determined by Vienna package RNAfold 2.1.1 and identification of possible SBS were determined following guidelines described in the materials and methods.

Table 3. Analysis of Stau1-regulated ASEs associated with disease.

Gene symbol	Gene name	ASE	Stau1 OE WT	Stau1 OE DM1	Predicted by screen	Variant previously reported	Reported variant name (Δexon)	Splice variant disease related?
INSR	Insulin Receptor	Exon 11	Inclusion	Inclusion	Y	Y - Savkur et al., 2001	IR-A	Y – DM1 - Savkur et al., 2001
hnRNP A2B1	Heterogeneous nuclear ribonucleoprotein A2B1	Exon 2	Skipping	Skipping	Y	Y – Koz et al., 1995	hnRNP B1	Y – Lung cancer - Sueoka et al., 1999; Kamma et al., 1999
LRRC23	Leucine-rich repeat-containing protein 23	Exon 3	Inclusion	Inclusion	Y	N	-	N
NRG1	Neuregulin 1	Exon 8	Skipping	Skipping	Y	?	?	? – NRG1 disregulation disrupts synaptic plasticity - Agarwal et al., 2014
HIF1α	Hypoxia-inducible factor 1-alpha	Exon 14	Inclusion	Inclusion	Y	Y - Gothie et al., 2000	HIF1α^{736} and sHIF1α	Y- higher expression levels of HIF1α^{736} in OR-negative carcinomas - Dales et al., 2010
FN1	Fibronectin	Exon 33	Skipping	Skipping	Y	Y - Goossens et al.,2009	EDA or EDI	Y – DM1 - Ohsawa et al., 2011
ACCN3	acid sensing (proton gated) ion channel 3	Exon 6	--	Inclusion	Y	?	?	N
CLCN6	Chloride Channel, Voltage-Sensitive 6	Exon 4	Inclusion	Skipping	Y	Y – Eggermont et al., 1997	CIC6b and Exon y+3	N

Y- Yes; N- No; ? - Variant reported in databases (NCBI) however could not find variant in publication; - - No information

Furthermore, our previous work found no protein-protein interactions between Stau1 and MBNL1 or CUGBP1 (Ravel-Chapuis *et al.*, 2012). Taken together, these findings support the idea that Stau1 regulates splicing without direct protein interactions or modulation of the expression levels of these other key splicing factors. Nevertheless, Stau1 may still affect the functional activity of these splicing factors in ways that are more indirect.

Alu elements serving as cis-regulatory elements for splicing have been described. For example in the *RABL5* pre-mRNA, two Alu elements that were in opposite orientation in the upstream intron were shown to affect the splicing patterns of the downstream exon 3 (Lev-Maor *et al.*, 2008). Another example is the regulation of the alternative splicing in the Ataxia Telangiectasia Mutated (*ATM*) pre-mRNA. In this case, an intronic splicing element derived from an Alu element was found to modulate the inclusion of a cryptic exon (Pastor *et al.*, 2009). In a number of cases, binding of trans-acting factors to Alu elements is required to mediate an effect on splicing (Kelley *et al.*, 2014; Zarnack *et al.*, 2013). For example, Zarnack and colleagues demonstrated that, in the absence of hnRNP C, thousands of Alu elements were included as exons in mRNA transcripts (Zarnack *et al.*, 2013). Splicing analysis of IR-minigenes in HeLa cells demonstrated a drastic shift in the splicing pattern to exon 11 inclusion occurred when the Alu elements in intron 10 were deleted. These findings agree with previous literature reporting similar results in HepG2 cells (Kosaki *et al.*, 1998). Thus, we propose that the presence of Alu elements serves to inhibit the inclusion of exon 11, perhaps through the recruitment of one or more Alu-element binding trans-acting factor(s), in addition to Stau1, that would then interfere with recruitment of constitutive splicing factors (see model in Fig 16). It may be informative to examine any potential interactions between Stau1 and known Alu-binding splicing proteins to further define the mechanism by which Stau1 regulates alternative splicing.

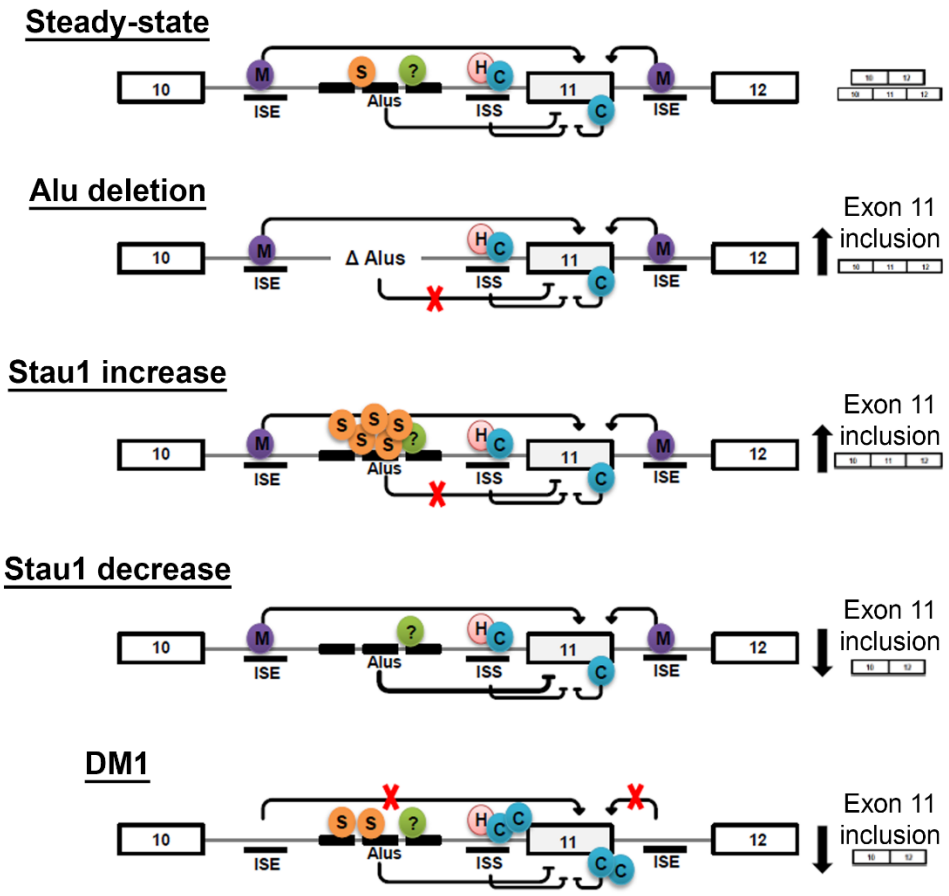

Figure 16. Proposed model of how Stau1 may contribute to *INSR* exon 11 alternative splicing.

At steady-state, Stau1's binding to Alu elements participates in the integration of several regulatory mechanisms towards establishing the level of utilisation of exon 11. Introns are denoted as grey lines and exons as large boxes. The splicing factors MBNL1 (M), CUGBP1 (C), hnRNP H (H), and Stau1 (S) are depicted binding to various known cis-regulatory elements (i.e. intronic splicing enhancer (ISE) and intronic splicing silencers (ISS)) and to the Alu elements (Alus). Complete deletion of the Alu elements results in increased exon 11 inclusion, suggesting they normally contribute towards a repressive influence on exon inclusion. Upon overexpression of

Stau1, increased exon 11 inclusion is also observed, suggesting Stau1 somehow disrupts the repressive influence of the Alu elements (see Discussion for details). Upon knockdown of Stau1 expression, the full inhibitory effect of the Alu elements would be observed. In DM1, the combined effects of reduced MBNL1 levels increased CUGBP1 activity, and a moderate increase in Stau1 would result in decreased exon 11 inclusion.

Our results show that increased Stau1 levels correlate with increased inclusion of exon 11. The specific details of what occurs once more Stau1 binds to the Alu elements remains unclear. As depicted in Fig 16, this could somehow prevent the inhibitory effect of the Alu repeats on exon 11 inclusion, either by preventing recruitment of Alu binding factors or through recruitment of a distinct factor that would instead favour inclusion. Conversely, upon Stau1 knock down, this would allow for the full inhibitory effect of the Alu elements on exon 11 inclusion (Fig 16). A number of additional factors have been reported to regulate alternative splicing of exon 11 (e.g. hnRNP H, DDX5, etc) (Mahadevan, 2012) and we cannot rule out a mechanism whereby Stau1 binding to the Alu elements may modulate the action of one or more of these factors. Finally, in the context of DM1, where we know the positive contribution of MBLN1 is lost and the inhibitory effects of CUGBP1 are enhanced, the moderate increase in Stau1 levels that we have documented in patient tissues, may not be enough to completely prevent the inhibitory contribution of the Alu elements on exon 11 inclusion (Fig 16). Alternatively, Stau1's activity might be misregulated in DM1 (e.g. through post-translational modification(s) and/or interaction with distinct factors). Further experimentation will be required to determine the precise mechanism(s) by which Stau1 contributes to *INSR* splicing under both normal and DM1 settings. Nevertheless, to our knowledge, this is the first example showing that the binding of Stau1 to Alu elements can regulate alternative splicing.

Stau1 can affect the alternative splicing of a broad range of pre-mRNAs

Strikingly, our high-throughput screen has revealed that Stau1 can regulate alternative splicing to an extent that is comparable to well-established splicing regulators like MBNL1 and RBFOX1 (see Fig 11D). An analysis of all ASEs examined in this study revealed that Stau1-regulated ASEs were more likely to harbour one or more Alu elements than those that were not Stau1-sensitive

(80.5% vs. 65.7%, respectively). Amongst Stau1-regulated pre-mRNAs Alu elements were found within both introns flanking the ASE for ~50% of cases. Recent studies that investigated SBS uncovered that Stau1 preferentially binds complex, imperfectly paired duplex structures formed by the pairing of at least two Alu elements (Gong and Maquat, 2011; Ricci *et al.*, 2014). Thus, it would be interesting in future work to investigate whether Alu elements found on either side of an ASE need to form duplexes in order to influence splicing decisions. Genome-wide occupancy assessment of splicing regulators, using, for example, HITS-CLIP or similar approaches, has revealed that binding of splicing regulators either upstream or downstream of an ASE is often correlated with whether it will mediate a positive or negative effect on that splicing event (Wang *et al.*, 2012b). Using a limited dataset of ASEs most significantly affected by Stau1, we determined that when Alu elements (and thus potential SBSs) were not present in both flanking introns, they were most often found in introns upstream of the ASE (~twice as often than in downstream intron; see Fig 14). However, we found no correlation between Alu elements being present upstream or downstream of ASEs and whether the effect of Stau1 on that ASE was the induction of skipping or inclusion. We did not document within this limited datas*et al*u elements positioned in very close proximity or overlapping with splice sites, but this is obviously another aspect that would need to be considered. These questions will require analysis of larger datasets in order for more conclusive patterns to emerge.

Although a majority of Stau1 ASEs harboured Alu elements in introns flanking the ASE, we observed that ~20% ASEs affected by Stau1 level modulation did not contain any Alu elements. Further analysis into the RNA secondary structure of non-Alu containing introns flanking ASEs revealed the presence of RNA duplex structures, which may represent potential SBSs. Interestingly, the three potential non-Alu SBSs in the Stau1 targets *hnRNPA2B1*, *NRG1* and

LRRC23 (Fig 15) are all located close to the 3'SS, which could thus interfere with recognition of this site by the basic splicing machinery; a phenomenon that Stau1 could then either promote or interfere with. Several pre-mRNAs containing ASEs that were sensitive to Stau1 level modulation have been previously identified as Stau1 targets. Specifically, Stau1 binds to these targets through non-Alu element binding sites located in the coding sequence of the transcripts, including: *ADPGK*, *AKT2*, *ANK1*, *APOBEC3C*, *ARF1*, *ARFRP1*, *ENSA*, *FN1*, *JAG2*, *NUTF2*, *OGDH*, *SHKBP1*, *SORBP1*, *TBC1D12/13*, and *THRA* (de Lucas *et al.*, 2014; Ricci *et al.*, 2014; Sugimoto *et al.*, 2015).

Stau1 as a disease modifier in DM1

Over the past decades, the major emphasis in defining the molecular pathogenesis of DM1 has focused on the role of a very few specific RNA-binding proteins, such as MBNL1 and CUGBP1, in aberrant alternative splicing events in DM1. Although animal models of MBNL1 and CUGBP1 (Ho *et al.*, 2005; Timchenko *et al.*, 2004) do reproduce many DM1 symptoms (Kanadia *et al.*, 2003; Suenaga *et al.*, 2012), some are not recapitulated, suggesting that other factors, such as disease modifiers are involved. Indeed, several disease modifier proteins have recently been identified and shown to have an impact on the DM1 pathology. For example, in a Drosophila DM1 model the RNA-binding proteins TBPH (homolog of human TAR DNA-binding protein 43 or TDP-43) and BSF (Bicoid stability factor; homolog of human LRPPRC) were found be misregulated with the expression of CUG expansions resulting in altered muscle sarcomere location of these proteins (Llamusi *et al.*, 2013). Another study, done by Huin and colleagues, reported that several genetic variants of the *MBNL1* gene could be associated with the severity of the disease, suggesting that these variants were acting as disease modifiers in DM1. (Huin *et al.*, 2013). Finally, the DEAD-box RNA helicase, DDX5/p68, found to be reduced in DM1 biopsied

skeletal muscle (Jones *et al.*, 2015), was shown to allow increased MBNL1 binding to mutant repeats which can influence splicing events misregulated in DM1 (Laurent *et al.*, 2012).

In agreement with our initial study (Ravel-Chapuis *et al.*, 2012), the data presented here show that overexpression of Stau1 resulted in several splicing events predicted to be beneficial for DM1, such as the rescue of the *INSR* exon 11. However, we also identified a number of detrimental splicing effects, which would likely exacerbate the DM1 pathology (see Fig 11A-B). This suggests that the upregulation of Stau1 may not represent a protective role in the DM1 pathology as previously suggested but, instead, shows Stau1 likely acts as a disease modifier for DM1 whose splicing impact can result in both beneficial and detrimental effects on the DM1 phenotype. Additionally, it is possible that Stau1 may act as a disease modifier in DM1 through non-splicing related activities. For example, we have recently shown that Stau1 negatively regulates skeletal muscle differentiation, at least in part through its regulation of c-myc translation (Ravel-Chapuis *et al.*, 2014). As such, Stau1 may thus contribute to the impaired differentiation/developmental program observed in DM1 (Amack and Mahadevan, 2004). The precise overall contribution of Stau1 to the DM1 phenotype thus remains to be fully explored, but our results to date strongly indicate that Stau1 needs to be considered amongst the gene products that modulate the complex DM1 pathophysiology and its response to future therapeutic interventions.

2.6 Materials and Methods

Cell lines

The following cell lines were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research: WT cell lines were represented by two cell lines with 0-5 CTGs repeats: GM03377 (Splicing screen) and GM01653 (Validation). DM1 cell lines used were GM03132 (1700 CTGs), GM03987 (500 CTGs) and GM03991 (50-80 CTGs). HeLa cell lines were obtained from ATCC (ATCC CCL-2) and HEK293-T cell lines were obtained from ATCC (ATCC CRL-1573).

Plasmid constructs, cell transfections and infections and cell lysis

Constructs: IR-minigenes WT and ΔAlus (aka IR-B and IR-E) were generously donated by Nicholas Webster and previously described in (Kosaki *et al.*, 1998), MyoD virus (pBRIT-MyoD-(His-TEV-3FLAG)) , GFP virus, hStau1^{5}-HA plasmid (described in (Wickham *et al.*, 1999)), shStau1 plasmid mix made up of two shRNAs against human Stau1 mRNA (referred to in text as shStau1) (Open Biosystems GE Dharmacon: sh06 [Clone ID:TRCN0000102306] + sh09 [Clone ID:TRCN0000102309]). Cells to be transfected were grown to ~70% confluency and transfected with 1-3 μg of DNA using Lipofectamine with plus reagent (Life Technologies:15338100), according to manufacturers protocol, for 48 hours. Virus production consisted of using plasmids previously described (Ravel-Chapuis *et al.*, 2012) pcDH-CMV-MCS-EF1-copGFP and pcDH-Stau1. Viral particles were produced by transient transfection of HEK-293T cells with lentiviral packaging vectors psPAX2 (Addgene:12260) and pMD2.G (Addgene:12259) using Lipofectamine 2000 reagent (Life Technologies:11668027) according to manufactures protocol. The conditioned medium containing viral particles was collected and used to transduce control and DM1 myoblasts overnight in the presence of 8ug/ml Hexadimethrine Bromide (SIGMA:H9268).

Subsequent infections were performed the following day, and cells were grown for several days before analyses. Infection of cells involved growing cells until ~70% confluency, infecting first with MyoD virus, selection with Puromycin (Wisent Bio Products:400-160-EM) (1μg/mL) for 5 days, infection with either the GFP or hStau1[55]-HA virus, confirming GFP expression after 48 and harvesting cells for RNA and protein 72 hours after initial second infection. Cells were washed with 1XPBS, scraped and lysed in 1 mL of RIPA buffer with Protease Inhibitor added prior to use. Cells with incubated in RIPA buffer for 30 min on ice and centrifuged for 15 minutes at 13,200 rpm. The supernatant was collected and stored at -20°C until use.

Western blotting analysis and antibodies

Following cell lysis, protein concentration was assessed using the Bio-Rad DC Protein Assay (Bio-Rad:500-0111) and protein (2-40 μg) was resolved by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PDVF) membranes (Immunobilon Transfer Membranes:IPVH00010). Transferred membranes were blocked with 5% milk for 30 minutes and probed with appropriate antibody in 1% milk solution for either 1 hour at room temperature or 12 hours at 4°C, with three 10 min washes with 1XPBS-0.05% Tween 20 between each antibody incubation. Antibodies included: Anti-Stau1 [1:1000] (Abcam:ab73478), Anti-GAPDH [1:10,000] (Abcam:ab8245), Anti-β-Actin [1:500] (Santa Cruz:sc-47778), Anti-CUGBP1 [1:1000] (Santa Cruz:sc-20003), Anti-hnRNP H [1:5000] (Abcam:10374), Anti-MyoD [1:300] (BD Pharmingen: 554130), Anti-MBNL1 antibody [1:300] (Abnova:H00004154), Anti-HA F7 probe [1:1000] (Santa Cruz:sc-7392). Secondary antibodies included: Mouse-anti-Rabbit HRP [1:20,000] (Jackson ImmunoResearch:211-032-171) and Goat-anti-Rabbit HRP [1:10,000] (Molecular Probes:MP 02764). Proteins on membranes were detected with Millipore-Luminata

Crescendo Western HRP Substrate (WBLUR0500) and visualized on film (HyBlot CL Autoradiography Film:E3018).

RNA extraction and cDNA synthesis

RNA was isolated from whole cell lysates using Ambion TRIzol Reagent (E3018) and 2μL of collected RNA was assessed on the Take3BioPlate Reader to determine quantity (ng/μL) and quality (RNA with 260/280 ~ 2.0 was used). 500 ng of RNA was used to synthesize cDNA with random hexamers (10mM) and the Promega AMV cDNA synthesis (Promega:M5101) was carried out following manufacturer's protocol. cDNA was diluted 1:20 and 5μL (~100 ng) was used for each RT-PCR and RT-qPCR reaction. All cDNA and RNA was stored at -20°C short-term and -80°C long term.

Reverse Transcription (RT)- PCR and quantitative RT- PCR (RT-qPCR)

RT-PCR was performed using Promega GoTaq® DNA Polymerase (Promega:M5101) according to manufacturer's protocol. RT-PCR conditions were as follows for validation of splicing screen: 95°C for 2 min, (95°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec)x32 cycles, 72°C for 10 min. Specific RT-PCR conditions used for particular primers are available upon request. Amplicons were run on a 2% agarose gel (containing 3-5 μL of EtBr 20 mg/mL) and visualized under UV light. All RT-qPCR reactions were performed using BioRad iQ SYBR® Green Supermix (BioRad:170-8882) according to manufactures protocol and run with a Chromo 4 Real-Time PCR Detector. RT-qPCR conditions for all primer sets used were carried out as follows: 95°C for 2 min, (95°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec)x40 cycles, 72°C for 10 min. Technical replicates of 3 were done for all RT-qPCR experiments and the average Ct values were normalized to either *GAPDH* or 18S (indicated in descriptions). The ΔΔCt method was used to analyze fold change of transcripts. Primers for *INSR* splicing analysis were previously described (Ravel-

Chapuis *et al.*, 2012), and any of the 487 primer sets used in our RT-PCR splicing screen are available upon request. Biological replicates of $\geq$3 samples were done for all PCR reactions.

High-throughput RT-PCR Splicing Screen

RNA from (WT) GM03377 and DM1-1700 CTG (GM03132) was synthesized to cDNA and subject to the screen as previously described (Klinck *et al.*, 2014). Raw data from the screen is included in Table 2. Analysis of data to determine top ASE included rankings of events that had the greatest PSI change (Δ) between two conditions, i.e. the ΔPSI between WT+GFP virus and WT+Stau1 virus:

$$\Delta PSI = PSI^{GFP} - PSI^{Stau1\text{-}HA} \quad (1)$$

Only values with a ΔPSI $\geq$10% between conditions were selected for additional analysis:

$$\left[(PSI(WT)^{GFP} - PSI(DM1)^{GFP}) \left(\sqrt{-(n)^2} \right) \right] * 100 \geq 10\% \quad (2)$$

ASEs which contained PSI values with no data (no isoforms detected), were not included in analyses.

Validation of ASEs regulated by Stau1

A minimum of three biological replicates were used to validate a change in the splicing patterns (ΔPSI) for all cell lines tested. Semi-quantitative RT-PCR was carried out with the required primers to obtain two isoforms describing an ASE for the splicing screen. Splicing patterns in each condition (WT+GFP, WT+Stau1-HA, DM1+GFP, DM1+Stau1-HA) was analyzed and classified as successfully tested and validated if a change in splicing pattern was detected in all three biological replicates and followed the same splicing pattern as predicted by our screen for all replicates (n=3). ASEs were classified as successfully tested but not validated if the splicing pattern detected in all three biological replicates did not change between conditions or did not follow the splicing pattern predicted by the screen.

RNA Immunoprecipitations (RIPs)

48 hours after transfection cells were treated with 1% formaldehyde to induce cross-link *in vivo* Stau1-HA-RNA complexes for 10 minutes at RT and reaction was quenched with 0.25 M glycine in PBS. Cells were suspended in 1 mL of RNase-free RIPA buffer and centrifuged for 15 minutes at 13, 200 rpm at 4°C. The supernatant was collected and centrifuged twice more. 40 μL of Santa Cruz Protein A/G PLUS agarose beads (Santa Cruz:sc-2003) suspended in RNase-free RIPA buffer was added to lysate was incubated with gentle rotation for 1 hour at 4°C to pre-clear. Beads were removed by centrifugation and the pre-cleared lysate was aliquoted by volume into 10% input, IgG and IP. Normal mouse IgG antibody (Santa Cruz:sc-2025) or mouse-anti HA antibody (4 μg) was added to IgG or IP, respectively, and incubated for 16 hours at 4°C with gentle rotation. 40 μL of A/G plus beads were added to IgG and IP samples and incubated for 1 hour at 4°C with gentle rotation. Stau1-RNA complexes bound to beads were pelleted at 2,500 rpm for 30 sec, supernatant removed and the pellet was resuspended in 1 mL of RNase-free RIPA buffer. These washes were repeated three times. Following final resuspension of pellet, crosslinking was reversed (1 hour at 70°C). Trizol was then directly added to the bead-RIPA solution and RNA isolation protocol was followed (as described above).

Identification of SBS (Alu elements and potential non-Alu elements) flanking ASEs

Primer pairs in the high-throughput RT-PCR splicing screen were used in a BLAST to identify the mRNA transcript ASE that Stau1 was suspected to regulate. Using CLC MainWorkbench, alignments of the DNA and mRNA transcripts revealed the exon(s) and flanking introns, which defined the ASE. Flanking introns were then analyzed with RepeatMasker (v.4.0.6) to identify the presence and subfamily of Alu element(s). Exons that made up the ASE were also analyzed for the presence of Alu elements however, none was found at that time. A total of 23 cassette exon

type Alu element containing Stau1-regulated ASE targets were examined to identify the location of the Alu(s), either upstream or downstream of the ASE. These were then categorized by whether Stau1 overexpression induced exon inclusion or skipping of the cassette exon. If an intronic sequence did not contain any Alu elements, the MFE structure of the intronic sequences were manually searched for potential duplexes resembling SBS. These potential SBS were identified based on previous reports describing identified SBS, (de Lucas *et al.*, 2014; Ricci *et al.*, 2014) i.e. average size of stem length (duplex) (between 5-19 base pairs), high base pair probability and varying degrees of imperfect base pairing (preference given to longest continuous duplex structure formation). If the intronic sequence was >7,500 bps (current limit of RNAfold partition function calculations), then intron was divided into equal segments, each $\leq$7,500 bps, and subsequent predicted RNA secondary structure was used.

Statistical Analysis

To identify statistical significance between two groups one-tailed student's t-tests were carried out on data with biological replicates $n \geq 3$. The level to determine a value as significant was set as $p<0.05$. Significance was denoted as follows: * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

2.7 Acknowledgements

The authors would like to thank Nicholas J. G. Webster for providing the IR-minigene constructs, Mathieu Durand and Philippe Thibault for discussions about the RT-PCR screen and Guy Bélanger for technical assistance.

Chapter 3 - Manuscript #2

A Novel Role for CARM1 in Promoting Nonsense-Mediated mRNA Decay: Potential Implications for Spinal Muscular Atrophy

Publication Information:

Sanchez, Gabriel*, Emma Bondy-Chorney*, Janik Laframboise, Geneviève Paris, Andréanne Didillon, Bernard J. Jasmin, and Jocelyn Côté. "A novel role for CARM1 in promoting nonsense-mediated mRNA decay: potential implications for spinal muscular atrophy." *Nucleic acids research* 44, no. 6 (2015): 2661-2676.

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Author's contribution:

Conceived and designed the experiments: EBC, GS, JL, GP, AD, BJJ, JC. Performed the experiments: EBC, GS, JL, GP, AD. Analyzed the data: EBC, GS, JL, GP, AD, BJJ, JC. Contributed reagents/materials/analysis tools: EBC, GS, JL, GP, AD, BJJ, JC. Wrote the paper: EBC, GS, BJJ, JC.

Contributions by figure: Fig 17 (EBC, GS, JL), Fig 18 (EBC, GS, JL), Table 4 (EBC, GS), Fig 19 (GS), Fig 20 (EBC, GS), Fig 21 (EBC, JL), Fig 22 (EBC), Fig 23 (EBC, JL), Fig 24 (EBC, GS, JL), Fig 25 (GP, JL), Fig 26 (GP), Fig 27 (EBC, JL, GP)

A Novel Role for CARM1 in Promoting Nonsense-Mediated mRNA Decay: Potential Implications for Spinal Muscular Atrophy

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3.1 Abstract

Loss of 'Survival of Motor Neurons' (SMN) leads to spinal muscular atrophy (SMA), a disease characterized by degeneration of spinal cord alpha motor neurons, resulting in muscle weakness, paralysis and death during early childhood. SMN is required for assembly of the core splicing machinery, and splicing defects were documented in SMA. We previously uncovered that Coactivator-Associated Methyltransferase-1 (CARM1) is abnormally up-regulated in SMA, leading to mis-regulation of a number of transcriptional and alternative splicing events. We report here that CARM1 can promote decay of a premature terminating codon (PTC)-containing mRNA reporter, suggesting it can act as a mediator of nonsense-mediated mRNA decay (NMD). Interestingly, this pathway, while originally perceived as solely a surveillance mechanism preventing expression of potentially detrimental proteins, is now emerging as a highly regulated RNA decay pathway also acting on a subset of normal mRNAs. We further show that CARM1 associates with major NMD factor UPF1 and promotes its occupancy on PTC-containing transcripts. Finally, we identify a specific subset of NMD targets that are dependent on CARM1 for degradation and that are also misregulated in SMA, potentially adding exacerbated targeting of PTC-containing mRNAs to the already complex array of molecular defects associated with this disease.

3.2 Introduction

Autosomal-recessive proximal spinal muscular atrophy (SMA) is a progressive neuromuscular disorder characterized by the selective loss or dysfunction of α-motoneurons in the anterior horn of the spinal cord (Markowitz *et al.*, 2012). With a prevalence of at least 1 in 10000 live births and a carrier frequency of ~1 in 40, SMA is amongst the leading genetic cause of infant mortality (Ogino and Wilson, 2004; Swoboda, 2011). Based on the time of onset of the disease and its severity, SMA can be divided into five types, with Type 0 and Type I (Werdnig–Hoffman syndrome) being the most severe forms (Kostova *et al.*, 2007). Patients with severe Type I SMA will usually develop weakness of the proximal muscles of the trunk and body, ultimately leading to muscle atrophy and death from respiratory distress within ~2 years of age, depending on the choice of palliative care (Gregoretti *et al.*, 2013). SMA is caused by disruption of the survival of motor neuron (SMN1) gene (Lefebvre *et al.*, 1995). In humans, a second copy of the *SMN* gene exists but naturally harbors a non-polymorphic C→T transition that interferes with the normal splicing of exon 7, resulting in the expression of a truncated and unstable form of the protein (Burnett *et al.*, 2009; Cho and Dreyfuss, 2010; Lorson *et al.*, 1999; Monani *et al.*, 1999). The low level of full-length functional SMN protein produced in human SMA patients is sufficient to sustain embryonic development and survival of all cells, except lower motoneurons, which seem to have a lower 'tolerance threshold' for SMN levels, a phenomenon which still remains one of foremost questions in the field. SMN exists in cells as part of a stable ~50S macromolecular complex consisting of at least eight tightly associated components that include Gemins 2-8 and unrip (Eggert *et al.*, 2006; Kolb *et al.*, 2007; Pellizzoni, 2007). The best understood function for the core SMN complex is its essential role in promoting the efficiency and specificity of the cytoplasmic assembly of Sm proteins and U snRNAs into small nuclear ribonucleoprotein particles

(snRNPs), the core components of the pre-mRNA splicing machinery (Eggert *et al.*, 2006; Fischer *et al.*, 2011; Kolb *et al.*, 2007; Pellizzoni, 2007). Accordingly, a number of studies have now reported lower levels of specific U snRNAs and widespread splicing defects in SMA tissues, including a recently uncovered feedback loop affecting the splicing of *SMN2* exon 7 and misregulation of a subset of U12-dependent introns (Baumer *et al.*, 2009; Boulisfane *et al.*, 2011; Campion *et al.*, 2010; Gabanella *et al.*, 2007; Huo *et al.*, 2014; Jodelka *et al.*, 2010; Lotti *et al.*, 2012; Ruggiu *et al.*, 2012; See *et al.*, 2014; Zhang *et al.*, 2008).

Parallel work also supports additional and distinct roles for SMN in motoneurons, where it localizes to so-called RNA granules along axonal processes and interacts with a number of RNA binding proteins such as FUS/TLS, IMP1/ZBP, TDP-43, hnRNP R/Q, FMRP, KSRP and HuD (Akten *et al.*, 2011; Dombert *et al.*, 2014; Fallini *et al.*, 2014; Fallini *et al.*, 2011; Freibaum *et al.*, 2010; Hubers *et al.*, 2011; Peter *et al.*, 2011; Piazzon *et al.*, 2008; Rossoll *et al.*, 2003; Rossoll *et al.*, 2002; Sun *et al.*, 2014; Tadesse *et al.*, 2008). RNA granules are responsible for the transport, along microtubules, of specific mRNAs in dendrites and axons, and contribute to the regulation of mRNA stability and local translation at synapses and growth cones, which in turn is crucial for neuronal differentiation, axon outgrowth, and synaptic function (Kiebler and Bassell, 2006; Liu-Yesucevitz *et al.*, 2011; Thomas *et al.*, 2011). Recent studies from our group and others have provided evidence suggesting SMN is somehow required for the proper assembly of RNA granules (Akten *et al.*, 2011; Fallini *et al.*, 2011; Hubers *et al.*, 2011). Therefore, defects in RNA granules assembly and/or function may account for the various neurite outgrowth and synaptic maturation, stability and functional phenotypes documented in SMA (Biondi *et al.*, 2008; Bowerman *et al.*, 2007; Cifuentes-Diaz *et al.*, 2002; Kariya *et al.*, 2008; Kong *et al.*, 2009; Liu *et al.*, 2011; McGovern *et al.*, 2008; Mentis *et al.*, 2011; Murray *et al.*, 2008; Rossoll *et al.*, 2003; Shafey *et*

al., 2008; Tadesse *et al.*, 2008). It was also demonstrated for the first time recently that local translation of β-actin mRNA was deregulated in motorneurons from a severe mouse model of SMA (Rathod *et al.*, 2012), although a direct involvement of SMN in this process was not investigated.

In a recent study (Sanchez *et al.*, 2012), we demonstrated that SMN co-fractionates with polyribosomes and represses translation *in vitro*. In this work, we further identified the protein arginine methyltransferase CARM1 as a target that is repressed by SMN at the translational level in motorneuron-derived MN-1 cells. Accordingly, we documented that CARM1 is abnormally up-regulated at the protein level in spinal cord tissue from SMA mice and in severe Type I SMA patient cells. CARM1 is best-known as a transcriptional regulator through its methylation of histones and transcription factors/co-regulators (Ackermann *et al.*, 2013; Di Lorenzo and Bedford, 2011; Kawabe *et al.*, 2012; Lee and Stallcup, 2009; Wu and Xu, 2012), but has also emerged as a factor able to influence post-transcriptional processes, including alternative pre-mRNA splicing and mRNA stability, through methylation of specific splicing factors and RNA-binding proteins (Calvanese *et al.*, 2010; Cheng *et al.*, 2007; Fauquier *et al.*, 2008; Feng *et al.*, 2006; Fujiwara *et al.*, 2006; Hubers *et al.*, 2011; Yang and Bedford, 2013). Based on these observations, we used genome-wide exon array technology to identify specific transcriptional and splicing targets of CARM1 in motoneuron-derived MN-1 cells and found that a number of these targets were also misregulated in a SMA cell culture model (Sanchez *et al.*, 2012), strongly suggesting that CARM1 up-regulation contributes to altered gene expression profiles observed in the pathology.

Nonsense-mediated mRNA decay (NMD) is a surveillance mechanism that in addition to preventing production of truncated protein isoforms with potentially noxious consequences for the cell (Wagner and Lykke-Andersen, 2002), has also been shown to act on numerous mRNAs thereby contributing to general post-transcriptional gene regulation (Yepiskoposyan *et al.*, 2011).

In the present study, we report that CARM1 interacts with the essential NMD factor UPF1 in an RNA-dependent fashion and can promote NMD on a generic β-globin pre-mRNA reporter. Additionally, CARM1 was required for interaction of UPF1 with a premature termination codon (PTC)-containing mRNA. We identified a number of well-established endogenous NMD targets that were misregulated in the face of modulated CARM1 levels and in SMA conditions. Since there is an aberrant upregulation of CARM1 in SMA, we believe that this (and potentially other mechanisms) induce NMD misregulation of several key mRNAs that could contribute to the SMA pathophysiology.

3.3 Materials and Methods

Cell Culture, treatments, and transfection experiments

Motoneuron-derived Ctrl MN-1 cells, SMN stable knockdown MN-1 cells (ΔSMN) and CARM1 stable knockdown MN-1 cells (ΔCARM1) are described previously (Hubers *et al.*, 2011). Cells were cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum and maintained in 2 μg/ml of puromycin. For transient transfection, Lipofectamine and Plus Reagents from Invitrogen were used. Cells were harvested for biochemical analyses 48h after transfection. For the USPL1 depletion experiment, the pGIPZ (OpenBiosystems) control vector and the pGIPZ-shRNA-UPF1 (RHS4430-101031100, V3LHS-352951, OpenBiosystems) were transiently transfected. For Forskolin treatment, cells were treated for 6h with 100 μM. For cycloheximide (CHX) treatment, cells were treated 4h with 10 μg/ml. For wortmannin treatment, cells were treated 6h with 5μM. The β-Globin WT and MT (NMD) reporters (Sinha *et al.*, 2010) were a kind gift from Dr Adrian R Krainer (Cold Spring Harbor Laboratory, USA) and the CARM1-WT and CARM1-E266Q plasmids (Cheng *et al.*, 2007) used for the rescue experiments were a generous gift from Dr Mark T. Bedford (University of Texas M.D. Anderson Cancer Center, USA).

Animals

Spinal cord tissue was harvested from 1-3 month old C57 Black 6 SMN+/- mice (obtained from Drs. Alex MacKenzie and Rashmi Kothary), flash frozen in liquid nitrogen and stored at -80°C.

RNA purification, RT-PCR and RT-qPCR

RNA was extracted by using TRIzol (Invitrogen) and treated with DNase I (DNAfree, Ambion). Reverse-transcription (RT) was done by using AMV reverse transcriptase (Promega) and random primers. After completion of the RT reaction, cDNA samples were diluted to a final concentration of 2.5 ng/µl. 5 µl of cDNA samples were used per 25 µl PCR or qPCR reactions. PCR was performed by using GoTaq Flexi DNA Polymerase (Promega) and Quantitative PCR was performed by using iQ SYBR Green Supermix (BioRad) on a Chromo4 real-Time Detector (BioRad). The relative amounts of cDNA targets in samples were determined on the basis of the threshold cycle for each PCR product (Ct). All semi-quantitative RT- and RT-qPCR primers used in this study and resulting PCR amplicons are shown in Table 4.

Co- and RNA-immunoprecipitation experiments

Proteins were extracted from cells collected from 100 mm plates. Cells were first washed in 1× PBS and then incubated in lysis buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% NP40; 0.5% Sodium Deoxycholate; water and complete protease inhibitor cocktail from Roche Applied Sciences). Immunoprecipitations were done by using Protein A/G PLUS-Agarose beads (sc-2003, Santa Cruz) and 3µg of specific antibody or 3µg of control IgG (sc-2027, Santa Cruz). After an overnight incubation, beads were washed 4 times in lysis buffer. Subsequently, samples were subjected to SDS-PAGE and western blotting analysis. For RNAse A treatment (Qiagen, 19101), the cell pellets were first incubated with lysis buffer. Then, RNAse A (1µg/ml) was added to supernatants and the samples were incubated for 1h at 37°C prior to realize an

immunoprecipitation with an UPF1 antibody. RNA-immunoprecipations were performed as described previously (45). RNA was immunoprecipitated using 4 µg of UPF1 anibody and 4 µg of control rabbit IgG (sc-2027, Santa Cruz).

Immunoblotting

The following antibodies were used: CARM1 (A300-421A, Bethyl Laboratories); GAPDH (MMS-580S, Covance); β-Actin (sc-47778, Santa Cruz Biotechnology) ; Tubulin (T6199, Sigma-Aldrich); UPF1 (07-1014, Millipore); UPF3b (sc-48800, Santa Cruz Biotechnology); Magoh (Ab10686, Abcam); eIF4A3 (Ab32485, Abcam); RBM8a (NB100-55326, Novus Biologicals); Casc3 (LS-C100827, Lifespan Biosciences). Quantitative analyses were done with the ImageJ software.

Statistical analysis

For statistical analysis, unpaired t-test was used. However, when three or more groups are compared, a one-way ANOVA and post hoc Tukey analysis was used. The minimum α-level of significance was set at 0.05. Data are presented as means ± SEM. In the figures, a single asterisk shows $P < 0.05$, a double asterisk shows $P < 0.01$, a triple asterisk shows $P < 0.001$. Analyses were realized with Graphpad Prism 6 software.

Table 4. NMD targets tested in this study.

Gene	ASC-NMD	Proposed NMD inducing feature	Reference(s) of work demonstrating transcript is subject to NMD	CARM1 sensitive?	NMD transcript decreased in SMA?
USPL1 E2-	Yes	PTC	This work	Yes	Yes
Sfrs10	Yes	PTC	McIlwain et al., 2010 This work	Yes	No
Ccar1	Yes	PTC	Saltzman et al., 2008 This work	Yes	Yes
hnRNPA2B1	Yes	PTC	McIlwain et al., 2010	Yes	N.D
Nfyb	Yes	PTC	McIlwain et al., 2010 This work	No	N.D
Cask	Yes	PTC	McIlwain et al., 2010	No	N.D
Alkbh3	Yes	PTC	McIlwain et al., 2010	No	N.D
Zyx	Yes	PTC	McIlwain et al., 2010	No	N.D
GADD45a	No	Unknown	Lykke-Andersen et al., 2014 Tani et al., 2013 Tani et al., 2012 Mendell et al., 2004	Yes	Yes
ARC	No	Two 3'UTR introns	Giorgi et al., 2007	Yes	Yes
ASNS	No	uORF	Morris et al., 2007 Mendell et al., 2004	Yes	Yes
ATF4	No	uORF	Morris et al., 2007 Weischenfeldt et al., 2008 Mendell et al., 2004	N.D	Yes
CARS	No	Unknown	Linde et al., 2007 Morris et al., 2007 Mendell et al., 2004	No	Yes
SARS	No	Unknown	Mendell et al., 2004 Morris et al., 2007	No	Yes
SLIT2	No	Unknown	Mendell et al., 2004 Morris et al., 2007	No	Yes
MAP3K14	No	uORF	Linde et al., 2007 Morris et al., 2007 Mendell et al., 2004	No	Yes
UPF1	No	Long 3'UTR	Huang et al., 2011	No	No
UPF2	No	Long 3'UTR/uORF	Huang et al., 2011	No	N.D
UPF3b	No	Long 3'UTR	Huang et al., 2011	No	N.D
SMG1	No	Long 3'UTR/uORF	Huang et al., 2011	No	N.D
SMG6	No	Long 3'UTR/uORF	Huang et al., 2011	No	N.D
SMG7	No	Long 3'UTR/uORF	Huang et al., 2011	No	N.D

3.4 Results

Aberrant splicing of *USPL1* pre-mRNA is observed in human SMA patients cells

A number of studies have assessed genome-wide gene expression profiles in various models of SMA (Baumer *et al.*, 2009; Campion *et al.*, 2010; Maeda *et al.*, 2014; Olaso *et al.*, 2006; Zhang *et al.*, 2008), but intriguingly, very little overlap has been found between these studies. Amongst the handful of genes identified as misregulated in the pathology is the ubiquitin-specific protease-like 1 (*USPL1*) gene, which was recently found to code for a SUMO isopeptidase shown to localize to Cajal bodies (Schulz *et al.*, 2012). The *USPL1* pre-mRNA is alternatively spliced within its 5' end to produce isoforms including or skipping 169 bp cassette-type exon 2. It has been reported that the isoform including exon 2 (E2+) is selectively over-represented in spinal cord tissues from mouse models of severe SMA, relative to the E2- transcript (Baumer *et al.*, 2009; Liu *et al.*, 2010; Zhang *et al.*, 2008). To determine if this aberrant splicing event is also observed in SMA patients cells, we assessed the inclusion of exon 2 in primary fibroblasts derived from a severe type I SMA patient. As shown in Figure 17A, increased inclusion of *USPL1* exon 2 was observed, indicating for the first time that this alteration is present in human SMA patient cells. We next quantified by RT-qPCR the *USPL1* E2+/E2- ratio in a stable motoneuron MN-1 cell line expressing either a shRNA against SMN (shSMN) or a control shRNA (Hubers *et al.*, 2011; Sanchez *et al.*, 2012). As expected, depletion of SMN resulted in a relative increase in *Uspl1* E2+ mRNA levels relative to E2- isoform, as described in the pathology (Figure 17B), indicating that we can recapitulate this molecular defect in a motoneuron-like cell culture model of SMA.

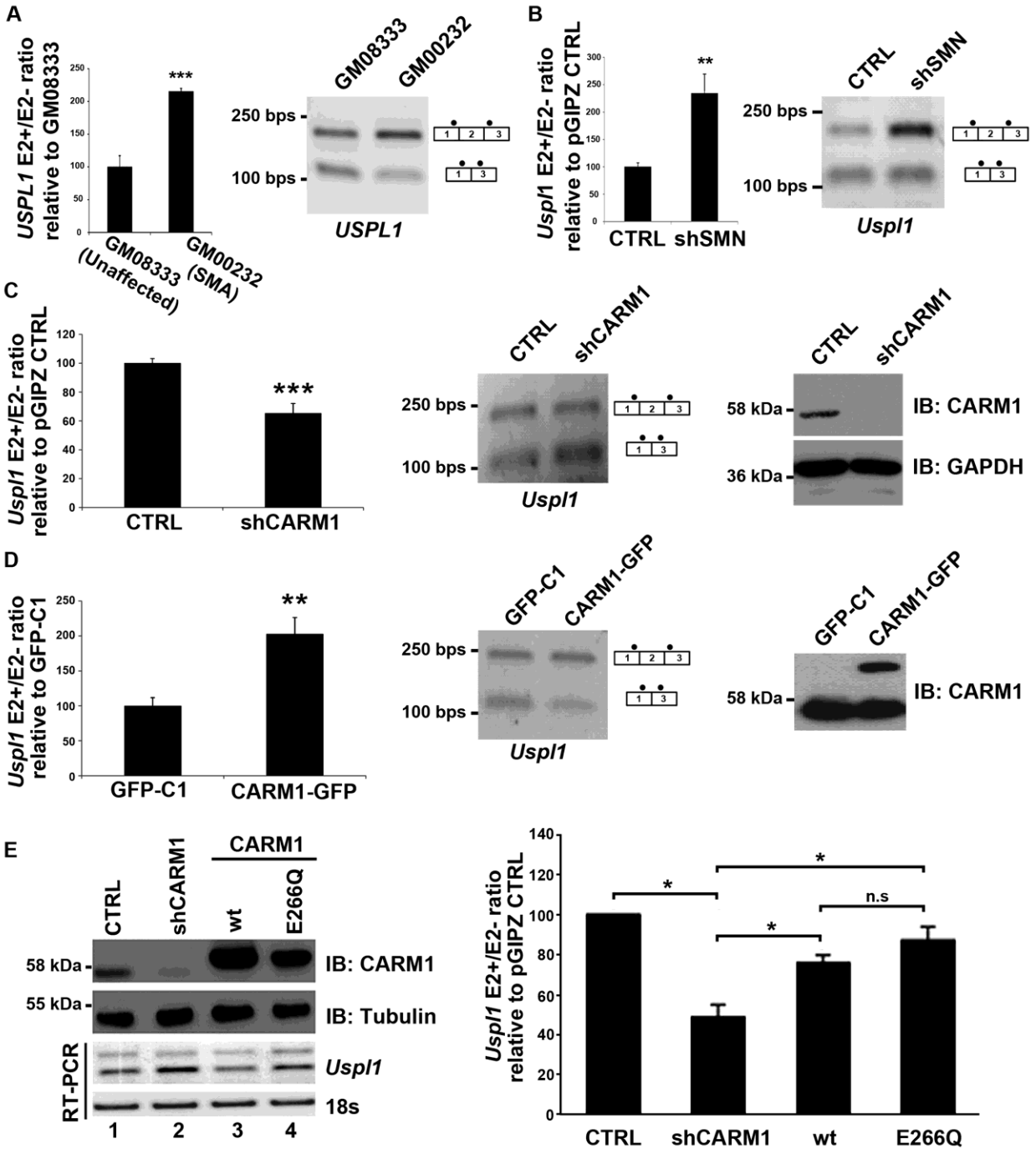

Figure 17. The ratio of USPL1 splicing variants is altered in SMA and by CARM1.

(**A**) *USPL1* E2+/E2- ratio obtained with the GM00232 primary fibroblasts from a SMA patient was expressed relative to GM08333 primary fibroblasts from an unaffected control. Data are means +/- SEM (n = 4). (**B**) Quantification of the *Uspl1* E2+/E2- ratio in the MN-1 pGIPZ CTRL

cell line and MN-1 shSMN cell line which stably expressed a shRNA against SMN (shSMN). Results obtained in the shSMN condition were expressed relative to the CTRL cell line. Data are means +/-SEM (n = 5). (**C**) CARM1 affects the *USPL1* E2+/E2- ratio. Total RNA was isolated from the MN-1 pGIPZ CTRL cell line and the MN-1 shCARM1 cell line which stably expressed a shRNA against CARM1. *Uspl1* E2+/E2- ratio levels obtained in the shCARM1 cells were expressed relative to CTRL. Data are means +/- SEM (n = 6). (**D**) Quantification of the *Uspl1* E2+/E2- ratio in the MN-1 stable cell lines expressing either the pEGFP-C1 empty vector (GFP-C1) or a CARM1-GFP construct. Data obtained in the CARM1-GFP condition were expressed relative to GFP-C1. Data are means +/- SEM (n = 6). CARM1 protein levels were assessed in the MN-1 GFP-C1 and MN-1 CARM1-GFP cell lines (upper band, CARM1-GFP). (**E**) MN-1 cell lines were transiently transfected with either a pGIPZ CTRL or shCARM1 plasmid for 24 h. A rescue was performed in the MN-1 shCARM1 cell line by transfecting with either the WT-CARM1 or the CARM1-E266Q mutant expression vectors for an additional 24 h. Protein lysate from transfected MN-1 lines confirm the knockdown and overexpression of CARM1 or the E266Q mutant, normalized to Tubulin. Total RNA was extracted and RT-PCR performed using *Uspl1* primers to amplify both the E2+ and E2- isoforms of the mRNA. 18s RNA was also shown as a loading control. Quantification of the *Uspl1* E2+/E2- ratios in the CARM1 rescue MN-1 cell lines. *Uspl1* mRNA levels are shown as relative to CTRL. Data are means +/- SEM (n = 3).

CARM1 regulates the relative expression of *Uspl1* alternatively spliced mRNA isoforms

Our previous work has uncovered that protein levels of the protein arginine methyltransferase CARM1 are up-regulated in spinal cord motor neurons of SMA mouse models and in severe Type I SMA patient cells (Sanchez *et al.,* 2012). This study also identified aberrant gene expression and alternative splicing profiles due to CARM1 up-regulation (Sanchez *et al.,* 2012). To assess if *Uspl1*

alternative splicing was also sensitive to CARM1 levels, the E2+/E2- ratio was quantified by RT-qPCR in a motoneuron MN-1 cell line stably expressing an shRNA against CARM1 (Hubers *et al.*, 2011; Sanchez *et al.*, 2012). Strikingly, CARM1 down-regulation resulted in a decrease of the E2+/E2- ratio (Figure 17C), thus shifting the ratio opposite to what is observed in the pathology. The E2+/E2- ratio was then quantified in a stable motoneuron MN-1 cell line overexpressing a CARM1-GFP construct. Accordingly, overexpression of CARM1 led to an increased E2+/E2- ratio and thus, mirrors what is seen in the SMA pathology (Figure 17D). To insure that the observed changes were not due to non-specific and/or off-target effects, a CARM1 expression rescue experiment was performed, using either wild type (wt) CARM1 or a mutant allele (E266Q) lacking methyltransferase activity (Cheng *et al.*, 2007) (Figure 17E). First, CARM1 levels were knockdown using an shRNA and confirmed by western blot in MN-1 cell lines. RT-PCR was used to confirm the decrease in the *Uspl1* E2+/E2- ratio as previously observed (Figure 17E, lanes 1 and 2), which accompanies the decrease in CARM1. As expected, re-introduction of wt CARM1 into the shCARM1 MN-1 cells rescued the *Uspl1* E2+/E2- ratios, returning them towards levels observed in the control condition (Figure 17E, lane 3). Intriguingly, re-introduction of the CARM1 E266Q mutant allele rescued E2+/E2- ratios as efficiently as the wt allele (Figure 17E, lane 4). Altogether, these results indicate that CARM1 can regulate the expression of *Uspl*1 E2+/E2- ratios, through a mechanism that is seemingly independent of its methyltransferase activity.

Skipping of *Uspl1* alternative exon 2 creates a premature termination codon that elicits NMD

Upon closer inspection of the nucleotide sequence surrounding *Uspl1* alternative exon 2, we noted that skipping of exon 2 causes a shift in the coding reading frame resulting in the creation of a premature termination codon (PTC; Figure 18A), which, based on its position >50-55 nt upstream of the exon junction is predicted to elicit the NMD pathway (Ishigaki *et al.*, 2001; Maquat, 2002).

We refer to this transcript as *Uspl1* E2- (Uspl1-006 - ENSMUST00000121416) and the WT transcript which includes exon 2 we denote *Uspl1* E2+ (Uspl1-001 - ENSMUST00000050472). We also observed an additional *Uspl1* isoform containing a "cryptic" exon upstream of exon 2 which we denoted as exon 1α (Uspl1-002 - ENSMUST00000122160). This isoform did not appear to be regulated by CARM1 and was not included in future analysis and specific qRT-PCR primers were designed to avoid amplification of transcripts containing this exon (Primer list - Table 5).

Since NMD is a process coupled to translation (Ishigaki *et al.*, 2001; Lejeune *et al.*, 2002; Lejeune *et al.*, 2004; Maquat, 2004), MN-1 cells were treated with an inhibitor of translation (cycloheximide, CHX) followed by RT-PCR analysis as above to determine the ratio of *Uspl1* isoforms. In response to cycloheximide treatment, the E2+/E2- ratio was decreased relative to carrier treatment (Figure 18B), consistent with stabilization of the E2- mRNA in the absence of translation-dependent NMD activity. To further confirm that the E2- transcript is regulated by NMD, a shRNA against UPF1 (Figure 18) was transiently transfected into MN-1 cells. As a result of the down-regulation of the main trans-effector of the NMD

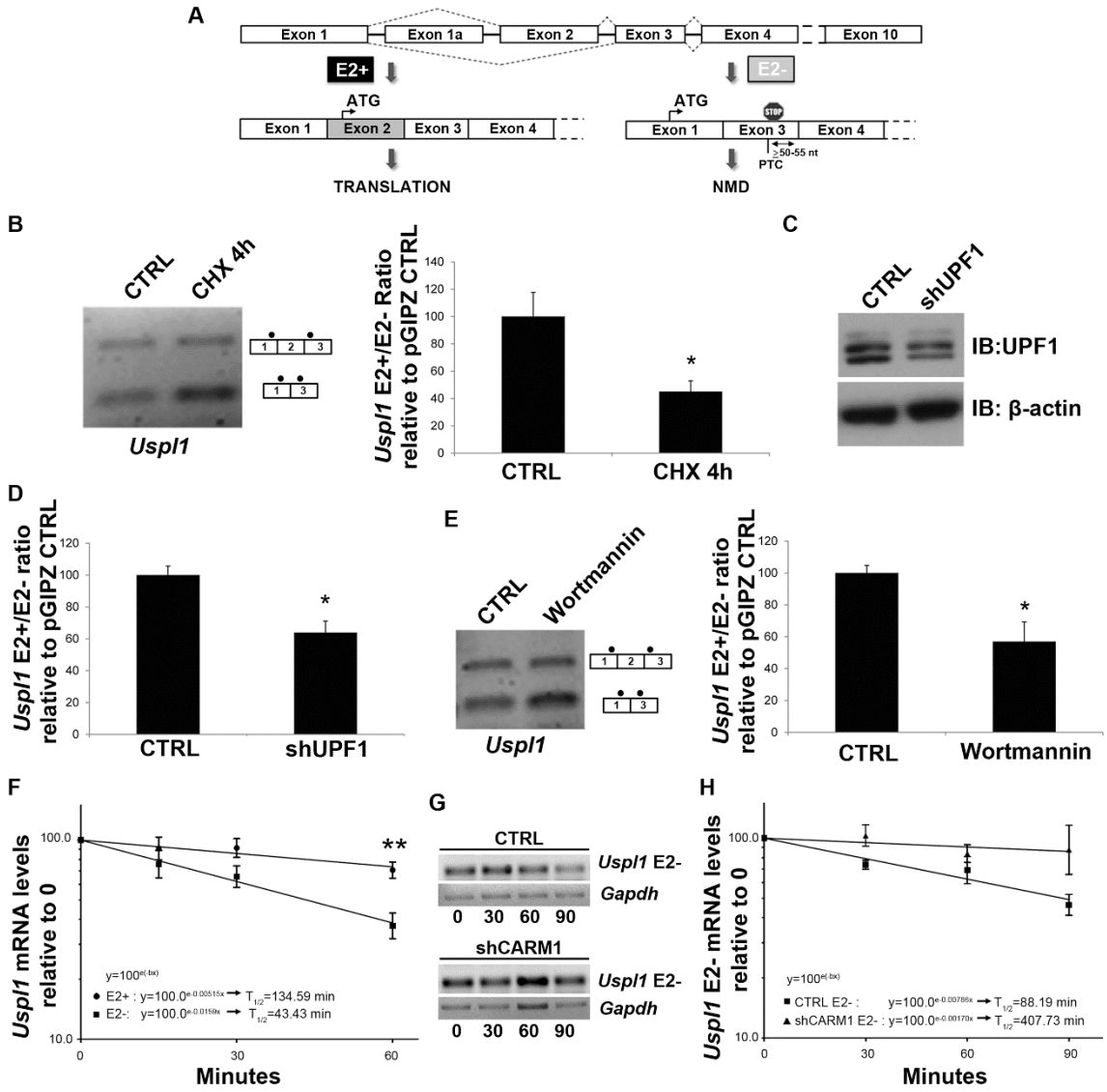

Figure 18. Uspl1 is a NMD-regulated target.

(A) Schematic representation of *Uspl1* E2+ and *Uspl1* E2- transcripts. As depicted in this illustration, the skipping of *Uspl1* exon 2 introduces a Premature Termination Codon (PTC) in exon 2 of the *Uspl1* E2- transcript. (B) Impact of the translational inhibitor cycloheximide on the *Uspl1* E2+/E2- ratio. Cells were treated 4 h with cycloheximide (CHX). Then, results obtained in response to CHX were expressed relative to a carrier CTRL. Data are means +/- SEM (*n* = 3). (C) UPF1 protein levels were assessed from cell transiently transfected with a control shRNA vector

CTRL or a shRNA against UPF1. β-actin was used as a loading control. (D) Effect of UPF1 down-regulation on the *Uspl1* E2+/E2- ratio. Data are means +/- SEM (n = 3). (E) Impact of the SMG-1 inhibitor wortmannin on the *Uspl1* E2+/E2- ratio. Cells were treated 6 h with wortmannin. Subsequently, results obtained in response to wortmannin were expressed relative to DMSO carrier CTRL. Data are means +/- SEM (n = 3). (F) Quantification of the *Uspl1* E2+ and *Uspl1* E2- mRNA half-lives. MN-1 wt cell lines were treated with actinomycin D (5 μg/ml) for 15, 30 and 60 min. Semi-logarithmic graph shows the decay rate of mRNAs calculated using formula: $(x) = ln(0.5)/b$, where b = slope obtained from the trendline formula: $y = ne^{-bx}$. Quantification shows the mean +/- SEM (n = 6), of *Uspl1* E2+/18S or *Uspl1* E2-/18S. (G) Representative RT-PCR gels showing time course of *Uspl1* E2- and *Gapdh* mRNA levels in DMSO carrier CTRL or shCARM1 conditions, treated with actinomycin D. (H) Quantification of the *Uspl1* E2- mRNA half-lives in MN-1 pGIPZ CTRL or shCARM1 cell lines. Quantification shows the mean +/- SEM (n = 3), of *Uspl1* E2-/*Gapdh*.

Table 5. Primers used in this study.

Gene	Primers	
	Forward (5'-3')	Reverse (5'-3')
RT-qPCR		
USPL1 E2-	GGTTTGCCACTGGTTGGAC	TGGAAGCTAATGCGGTAAGG
USPL1 E2+	GAGCGTACTTCGGGAGGAATT	GATCCTCACACAGAAAGACAG
hGADD45a	AGACCGAAAGGATGGATAAGGT	CCACATCTCTGTCGTCGTCC
hARC	ACAACAGGTCTCAAGGTTCCC	AGCCGACTCCTCTCTGTAGC
hASNS	ACGACCAAAAGAAGCCTTCAG	CCACTTGGGCATCCAGTAAT
hCARS	ATGACATGGAGGGCAAAGAG	TTAGGACCCAAGGGTGACTG
hSARS	CTGGCCTGTCTACCTGCTTC	CTGGCAGCATGATTCAAAGA
hMAP3K14	TCAGTGCAGAACCAGGTCAG	GGGGACTGAGAACCACTTCA
mGADD45a	AGACCGAAAGGATGGACACG	GGGTCTACGTTGAGCAGCTT
mARC	CTGAGATGCTGGAGCACGTA	GCCTTGATGGACTTCTTCCA
mASNS	GACCCAAAGAAGCCTTCAGTGAT	GATCCACTTAGGCATCCAATAATGAG
mCARS	TTCCCAGTGATGGCAGGTTC	CTGGCTGCCTCGTCACTAAT
mSARS	ACCAAGAAGATGATGGACAAGAAAA	GGGCGAAAAGAGGGAAGACA
mMAP3K14	TGTCTCAAGATTGCCAGCGA	ACTTCCTGTAGTGCCTTGCC
UPF2	CCCAGCTCCAGCAAACACTA	GATCAACGTCTCCTCCCACC
SMG1	CTCACCGAACAGGGAAGGAC	ATAGCCCACTGCCAAAGGAC
SMG6	CCCGTGTGGTGCAAGAAAAG	TCGTTGTTACCCAGCTGTCC
SMG7	TCTTTGTCCTCTCCCTGTCCC	CCTGTTGGCTGGTCTAAGGT
RT-PCR		
USPL1	GTCCACTGTATGCGAAGAAG	TTGCCTTTCGCTCTACAAGC
hUSPL1 E2-/E2+	AGATCAAGCTGGAACAGGAG	AGCAGGGCAATACTCATCTG
β-Globin mRNA	TGCATCTGACTCCTGAGGAGAA	GGACTTAGGGAACAAAGGAACCT
β-Globin pre-mRNA	TAGAAACTGGGCATGTGGAG	CATCAAGCGTCCCATAGACTC
Ccar1	GGAATGAAAGGCAAGGATGA	GGGTAGGAGTGGCGATCTCT
Sfrs10	GAGCTCCTCGCAAAAGTGTG	GATCCGTGAGCACTTCCACT
Nfyb	CCTCCCAGCTAGGGATTTCT	GTCTTCCGCTTCTCCTGATG
hnRNPA2B1	AGTGGTGGCTATGGTGGAAG	AAATGAAGGCACCAACAAGAA
Cask	AGGGAAATGCGAGGGAGTAT	GTCCTTTTGGTTGGGTGGTT
Alkbh3	ATCCTGGCTTTGTGGACTTG	GGTGTGGCTGGTGTTCTCTT
Zyx	GCCCAAAGTGAATCCTTTCC	AGAAGGGGTCTTCCAAGGAG

m: Mouse specific primers, h: Human specific primers

process, the *Uspl1* E2+/E2- ratio, as determined using RT-qPCR, was again decreased as compared to an shRNA CTRL (Figure 18D). Finally, to ascertain that the *Uspl1* E2- transcript is a NMD target, MN-1 cells were treated with wortmannin, a drug known to inhibit NMD by preventing the phosphorylation of UPF1 by SMG-1 (Isken and Maquat, 2008; Pal *et al.*, 2001). Again, this resulted in a decrease in the E2+/E2- ratio relative to carrier treatment (Figure 18E). To further confirm that the E2- transcript is indeed subjected to NMD and regulated at the level of mRNA stability, we determined the half-life of each *Uspl1* alternatively spliced isoform (Figure 18F). MN-1 cells were treated with the transcription inhibitor actinomycin D for 15, 30 and 60 minutes. Quantification of *Uspl1* E2+ and E2- RNA variants at each time points indicated that the putative E2- NMD transcripts has a considerably shorter half-life of approximately 43 minutes, relative to the E2+ mRNA at approximately 135 minutes (Figure 18F). Thus, together, these results strongly support the notion that the *Uspl1* E2- isoform is target of the NMD pathway. Since we determined that CARM1 levels influenced the relative ratio of *Uspl1* E2+/E2- isoforms, we next assessed whether this effect was due to an effect on mRNA stability of the E2- mRNA isoform. Thus, the same experiment as in Figure 18F was performed using MN1 cells stably transfected with a control shRNA vector or with a shRNA expression plasmid targeting CARM1. Coherent with our findings, the reduction of CARM1 levels resulted in a ~ 4.6 fold increase in the *Uspl1* E2- mRNA half-life compared to CTRL conditions (Figure 18G-H), although these results did not quite reach statistical significance. These results thus suggest that CARM1 can regulate *Uspl1* expression at the level of alternative splicing, and potentially to some extent also at the level of mRNA stability through regulation of the NMD pathway acting on the E2- mRNA isoform.

CARM1 can affect the fate of a generic NMD reporter

These results prompted us to investigate whether CARM1 has a general impact on NMD. To test this hypothesis, we used a well-characterized NMD reporter system (Zhang, 1998) consisting of the full-length β-globin pre-mRNA as well as a mutant version containing in exon 2, a nonsense mutation which introduces a PTC following splicing (Figure 19A). The PTC-containing β-globin mRNA has been thoroughly demonstrated to be a bone fide NMD-regulated target (Sinha *et al.*, 2010; Zhang, 1998; Zhang *et al.*, 2004). Wild type (WT) or mutant (MT) β-globin reporters were transiently transfected into MN-1 pGIPZ CTRL and MN-1 shCARM1 cell lines (Figure 19B, lanes 1-4). In order to quantify the effect of the CARM1 knockdown on the NMD process, levels of the WT and MT reporters were first normalized to their respective pre-mRNA levels (thus accounting for potential differences in transfection efficiency and/or expression levels). Then, the results obtained for the MT reporter were expressed relative to the WT reporter (Figure 19C). As shown in Figure 19B, the relative abundance of the PTC-containing MT reporter mRNA in the MN-1 pGIPZ CTRL cell line is lower than in its WT counterpart (Figure 19B lanes 1-2, and 19C). Remarkably, in the MN-1 CARM1 hypomorph cell line, the amount of the WT minigene is no longer significantly different from the WT (Figure 19B lanes 3-4, and 19C), suggesting CARM1 is required for recognition and/or degradation of the PTC-containing mRNA through NMD.

Since CARM1 is a well-known regulator of gene expression through various mechanisms (Ackermann *et al.*, 2013; Bedford and Richard, 2005; Covic *et al.*, 2005; Di Lorenzo and Bedford, 2011; Kawabe *et al.*, 2012; Lee and Stallcup, 2009; Stallcup, 2001; Wu and Xu, 2012), we wanted to rule out the possibility that CARM1 might be mediating its effect on NMD by

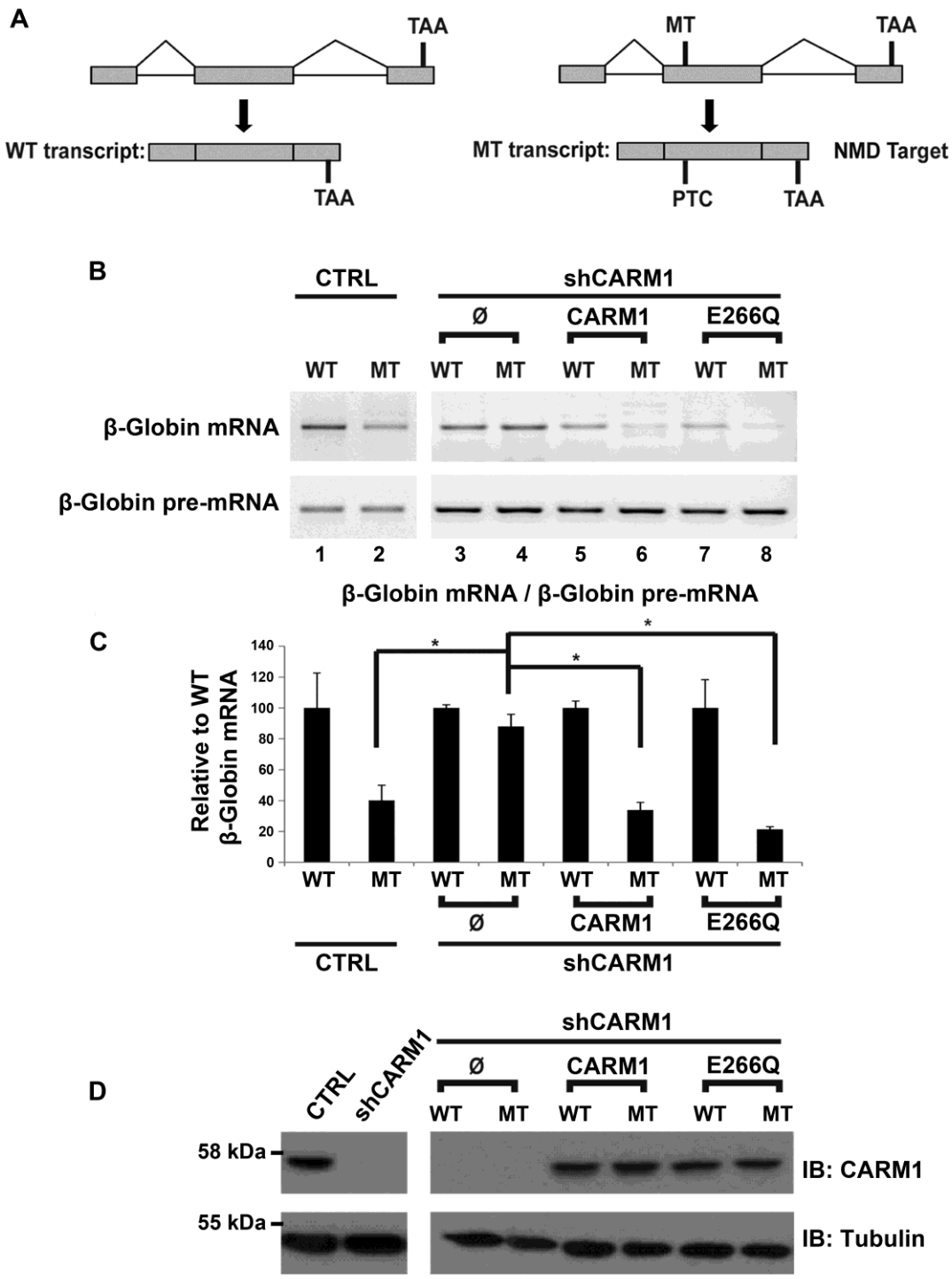

Figure 19. CARM1 can affect the fate of a generic NMD reporter.

(A) Schematic representation of the wild-type (WT) β-Globin plasmid and the mutant (MT) β-Globin reporter containing a PTC which elicit degradation by the NMD pathway. (B) The WT and

MT reporters were transiently transfected either into the MN-1 pGIPZ CTRL or the MN-1 shCARM1 cell line. Furthermore, the WT and MT reporters were also transiently co-transfected with a pcDNA3.1 empty vector, CARM1 or CARM1 E266Q expression vectors into the MN-1 shCARM1 cell line. Representative RT-PCRs of the β-Globin mRNA and β-Globin pre-mRNA are shown. (C) After normalization of the β-Globin mRNA to the β-Globin pre-mRNA levels in each condition, the data were expressed relative to the WT. Data are means +/- SEM, (MN-1 CTRL cell line, $n = 5$ and MN-1 shCARM1 cell line, $n = 3$). (D) CARM1 protein levels were assessed by western blotting in MN-1 pGIPZ CTRL and MN-1 shCARM1 cell lines. Tubulin was used as loading control.

regulating the expression of one or more of the core NMD factors. Consequently, protein levels of the tetrameric EJC core complex components (Tange *et al.*, 2005) as well as the NMD factors UPF1, UPF2 and UPF3b (aka. UPF3X) (Serin *et al.*, 2001) were compared in the MN-1 pGIPZ CTRL and MN-1 shCARM1 cell lines (Figure 20A and B). Additionally, the mRNA expression patterns measured by RT-qPCR, of the NMD factors *Smg1*, *Smg6*, *Smg7* and *Upf2* were also compared in the MN-1 pGIPZ CTRL and shCARM1 cell lines (Figure 20C). From these analyses, no significant difference in expression was observed for these key NMD factors upon depletion of CARM1. Altogether, these results strongly suggest that CARM1 can promote NMD, through a mechanism other than through transcriptional regulation of major known NMD factors. To ensure that the observed changes in mRNA levels are not due to off-target effects, a rescue experiment was also performed as above with either wt or E226Q CARM1 alleles. Consistent with our previous observation, re-expression of either WT or mutant CARM1 alleles was able to rescue the efficiency of the NMD process (Figure 19B lanes 5-8, and 19C-D). This observation suggests that CARM1 can promote NMD through a mechanism that is somehow independent of its methyltransferase activity.

CARM1 interacts with main NMD effector UPF1

In order to get some insights into the mechanism through which CARM1 regulates NMD, we assessed its potential interaction with core NMD factors. Since UPF1 is central to the NMD process, an endogenous UPF1 immunoprecipitation was performed in extracts from MN-1 cells and analyzed by western blotting for the presence of CARM1. As shown in Figure 21A, CARM1 was detected in the endogenous UPF1 immunoprecipitate. Immunoprecipitations with a UPF3 antibody, which detects UPF3b (aka. UPF3X) the first protein to associate with the EJC

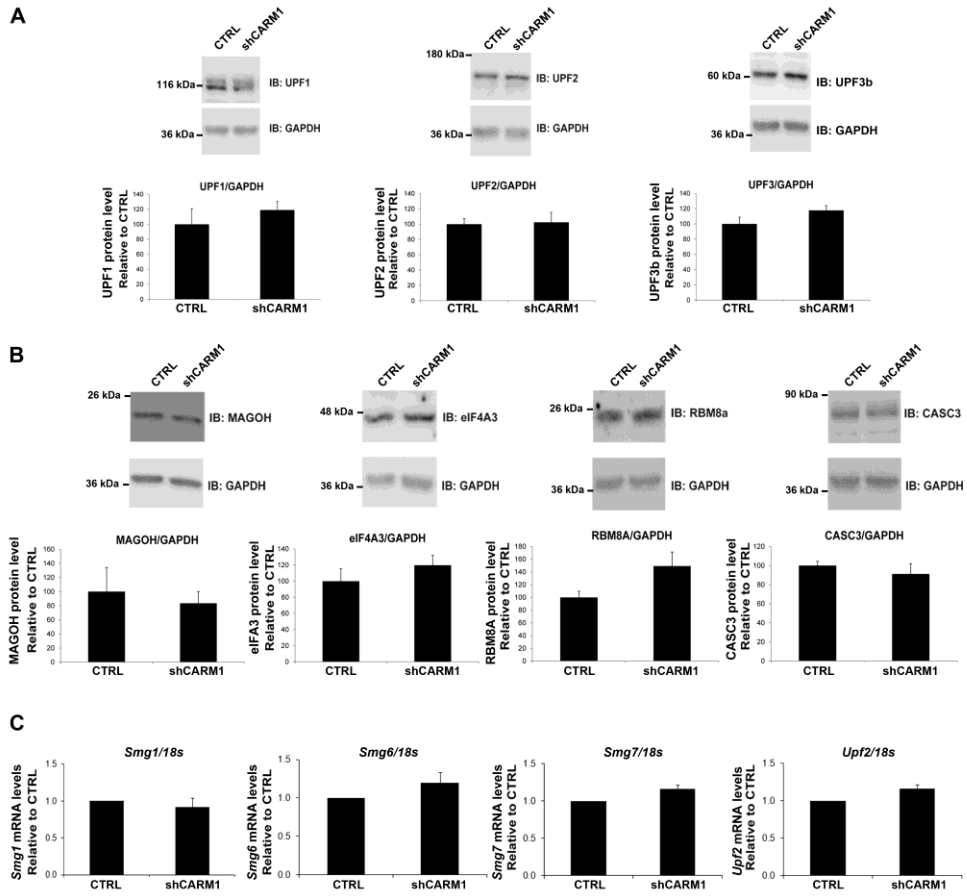

Figure 20. CARM1 depletion does not affect the expression level of NMD components including EJC core components.

(A-B) MAGOH (n=4), eIF4A3 (n=5), RBM8a (n=5) and CASC3 (n=5) protein levels were assessed in response to the depletion of CARM1. After optical density analyses and normalization to GAPDH protein levels, values were expressed relative to the MN-1 pGIPZ CTRL cell line. Data in the bar graph are means +/- SEM. (C) NMD component mRNA levels were gathered by RT-qPCR from total RNA isolated from MN-1 shCARM1 cell lines and compared to MN-1 pGIPZ CTRL. 18s RNA levels were used for expression normalization and values shown in the bar graph are means +/- SEM, n=3.

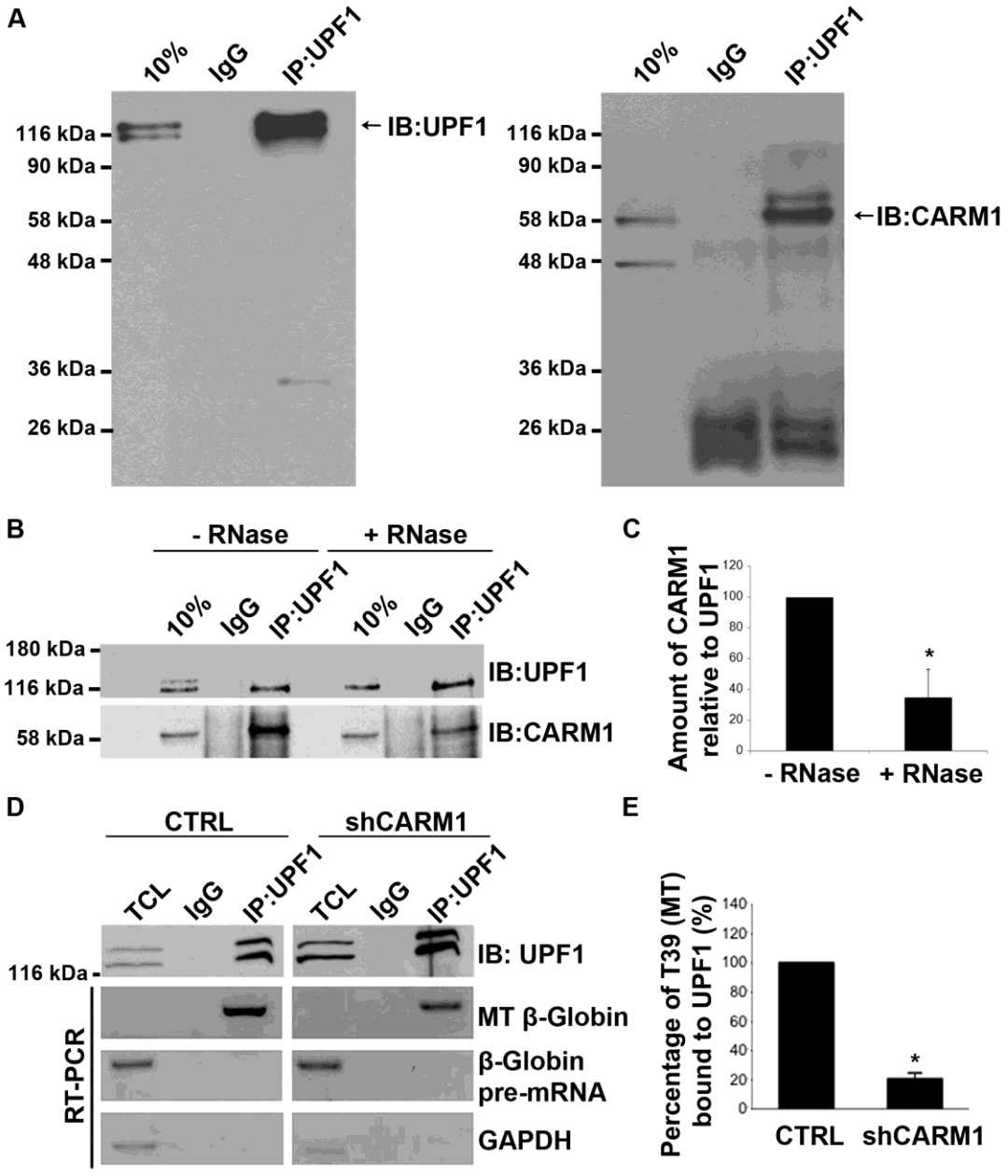

Figure 21. CARM1 can be co-immunoprecipitated with UPF1 and the interaction between UPF1 and the β-Globin T39 mutant (MT) decreases with CARM1 knockdown.

(A) Total cell lysates were prepared from MN-1 cells and subjected to immunoprecipitation with an IgG CTRL or UPF1 antibodies. Immunoprecipitated proteins were then analysed by western blot using antibodies against UPF1 and CARM1. (B) UPF1 immunoprecipitation experiments

were performed with or without (w/o) pretreatment of the cell lysate with RNase A (1 μg/ml) for 30 min at 37°C. (C) Then, the CARM1/UPF1 ratios in response to the RNase A treatments were assessed. Values shown in the bar graph are means +/- SEM (*n* = 3). (D) The MT reporter was transiently transfected either into the MN-1 pGIPZ CTRL or the MN-1 shCARM1 cell line. RT-PCR analysis was performed using primers specific for the MT mRNA or pre-mRNA, on total RNA extracted from the CTRL (left panel) or shCARM1 (right panel). (E) β-Globin mRNA levels, shown here as percent bound to UPF1, were normalized to overall immunoprecipated UPF1 levels and *Gapdh* mRNA was used as a loading control. Data are means +/- SEM (*n* = 3).

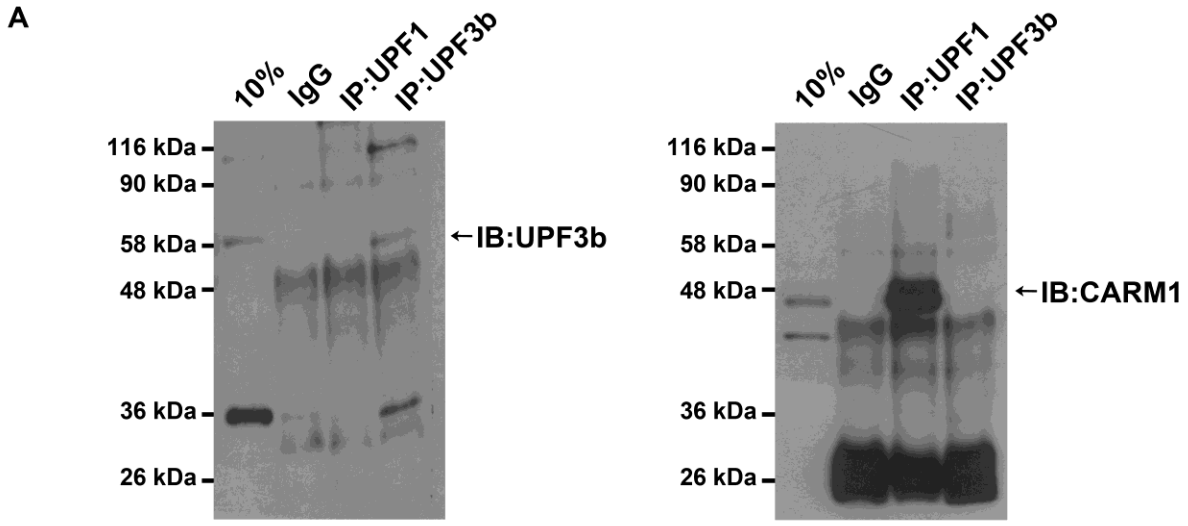

Figure 22. Immunoprecipitation of UPF3.

(A) Total cell lysates were prepared from MN-1 cells and subjected to immunoprecipitation with an IgG CTRL, UPF1, or UPF3 antibodies. Immunoprecipitated proteins were then analyzed by western blot using antibodies against CARM1.

and recruit UPF2 to the EJC (Isken and Maquat, 2008), were also performed, but failed to pull-down CARM1 (Figure 22).

We also wanted to assess whether CARM1 may interact with UPF1 through a mechanism independent of NMD, like perhaps Staufen1 (Stau1)-mediated decay (SMD). SMD is thought to be a process in which the double-stranded RNA binding protein, Stau1, binds to sites within the 3'UTR of a subset of transcripts and through interaction with UPF1, has been shown to promote the decay of those transcripts (Kim Y K, 2007; Kim *et al.*, 2005; Park *et al.*, 2013). However, we have not been able to detect any interaction between CARM1 and Stau1, and reducing Stau1 expression using shRNAs did not affect the relative ratio of *Uspl1* E2+/E2- isoforms (data not shown), strongly suggesting that CARM1 is not affecting mRNA fate through an impact on SMD. Because our attempts at demonstrating a potential direct interaction between CARM1 and UPF1 purified from E. coli have so far failed (F. Fiorini and H. Le Hir, personal communication), it appears that post-translational modifications might be required for the interaction to occur or, alternatively, that it may be mediated through an intermediate (e.g. protein or RNA). To test the idea that RNA may be necessary for the interaction between UPF1 and CARM1, we next performed co-immunoprecipitations with MN-1 extracts pre-incubated or not with RNase A and assessed the presence of CARM1 in the UPF1 immunoprecipitate by Western blotting (Figure 21B). Whereas an equivalent amount of UPF1 was immunoprecipitated in both conditions, less CARM1 protein was detected following RNase A treatment, thereby indicating that the interaction is likely mediated via a RNA moiety (Figure 21B-C).

UPF1 occupancy on a PTC-containing transcript is reduced in the absence of CARM1

Since CARM1 interacts with UPF1, and based on our observations indicating it plays more of a scaffolding role (i.e. independent of its methyltransferase activity), we sought to assess whether

CARM1 may influence steady-state levels of UPF1 on PTC-containing mRNAs. Using RNA-immunoprecipations, we investigated the effect of CARM1 depletion on the ability of UPF1 to bind to a generic NMD target. MN-1 cells were co-transfected with the PTC-containing β-globin MT reporter pre-mRNA and either a CTRL plasmid or shCARM1. Forty-eight hours after transfection, UPF1 was immunoprepicated and the amount of the MT reporter mRNAs associated with UPF1 was measured by RT-PCR. After normalization to the amount of UPF1 protein immunoprecipitated in each condition (Figure 21D; top panel), the amount of the β-globin MT reporter mRNAs bound to UPF1 was significantly less in cells with reduced CARM1 levels, as compared with CTRL (Figure 21D-E). It is worth noting that although some level of UPF1 occupancy was also detected on the WT β-globin reporter, consistent with previous reports (Lee *et al.*, 2015; Silva *et al.*, 2008) CARM1 had no impact in this context (Figure 23A). Moreover, no detectable occupancy of UPF1 was observed on an unrelated, non PTC-containing pre-mRNA minigene, whether CARM1 was present or not (Figure 23B). Together, these experiments strongly suggest that CARM1 promotes NMD, at least in part, by favouring UPF1 association with PTC-containing mRNA transcripts.

CARM1 regulates a diverse subset of known endogenous NMD targets

The impact of CARM1 on synthetic NMD reporters led us to speculate that it may have a broad impact on NMD-regulated transcripts. We therefore assessed the expression levels of several previously validated endogenous NMD targets (Giorgi *et al.*, 2007; Linde *et al.*, 2007; McIlwain *et al.*, 2010; Mendell *et al.*, 2004; Morris *et al.*, 2007; Saltzman *et al.*, 2008; Sharova *et al.*, 2009; Tani *et al.*, 2013) in the face of reduced CARM1 levels in MN-1 cells. These transcripts are targets of the NMD machinery because they either: (i) harbor a PTC following inclusion or

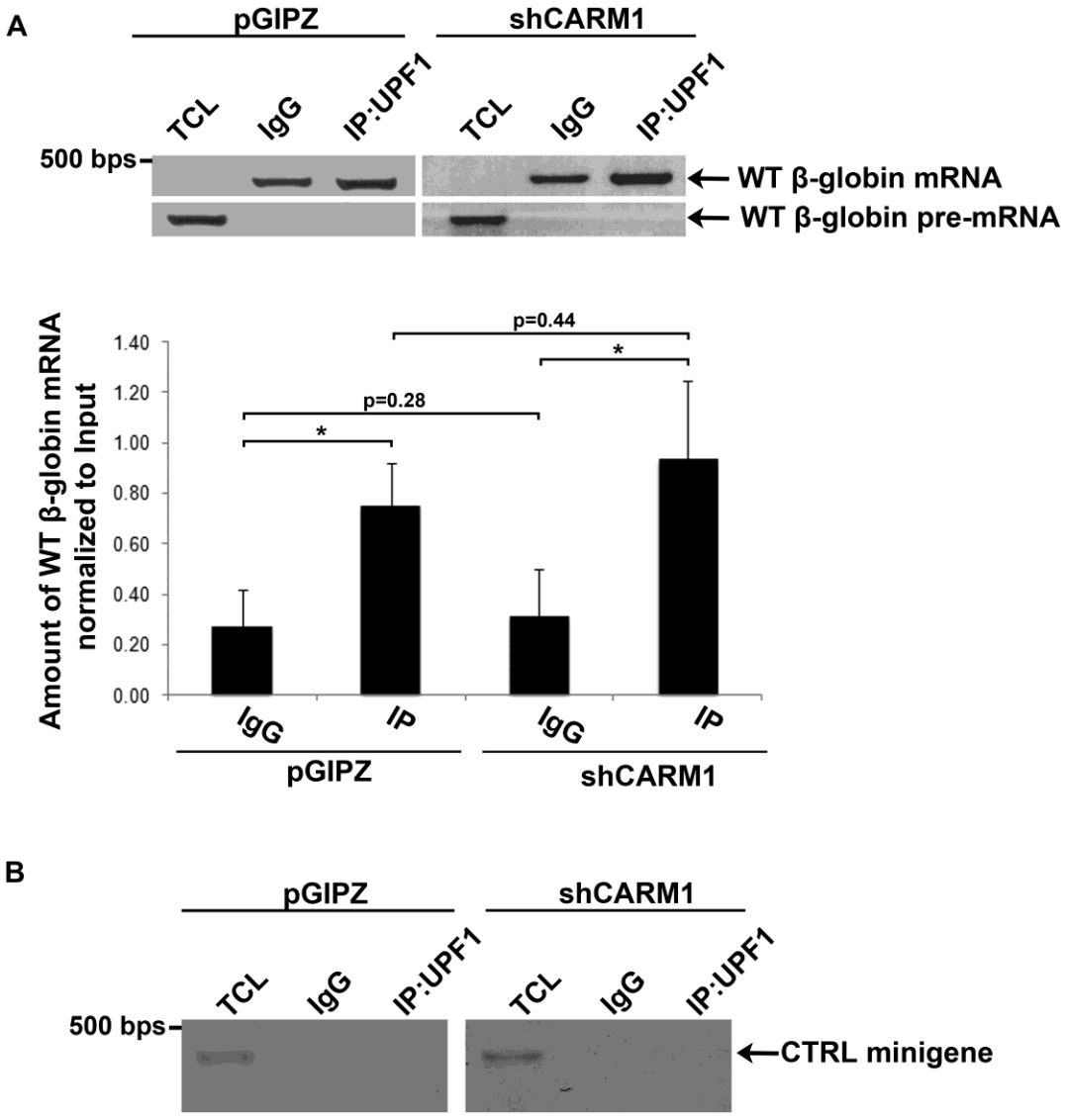

Figure 23. UPF1 Reporter assay WT β-Globin plasmid and CTRL minigene plasmid.

The WT and CTRL (*INSR* minigene) reporters were transiently transfected either into the MN-1 pGIPZ CTRL or the MN-1 shCARM1 cell line. Total cell lysates were prepared from MN-1 cells and subjected to immunoprecipitation with an IgG CTRL, or UPF1 antibodies. RT-PCR analysis was performed using primers specific for the WT mRNA or pre-mRNA, or CTRL reporters, on total RNA extracted from the CTRL or shCARM1. (A) RT-PCR of WT β-Globin mRNA after normalization to the β-Globin pre-mRNA levels in each condition, the data were expressed relative

to the input to demonstrate amount of WT β-Globin immunoprecipitated with IgG or UPF1. Data are means +/- SEM, (n=4). (B) RT-PCR with CTRL minigene demonstrating a transcript with no non-specific binding to IgG or UPF1 in this assay.

exclusion of an exon through alternative splicing; (ii) contain an upstream open reading frame (uORF); or (iii) contain introns within their 3'UTRs (Nagy E, 1998). Amongst eight Alternative Splicing-Coupled NMD transcripts that we tested, three (*Sfrs10*, *hnrnpa2b1*, and *Ccar1*) were sensitive to CARM1 levels (Figure 24A-B and Table 4), while for example, the *Nfyb* mRNA was not dependent on CARM1 (Figure 24C). As shown by semi-quantitative RT-PCR, the *Sfrs10* isoform containing a PTC upon exon inclusion is increased relative to the skipped isoform in response to CARM1 depletion (Figure 24A). However, for *Ccar1*, while the relative ratio of spliced isoform is affected in the face of reduced CARM1 levels (Figure 24B), total amount of transcripts appear to stay constant, suggesting that CARM1 likely influences this pre-mRNA at the alternative splicing level rather than through an effect on NMD. In any case, more experiments would be required in order to determine precisely whether CARM1 affects the expression of each of these genes at the level of splicing and/or NMD.

We next assessed eight transcripts with distinct NMD-inducing features, that all have been documented elsewhere to be subjected to NMD (Table 4), in order to determine whether their steady-state levels was influenced by CARM1. As before, rescue experiments were conducted to ensure that the observed changes were not due to off-target effects. Of the seven transcripts assessed, *Gadd45a* and *Arc*, showed significant dependence on CARM1 levels (Figure 24D-E). A general trend towards CARM1-sensitivity was also observed for *Asns*, although statistical significance was not quite attained using CARM1 shRNA knock-downs (see below). For *Gadd45a*, both wt and methyltransferase-dead CARM1 alleles were as efficient at rescuing mRNA levels to that of CTRL (Figure 24D-E). However, for *Arc*, the E266Q mutant did not fully rescue mRNA levels to that of CTRL, suggesting that methyltransferase activity could be involved in the mechanism by which CARM1 regulates this specific target (Figure 24D-E and see Discussion)

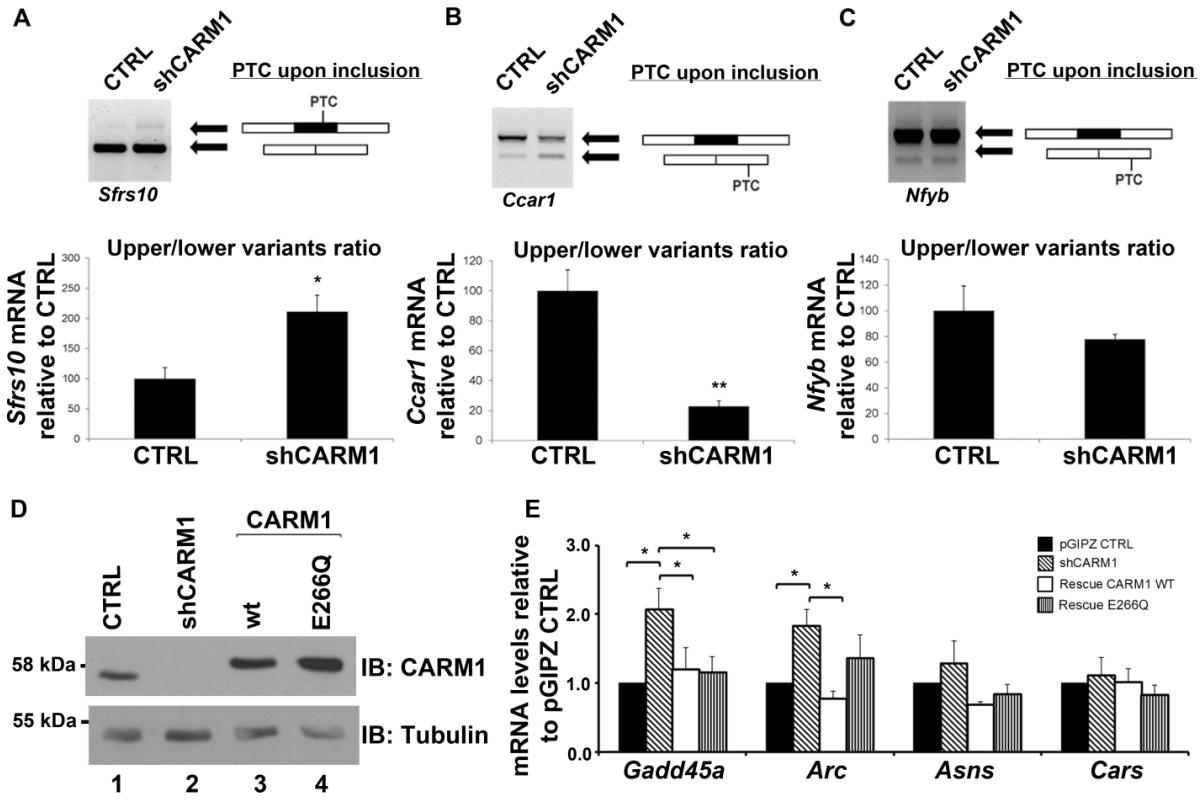

Figure 24. CARM1 regulates a diverse subset of known NMD targets.

(A) Total RNA was extracted from MN-1 pGIPZ CTRL and MN-1 shCARM1 cell lines. PCR primer sets were designed to flank the PTC resulting either by exon skipping or exon inclusion. For *Sfrs10*, PTC occurs upon exon inclusion. Values shown in the bar graph are means +/- SEM, *n* = 4. (B) For *Ccar1*, PTC is generated upon exon skipping. Values shown in the bar graph are means +/- SEM. (C) In the case of *Nfyb* gene, PTC is generated upon exon skipping. After optical quantification of the upper/lower splicing variants ratio, results are expressed relative to the MN-1 pGIPZ CTRL cell line. Values shown in the bar graph are means +/- SEM, *n* = 3 (D) A rescue was performed in two of the MN-1 shCARM1 cell lines by then transfecting with either the WT-CARM1 or the CARM1-E266Q mutant expression vectors for an additional 24 h. Protein lysate from transfected MN-1 lines confirm the knockdown and overexpression of CARM1 or the E266Q mutant, normalized to Tubulin. (E) Total RNA was extracted and RT-qPCR was used to measure

the mRNA levels of targets which were normalized to *18s* RNA levels and presented as relative to pGIPZ CTRL target mRNA levels. Data are means +/- SEM (n = 3).

To further and independently confirm that CARM1 is involved in regulating those specific endogenous NMD targets at the level of mRNA stability, we made use of mouse embryonic fibroblasts (MEFs) derived from *Carm1*$^{-/-}$ mice (Yadav *et al.*, 2003) (Figure 25). Using Actinomycin D treatments as described above, we measured the half-lives of the endogenous NMD targets *Arc, Gadd45a* and *Asns* (Giorgi *et al.*, 2007; Morris *et al.*, 2007), by RT-qPCR in wt and *Carm1*$^{-/-}$ MEFs. As predicted, all three NMD target mRNAs, this time including *Asns*, showed stabilization in *Carm1* $^{-/-}$ MEFs (Figure 25B-C and Figure 26). Specifically, for *Gadd45a*, the calculated half-life was of ~3.2 hrs in wt MEFs, compare with ~5 hrs in *Carm1* -/- cells (Figure 25B). Interestingly, this was comparable to the level of stabilization observed upon inhibition of NMD using Wortmannin in wt MEFs (see gel in Figure 25B). A similar phenomenon was also observed with *Asns*, with the half-life of the mRNA increasing by > 2-fold between wt and *Carm1* -/- MEFs (Figure 25C), and this difference also being reflected at the protein level (Figure 25A). However, for *Arc*, even if an ~2-fold increase in mRNA steady-state (Figure 24E) and protein (Figure 25A) levels was seen in CARM1-depleted cells, the measured difference in actual mRNA stability did not quite reach statistical significance (Figure 26), suggesting CARM1 may affect this particular target mostly at the level of transcription. Altogether, these experiments strongly suggest that, in addition to its well-known roles in regulating transcription and alternative splicing, CARM1 can also promote degradation of a specific subset of mRNAs through the NMD pathway.

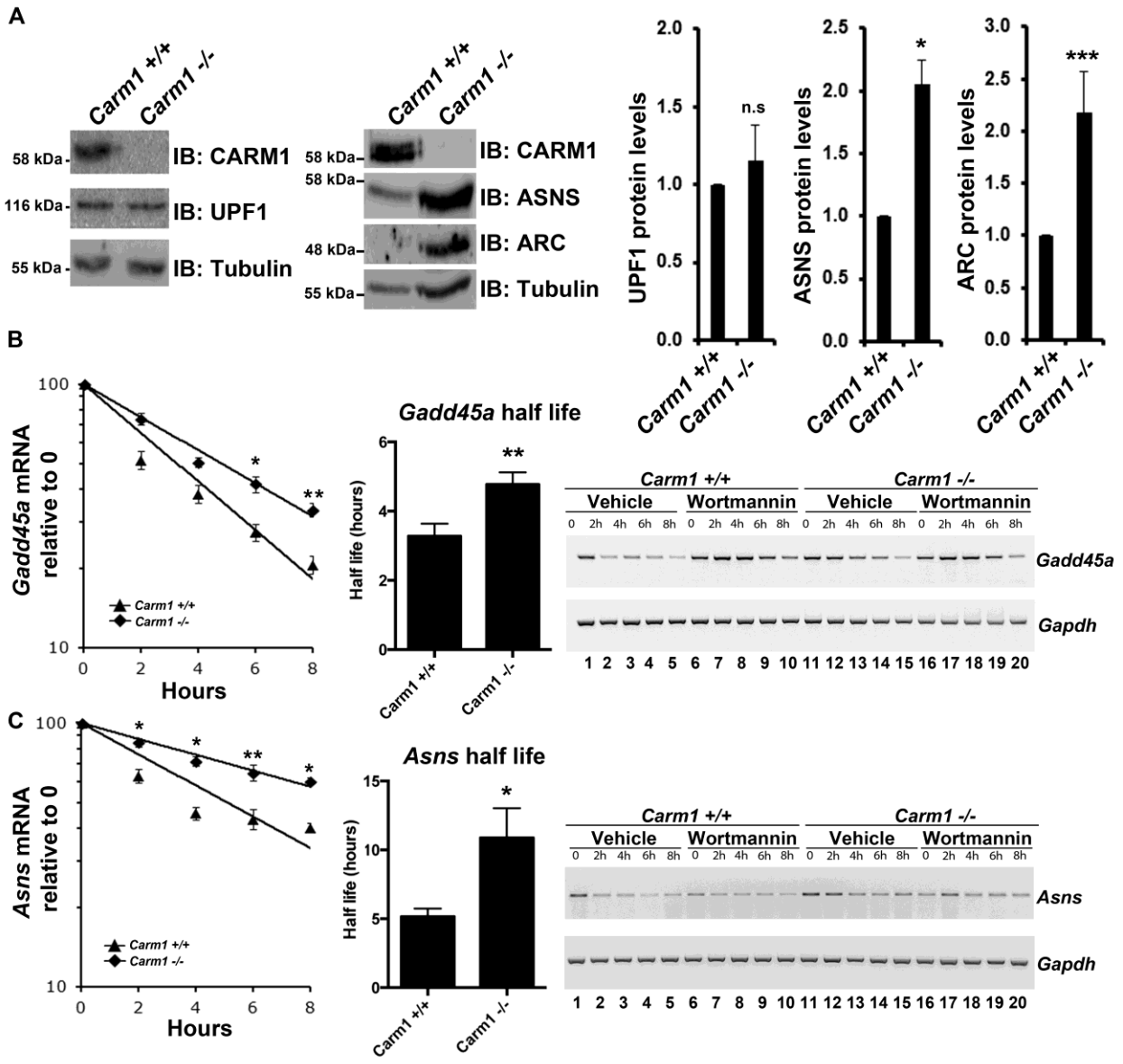

Figure 25. NMD target stability and protein expression in Carm1 +/+ and Carm1 -/- MEFs.

(A) Western blotting analysis and quantification shows levels of CARM1, UPF1 and NMD targets ASNS and ARC protein levels in *Carm1* +/+ (wt) and *Carm1* -/- MEFs. Tubulin was used as loading control. Data are means +/- SEM (*n* = 3). (B,C) Quantification of NMD transcripts *Gadd45A* and *Asns* mRNA half-lives normalized to *Gapdh*. Semi-logarithmic graph shows the decay rate of mRNAs calculated using formula: *(x) = ln(0.5)/b*, where *b* = slope obtained from the

trendline formula: $y = ne^{-bx}$. *Carm1* +/+ and *Carm1* -/- MEFs were pretreated with either a vehicle or Wortmannin (5 μM) and followed by treatment with actinomycin D (5 μg/ml) and RNA extraction at 0, 2, 4, 6 and 8 h. Quantification shows the means +/- SEM (n = 3) and data are presented as relative to zero time point.

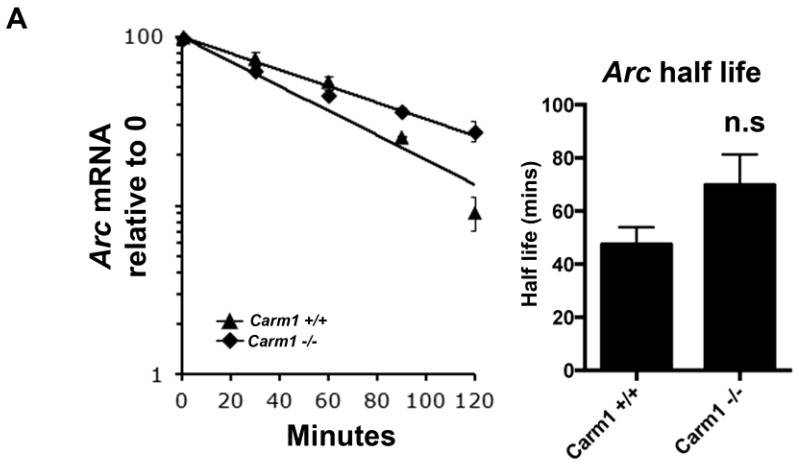

Figure 26. Arc mRNA stability in Carm1 +/+ and Carm1 -/- MEFs.

(A) Quantification of the NMD transcript *Arc* mRNA half-life was normalized to *Gapdh*. Semi-logarithmic graph shows the decay rate of mRNAs calculated using formula: $(x) = ln(0.5)/b$, where b = slope obtained from the trendline formula: $y = ne^{-bx}$. *Carm1 +/+* and *Carm1 -/-* MEFs were treated with actinomycin D (5µg/ml) and RNA extraction at 0, 20, 40, 60, 80, 100 and 120 minutes. Quantification shows the means +/- SEM (n=3) and data is presented as relative to zero time point.

CARM1-dependent NMD targets misregulated in SMA

Since we previously reported that CARM1 levels are up-regulated in SMA (Sanchez *et al.*, 2012), we reasoned that NMD targets found here to be CARM1-sensitive should be down-regulated in SMA-like settings. We first extended our findings to an *in vivo* setting by investigating the expression levels of the known endogenous NMD targets which we found to be CARM1 sensitive, in $Smn^{+/-}$ mice. Mice heterozygous for Smn show ~50% motor neuron attrition by ~6 months of age and are used as a model for mild SMA in humans (Balabanian S, 2007). Western blot analysis confirmed upregulation of CARM1 in these mice at pre-symptomatic time-points, and we observed no significant changes in UPF1 protein levels (Figure 27A-B). RT-qPCR analysis of CARM1-sensitive NMD targets *Gadd45a, Arc,* and *Asns,* revealed a significant decrease in mRNA expression levels in $Smn^{+/-}$ mouse spinal cord tissue as compared to wt (Figure 27C). These data, in conjunction with the increased CARM1 levels seen in the $Smn^{+/-}$ mice and the steady UPF1 levels in both conditions, are consistent with these targets being misregulated in SMA, at least in part, through a CARM1-dependent decay mechanism, although further experiments would be warranted to clearly demonstrate this link. Finally, we observed misregulation of *ARC, ASNS* and *ATF4* (another known endogenous NMD target) mRNAs in two independent type I SMA patients fibroblast cell lines, when compared to a fibroblast line derived from an unaffected carrier (Figure 27D-E). Interestingly, treatment of SMA fibroblasts with Wortmannin to inhibit the NMD pathway led to an increase in the levels of these mRNAs (Figure 27D-E), consistent with these mRNAs being misregulated in SMA due to a defect in NMD. Altogether, these results suggest that at least a subset of CARM1-sensitive NMD targets are decreased in SMA-like settings, supporting the notion that the NMD pathway may somehow be exacerbated in SMA.

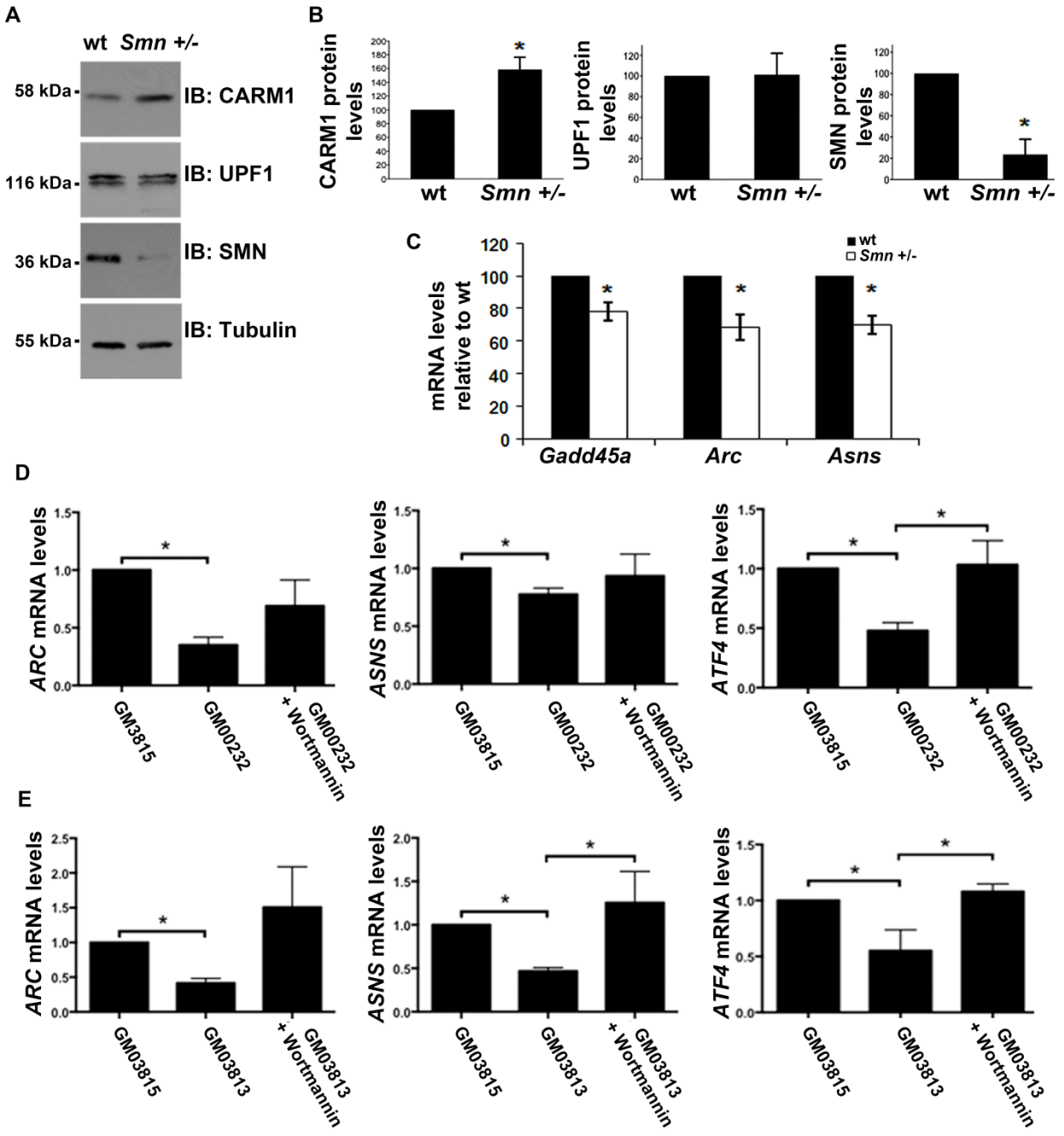

Figure 27. Endogenous NMD targets are mis-regulated in Smn +/- mice and SMA type I fibroblasts.

(A) Protein lysate was collected from the spinal cord tissue of wt and *Smn* +/- mice. CARM1, UPF1 and SMN protein levels were assessed by western blotting. (B) Quantification of protein

levels were gathered by normalizing to tubulin levels. Data are means +/- SEM (n = 3) (C) Total RNA was extracted from spinal cord tissue of wt and *Smn* +/- mice. RT-qPCR was used to measure the mRNA levels of targets which were normalized to *Gapdh* mRNA levels and presented as relative to wt target mRNA levels. Data are means +/- SEM (n = 3). (D,E) Fibroblast cell lines from two independent human SMA type I patients (GM00232, GM03813) were treated with Wortmannin (5 μM) for 6 h. Untreated fibroblast cell lines from a carrier (GM03815) were used as CTRL. Total RNA was isolated from the cell lines and three non-ASC-NMD target mRNA expression levels were measured by RT-qPCR: *ARC*, *ASNS* and *ATF4* using GAPDH as a normalization control. Data are means +/- SEM (*ARC*, *ATF4n* = 6; *ASNSn* = 5).

3.5 Discussion

We report in the present study that CARM1 promotes down-regulation of a specific subset of NMD substrates. Specifically, we found that CARM1 is required to elicit NMD on a generic NMD reporter, through a mechanism that is independent of its methyltransferase activity. We also demonstrate that CARM1 interacts with major NMD factor UPF1, in an RNA-dependent fashion, and that occupancy of UPF1 on a PTC-containing transcript is significantly decreased in CARM1-depleted cells. Finally, consistent with the fact that CARM1 is upregulated in SMA-like settings, we provide evidence that the NMD pathway may somehow be exacerbated in SMA.

CARM1 promotes down-regulation of mRNAs with different NMD-inducing features

We have initially focussed our efforts on *Uspl1* in the present study and uncovered that an alternatively spliced isoform resulting from the skipping of Exon 2 was a target for NMD. This finding was also corroborated by the fact that *Uspl1* was identified in a recent unbiased, genome-wide screen for novel NMD targets (Hurt *et al.*, 2013). While we clearly demonstrated that reduced levels of CARM1 affected the relative ratio of *Uspl1* alternatively spliced isoforms, our mRNA stability assessments suggested that this effect was likely mediated principally at the splicing level, although we can not rule out some contribution at the level of NMD. We also observed an effect of CARM1 on the relative ratio of spliced isoforms for three other genes, *Sfrs10, HnRNPa2b1* and *Ccar1* (Table 4), although additional experiments would be required to determine precisely whether CARM1 affects splicing and/or NMD for those targets. Because it is often more difficult for splicing-coupled NMD targets to discern between an effect on splicing and/or NMD, we next decided to focus on well-established endogenous NMD targets with distinct NMD-inducing features. We documented three such endogenous transcripts (*Gadd45a*, *Arc*, and *Asns*) which are targets of NMD due to features unrelated to alternative splicing (e.g. uORFs or introns in the

3'UTR), that are CARM1-sensitive. This suggest that CARM1's influence on NMD targets is not dependent on the mechanism through which NMD is elicited. Nevertheless, not all previously validated endogenous NMD targets we assayed were CARM1-sensitive (see Table 4), suggestive of a significant degree of target specificity for CARM1's regulation of NMD targets. For example, we have found that CARM1 did not influence mRNA or protein levels of major NMD factors (Figure 20), many of which are known to be regulated through the NMD pathway in a negative feedback regulatory loop involved in establishing and maintain NMD homeostasis (Huang *et al.*, 2011; Yepiskoposyan *et al.*, 2011). There is mounting evidence in the literature that several NMD branches exist (Chan *et al.*, 2007; Gehring *et al.*, 2005; Metze *et al.*, 2013), each with distinct substrate specificity features, so it would be interesting to determine whether this may explain CARM1's NMD target discrimination. Identification of additional CARM1-dependent NMD target in future studies, ideally through unbiased genome-wide approaches, should allow to determine the precise mechanism through which CARM1 target specificity is arising.

CARM1 interacts with major NMD factor UPF1

We have observed that CARM1 interacts with UPF1, in an RNA-dependent fashion, using co-immunoprecipitation experiments, with endogenous proteins. However, we have not been able to detect a signal for CARM1 upon UPF3b or UFP2 immunoprecipitations (Figure 22 and data not shown, respectively). Nevertheless, both these antibodies were not very efficient at immunoprecipitating their respective target, so we can not rule out at this point that a lack of signal for CARM1 in these experiments could be due to technical issues, like e.g. insufficient sensitivity or masking of an interaction domain by the antibodies. In an attempt to address the functional relevance of the CARM1/UPF1 interaction, we used RIP assays to measure the occupancy of UPF1 on a PTC-containing transcript, and found that occupancy was reduced upon CARM1 depletion

by RNA interference. This provides further evidence that CARM1 promotes NMD, and suggest that it may play a scaffolding role at some point along the NMD pathway. Nevertheless, it was recently shown that target discrimination is likely determined by the ATPase-dependent duration of UPF1 occupancy (off-rate) on mRNAs (Lee *et al.*, 2015), so it will be interesting to investigate whether CARM1 affects this process. Such a scaffolding role for CARM1 would be consistent with our observation that CARM1's methyltransferase activity is dispensable for its function in promoting down-regulation of NMD substrates. This is also in line with the fact that we were unable to detect any methylation mark in UPF1 using mass spectrometry (data not shown). Interestingly, the methyltransferase-DEAD E266Q CARM1 mutant was as efficient as wt CARM1 in rescue experiments, for all targets tested except for *Arc*, which we in fact showed is likely regulated by CARM1 mostly at the level of transcription. There are other documented cases of activity-independent roles for PRMTs. For example, Jayne *et al*. have reported that CARM1 methyltransferase activity was dispensable for the expression of a subset of NF-κB target genes in response to either TNFα or PMA/ionomycin stimuli (Jayne *et al.*, 2009). In the case of Rmt3, the homolog of human PRMT3 in *Schizosaccharomyces pombe*, Perrault *et al*. (Perreault *et al.*, 2009) demonstrated that enzymatic inactive mutants of Rmt3 were able to rescue the ribosomal 40S formation defect in Rmt3-null cells. Interestingly, they also observed that an Rps2 mutant in which the methylated arginines were substituted by lysines (which are not substrates for PRMTs but still have a positive charge) was still able to rescue the defects whereas Rmt3 mutants failing to interact with Rps2 did not. Altogether, these data argue that in addition to their enzymatic activity, PRMTs can also have methyltransferase-independent roles in scaffolding and/or stabilization of complexes through protein-protein and protein-RNA interactions. Nevertheless, it is known that transcriptional events can impact on the efficiency of NMD (Bühler *et al.*, 2005; Enssle *et al.*,

1993; Gudikote *et al.*, 2005), so considering that CARM1 is a well-known transcriptional and splicing regulator, further investigations will be required in order to determine the precise mechanism by which it can promote NMD.

Potential contribution of misregulated NMD targets to the pathophysiology of SMA?

In the current study we have used the *Smn*$^{+/-}$ mice to confirm our results in an *in vivo* setting that would allow us to assess protein expression profiles at a pre-phenotypic or disease onset time-point. Using this model, we reproduced our previous observation that CARM1 was upregulated (~60%) in spinal cord tissues, with no significant difference in main NMD mediator UPF1 levels, thus providing an ideal *in vivo* model to validate CARM1-sensitive NMD targets identified in our cell culture models. We were also able to corroborate some of our findings in two SMA Type I patient fibroblast cell lines, importantly showing that treatment with NMD inhibitor Wortmannin was able to restore steady-state expression levels of a subset of NMD substrates found to be misregulated in these cells. However, these experiments were performed with a very limited number of lines and lack of proper, genetically matched, control to allow for a stringent comparative analysis. It is interesting to note that amongst the NMD targets that we have found to be misregulated in SMA, both *ATF4* and *GADD45a*, are well known mediators of cellular stress response (Ebert *et al.*, 2012; Jiang *et al.*, 2007). More specifically, for example the ATF4/GADD45a axis was recently shown to contribute to skeletal muscle atrophy (Bongers *et al.*, 2013; Ebert *et al.*, 2012). Nevertheless, the fact that we observe a decrease in *ATF4* and *GADD45a* levels may seem counter-intuitive, but it will be important to investigate this further in relevant tissues (i.e. motorneurons, skeletal muscles, glial cells) and at various developmental stages in more severe SMA models. Another known NMD target that we have found to be downregulated in SMA, and which might have important implications for the pathophysiology of the disease, is

the immediate-early gene *Arc*. It was elegantly shown by Melissa Moore's group in 2007 that *Arc* mRNA are subjected to 'translation-dependent decay (TDD)' in dentrites of neuronal cells, as part of the mechanisms involved in maintaining its restricted protein expression at synapses (Giorgi *et al.*, 2007). The Arc protein, through is dynamic expression, is a crucial mediator of homeostatic synaptic scaling of AMPA type glutamate in order to exert minute control over individual synaptic strength and overall cellular excitability (Béïquea, 2011; Shepherd *et al.*, 2006). Strikingly, the existence of sensory-motor circuitry defects was recently demonstrated in SMA model mice, which among other things involved intrinsic hyperexcitability of SMA motoneurons (Mentis *et al.*, 2011). This hyperexcitability phenotype is in fact consistent with the reduced *Arc* levels that we have documented in the current study, suggesting misregulated expression of Arc in motoneuron dentrites may contribute, at least in part, to defects in the SMA sensory-motor circuit. Altogether, our results are consistent with the notion that the NMD pathway may somehow be exarcebated in SMA, although obviously, additional experiments, in more relevant models of the disease, will be necessary to clearly establish whether this contributes to SMA pathophysiology.

3.6 Funding

This work was supported by the Canadian Institutes of Health Research (CIHR) (MOP-86746 to J.C.) and by Families of SMA (FSMA). G.S. was supported by a PDF fellowship from the Association Française contre les Myopathies (AFM). E.B.-C. and A.D. were supported by STaR Awards from the University of Ottawa Centre for Neuromuscular Disease (CNMD) and the University of Ottawa Brain and Mind Research Institute (uOBMRI). J.C. is the recipient of a Canada Research Chair (Tier II) in RNA Metabolism

3.7 Acknowledgements

We thank Dr Adrian Krainer (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA) for generously providing the WT and MT β-Globin reporters. We also thank Dr Mark Bedford (The University of Texas MD Anderson Cancer Center, Smithville, TX, USA) for the generous gift of the CARM1 and CARM1 E266Q methyl dead expression vectors and CARM1$^{-/-}$ MEFs, and Dr. Jens Lykke-Andersen for reagents to perform tethering experiments. Finally, we thank members of the Côté and Jasmin labs for helpful discussions and technical advice, as well as Dr. Jean-Claude Béïque for his insights into the function of Arc in synaptic plasticity.

Conflict of Interest statement. None declared.

Chapter 4 - Manuscript #3

Altered Expression of Splicing Factors SNW1 and RALY Leads to Misregulation of Protein Arginine Methyltransferase 1 Exon 2 Alternative Splicing and Increased Invasion in Breast Cancer Cells

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Manuscript Information:

Bondy-Chorney, Emma, R. Mitchell Baldwin, Andréanne Didillon, Benoit Chabot, Bernard J. Jasmin, and Jocelyn Côté. "Altered Expression of Splicing Factors SNW1 and RALY Leads to Misregulation of Protein Arginine Methyltransferase 1 Exon 2 Alternative Splicing and Increased Invasion in Breast Cancer Cells". Manuscript in prep – to be submitted to the International Journal of Biochemistry and Cell Biology.

Author's contribution:

Conceived and designed the experiments: EBC, RMB, AD, BC, BJJ, JC. Performed the experiments: EBC, AD, BC. Analyzed the data: EBC, RMB, AD, BC, BJJ, JC. Contributed reagents/materials/analysis tools: BC. Wrote the paper: EBC, RMB, BJJ JC.

Contributions by figure: Fig 28 (EBC, BC, JC), Table 6 (BC, JC), Fig 29 (EBC), Fig 30 (EBC), Fig 31 (EBC, AD), Fig 32 (EBC), Fig 33 (EBC), Fig 34 (EBC, RMB), Fig 35 (EBC), Fig 36 (EBC, RMB, JC).

4.1 Abstract

Several alternatively spliced isoforms are generated from the Protein Arginine Methyltransferase 1, *PRMT1* gene that have distinct substrate specificity and subcellular localization. Previously, we reported that *PRMT1v2,* an isoform that includes the alternative exon 2, was increased in breast cancer. Moreover, overexpression of PRMT1v2 promoted breast cancer cell growth, survival, and invasion. Here, we investigated the underlying mechanism as to why the *PRMT1v2* isoform is selectively upregulated in breast cancer and whether we could potentially target key regulators of this pathway as a therapeutic strategy. A high-throughput RNAi RT-PCR screen identified several key splicing factors, namely SNW1 and RALY, that upon depletion either led to an increase or decrease in the inclusion of PRMT1 exon 2, respectively. These findings provide the first evidence to support the notion that the altered expression of specific splicing regulators leads to changes in the alternative splicing profile of *PRMT1* exon 2. Expression profiles SNW1 and RALY revealed altered mRNA and protein expression of these splicing factors in a panel of breast cancer cell lines and patient tumours. Importantly, we found that RALY depletion was sufficient to induce a significant decrease in invasive potential of highly aggressive breast cancer cells. Kaplan-Meier plots also showed a correlation between high RALY levels and decreased overall survival of patients, indicating RALY as potential prognostic indicator. Our work reveals crucial insights into the mechanisms regulating PRMT1 exon 2 alternative splicing and provides a proof-of-principle demonstration that targeting this splicing event may represent a promising therapeutic strategy for breast cancer.

4.2 Introduction

Arginine methylation is a common post-translational modification (PTM) involved in cellular processes including signal transduction, transcription, DNA repair, and pre-mRNA splicing (Bedford and Clarke, 2009; Blackwell and Ceman, 2012; Chen *et al.*, 2011a; Hata *et al.*, 2016; Herrmann *et al.*, 2009; Lake and Bedford, 2007). Arginine methylation is carried out by a small family of enzymes called protein arginine methyltransferases (PRMTs) composed of nine members that are classified into three groups based on the type of arginine methylation reaction each member catalyzes. The first group, Type I PRMTs, comprises PRMT1, 2, 3, 4 (CARM1), 6, and 8, and can catalyze both mono-methyl and asymmetric dimethyl arginine reactions. The type II PRMT group is made up of two members, PRMT 5 and 9, which catalyze both mono-methyl arginine and symmetric dimethyl arginine. Finally, PRMT7 is so far considered to be the sole type III PRMT, able to generate only mono-methyl arginines(Hadjikyriacou *et al.*, 2015; Morales *et al.*, 2016). The majority of the PRMTs are ubiquitously expressed except the neuron-specific expression of PRMT8(Lee *et al.*, 2005).

Not surprisingly, considering the essential roles that PRMTs play in normal cellular functions, their dysregulation has been linked to numerous human diseases (Akter *et al.*, 2016; Mastronardi and Moscarello, 2005; Sanchez *et al.*, 2016; Tradewell *et al.*, 2012; Tutarel *et al.*, 2012; Yang and Bedford, 2013). For example, PRMTs are commonly found to be overexpressed in various cancers (Yang and Bedford, 2013). PRMT2, PRMT5, and PRMT6 are overexpressed and/or have altered activity in cancers of several origins, including breast, bladder, gastric, lung, and colorectal (Cho *et al.*, 2012; Wei *et al.*, 2012; Yang and Bedford, 2013; Yoshimatsu *et al.*, 2011; Zhong *et al.*, 2012). PRMT7 has been identified as a candidate gene associated with breast cancer metastasis and the decreased survival of breast cancer patients(Baldwin *et al.*, 2015b; Thomassen *et al.*,

2009). PRMT1, the most abundantly expressed PRMT, is aberrantly expressed in colorectal, prostate, lung, bladder, leukemia, and breast cancer (Avasarala *et al.*, 2015; Baldwin *et al.*, 2012; Goulet *et al.*, 2007; Mathioudaki *et al.*, 2011; Tang *et al.*, 2000; Zou *et al.*, 2012). Interestingly, although the overall expression level of *PRMT1* mRNA is increased in breast cancer, we previously observed that the isoform with the highest relative increase in expression was *PRMT1v2* (Goulet *et al.*, 2007). From this work, a novel role for the almost exclusively cytoplasmic PRMT1v2 protein in promoting the survival and invasion of breast cancer cells was uncovered (Baldwin *et al.*, 2012). *PRMT1v2* isoform depletion by RNA interference (RNAi) resulted in an induction of apoptosis and decreased invasiveness of the aggressive breast cancer cell line MDA-MB-231. On the other hand, *PRMT1v2* overexpression was sufficient to confer to a weakly-aggressive, "luminal epithelial-like" breast cancer cell line, MCF7s, increased invasive properties (Baldwin *et al.*, 2012). We have also previously uncovered, using a quantitative proteomics approach, that the PRMT1 splice variants, PRMT1v1 and PRMT1v2, possess distinct protein interaction profiles (Baldwin *et al.*, 2015a).

Based on these studies, we set out to investigate the underlying mechanism at the origin of *PRMT1v2* upregulation in breast cancer cells. Here we report, by using a high-throughput RNAi RT-PCR splicing screen the identification of two splicing factors, SNW1 and RALY, that regulate *PRMT1* exon 2 inclusion. We show in two different breast cancer cell lines that modulating SNW1 and RALY expression levels are sufficient to modify *PRMT1* exon 2 alternative splicing. Initial mechanistic studies using a novel *PRMT1* minigene system also revealed several highly conserved intronic sequences, including a putative RALY binding site, which partially controlled *PRMT1* exon 2 alternative splicing. Analysis of a panel of breast cancer cell lines and tumour samples revealed aberrant down- and upregulation of both the mRNA and protein expression levels of

SNW1 and RALY, respectively, as compared to normal breast tissue controls. The altered expression profiles of SNW1 and RALY correlated with the increased *PRMT1* exon 2 inclusion splicing patterns in breast cancer and, in the case of RALY with overall survival of patients. Finally, we show that the regulation of *PRMT1v2* alternative splicing, via RALY depletion, can significantly decrease the invasive potential of MDA-MB-231s. Thus, this proof-of-concept study is the first to demonstrate that targeting the alternative splicing of *PRMT1* exon 2 represents a potential therapeutic avenue to treat highly aggressive, metastatic breast cancer.

4.3 Materials and Methods

Cell lines and tissue samples used

The following cell lines were obtained from ATCC: MCF10A (CRL-10317), Hs578Ts (HTB-126), MCF7 (HTB-22), MDA-MB-231 (CRM-HTB-26). RNA from breast tissue was obtained from Origene are as follows; RNA (5µg): Normal; (Origene:CR516938, CR518992, CR559063), Adenocarcinoma, ductal, primary (Origene:CR550145, CR559187; CR559317), Adenocarcinoma, ductal metastatic (Origene:CR560116, CR560023; CR560217), Adenocarcinoma, metastatic (Origene:CR560317, CR559424).

Plasmid constructs, Gibson assembly, and cell transformations

siRNAs: Non-targeting scramble (ON-target Tplus Control sRNAi:D001810-02-05), siSNW1 (Origene: SR307894), and siRNAs against human RALY (ON-target Tplus Control sRNAi: D001810-02-05, siRALY: custom seq "CUCUCGAGUCUUCAUUGGA"). PRMT1 minigenes generated in pcDNA3.1(-) backbone using Gibson assembly primers: pcDNA3.1(-)NotI-5'end of human PRMT1 exon 1 (cctctagactcgagcTGAAATCTTCCAGCGGGG) and 3'end of human PRMT1 exon 4-HindIIIpcDNA3.1(-) (atcagcggtttaaacttaTGTCCCCACTGACCTCGT), and additional overlapping primers of deleted regions, following Gibson assembly guidelines(Gibson *et al.,* 2009). Constructs were sequence verified (Génome Québec Innovation Centre).

Cell transfections and lysis

Cells were grown to ~50-70% confluency and transfected with 0.250-5 µg DNA using Lipofectamine with plus reagent (Life Technologies:15338100), according to manufacturer's protocol. RNA and protein were collected 48 hours after transfection followed by cDNA synthesis as previously described(Bondy-Chorney *et al.*, 2016b).

Western blotting analysis and antibodies

Cell lysis and western blotting was performed as previously described(Bondy-Chorney *et al.*, 2016b). Antibodies used: Anti-SNW1[1:1000](Bethyl:A300-785A-H), Anti-GAPDH[1:10,000](Abcam:ab8245), Anti-β-Actin[1:1000](Santa Cruz:sc-47778), Anti-RALY[1:1000](Millipore:AB12226), and Anti-PRMT1v2[1:500](Synthetic PRMT1v2 peptide, described previously(Baldwin *et al.*, 2012)), Mouse-anti-Rabbit HRP[1:20,000](Jackson ImmunoResearch:211-032-171) and Goat-anti-Rabbit HRP[1:10,000] (Molecular Probes:MP 02764).

RNA extraction and cDNA synthesis

To isolate total RNA from whole cell lysates TRIzol Reagent (Thermofisher Scientific:15596026) was used according to manufacturer's protocols. AMV cDNA synthesis kit (Promega:M5101) used to synthesize cDNA with random hexamers (10mM) from 500 ng of RNA. RT- and RT-qPCR reaction used 5μL (~100 ng) of diluted cDNA (1:20). RNA and cDNA was stored at -80°C.

Reverse Transcription (RT)- PCR and RT-quantitative PCR (RT-qPCR)

Promega GoTaq DNA Polymerase kit (Promega:M5101) was used for RT-PCR reactions according to manufacturer's protocol. RT-PCR conditions: 95°C 2 min, (95°C 30 sec, 55°C 30 sec, 72°C 45 sec)x32 cycles, 72°C 10 min. Gibson assembly PCR required increased annealing temperatures (~65°C) and extension times. PCR products visualized by gel electrophoresis as previously described(Bondy-Chorney *et al.*, 2016b). The percent of exon inclusion or Percent Splicing Index (PSI), was calculated using the following formula (1): % exon inclusion=(upper isoform/Σ upper isoform + lower isoform). RT-qPCR reactions were carried out using BioRad iQ SYBR® Green Supermix (BioRad:170-8882) according to manufacturer's protocol. Conditions

for RT-qPCR: 95°C 2 min, (95°C 30 sec, 60°C 30 sec, 72°C 45 sec)x40 cycles, 72°C 10 min. Three technical replicates were performed for all RT-qPCR experiments, Ct values were averaged and normalized to control gene as indicated in text. Fold change in transcript was analyzed using the ΔΔCt method. Biological replicates of ≥3 samples were done for all RT- and RT-qPCR reactions.

RNAi high-throughput RT-PCR screen

The RNAi high-throughput RT-PCR screen was carried out by RNomics Platform at Université de Sherbrooke using the following primers to amplify the PRMT1v1 and v2 isoforms as (Forward:GAGGCCGCGAACTGCATCAT and Reverse:TGGCTTTGACGATCTTCACC). The annotation and analysis of alternative splicing was determined using endpoint PCR coupled with microcapillary electrophoresis. PSIs were then calculated according to formula (1) and the change in PSI (dPSI) was determined by the following formula (2): $dPSI=PSI^{siRNA}-PSI^{LipoCTRL}$. All PSIs from all 56 siCTRL and siRNA MCF7 cell lines tested are included in Table 6. Any PSI values with no useable control, a PSI error, or a no assay signal were not included in analyses.

RNA Immunoprecipitations (RIPs)

RIPs were performed as previously described(Bondy-Chorney *et al.*, 2016b).

Immunohistochemistry (IHC) and K-M plot analysis

IHC analysis, including protein expression figures based on tissue stains, were obtained and presented here courtesy of Human Protein Atlas, www.proteinatlas.org, from the Human Protein Atlas version 15, Ensembl version: 78.38(Uhlén *et al.*, 2015). Protocol for IHC analysis used as previously described (http://www.proteinatlas.org/ download/IHC_protocol.pdf). Kaplan-Meier

(K-M) plotter was used to generate all K-M plots presented here (http://kmplot.com)(Győrffy *et al.*, 2010). In all cases, the parameter of overall survival (OS) was used in the km-plotter analysis for an endpoint (patient n[number of patients with available clinical data]=1117)(Győrffy *et al.*, 2010).

Invasion assays

Motility and invasion assays were performed as previously described(Baldwin *et al.*, 2012).

Statistical Analysis

Statistical significance between two groups, for example, the CTRL compared to a knockdown siRNA cell line, was analyzed using two-tailed student's t-tests (type 2) with biological replicates $n \geq 3$. The level to determine a value as significant was set as $p<0.05$. Significance was denoted as follows: * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

4.4 Results

High-throughput RNAi RT-PCR splicing screen reveals splicing factors that regulate *PRMT1* exon 2 splicing

We previously identified *PRMT1v2* as an alternatively spliced *PRMT1* isoform that plays a role in promoting breast cancer cell survival and invasion, however the mechanism by which generation of this isoform is regulated in cells is currently not known. Therefore, we first set out to identify splicing factors that are involved in the splicing regulatory pathway of the *PRMT1v2* isoform. A custom targeted RNAi screen coupled with high-throughput RT-PCR was used to identify splicing factors that regulate *PRMT1* alternative exon 2 inclusion (Fig 28A). A total of 56 known splicing factors were targeted by RNAi in the human breast carcinoma cell line MCF7 and the *PRMT1* exon 2 inclusion was determined for each. Here, we have shown the raw data output in the form of a heatmap that represents the change in the inclusion of exon 2 expressed as the change in PSI in the MCF7 knockdown cell lines compared to a non-targeting control (Fig 28B). Using a dPSI >5% we identified several splicing factors which altered the ratio *PRMT1* exon 2 inclusion (Fig 28C). Altogether, 8 splicing factors were found to regulate the inclusion of exon 2. Specifically, upon depletion of the splicing factors CDK11, CDC5L, SNW1, SFRS3, and SR-A1 an increase in exon 2 inclusion was observed (dPSI $\geq$5%) (Fig 28B). CTNNB1 was also identified as a hit, however, upon closer inspection it was removed due to a lack of detection of both *PRMT1* isoforms in the CTRL samples for this factor (Table 6). In contrast, the knock down of the splicing factors RALY, SF3A2, and RNPC2 resulted in decreased exon 2 inclusion (dPSI $\geq$5%) (Fig 28B).

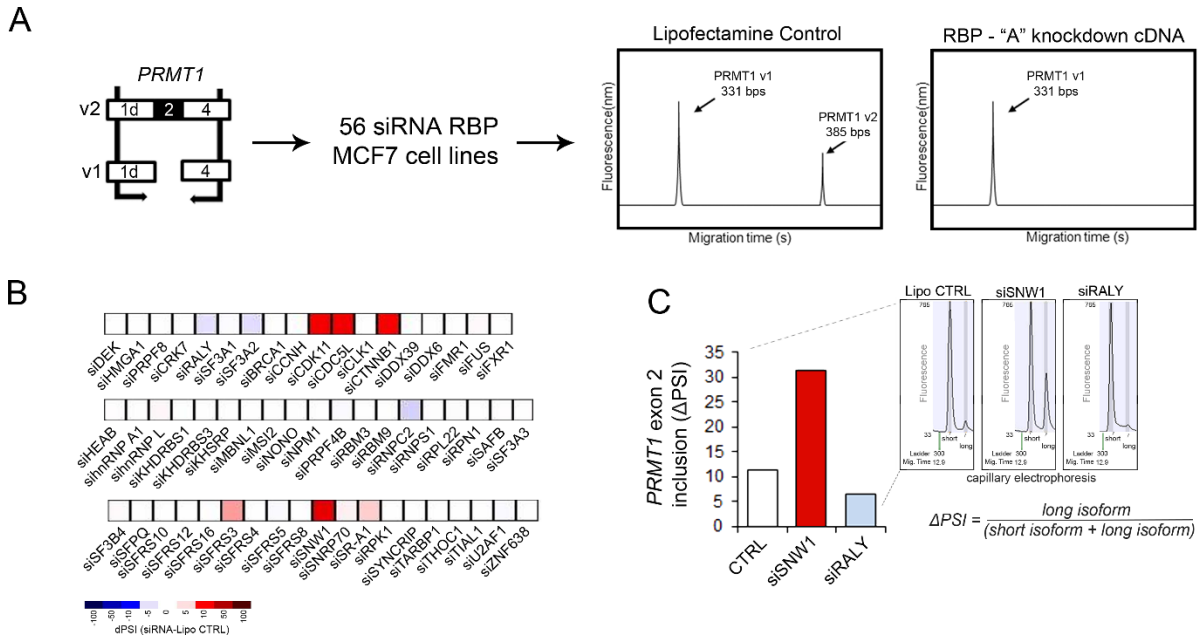

Figure 28. High-throughput RT-PCR screen reveals several splicing proteins regulate PRMT1 v1/v2 splicing.

(A) Representation of the *PRMT1* exon 2 splicing event. Primers located in exon 1d and exon 4 were used to amplify the *PRMT1v1* (-exon2) and *PRMT1v2* (+exon2) isoforms. High-throughput PCR was performed to amplify v1/v2 isoforms for in 56 MCF7 cell lines which each had a splicing factor knocked down. Expression of the isoforms is quantified by capillary electrophoresis of the PCR reactions. (B) Heatmap showing the difference in percent splicing index (dPSI) of the PRMT1 v1/v2 ratios (exon 2 inclusion) of 56 different splicing factor knockdown MCF7 cell lines. (C) The top three results from the screen represent the most apparent changes in splicing regulation of the PRMT1 exon 2 inclusion change.

Table 6. High-throughput RNAi RT-PCR Screen

* Due to the size of this table it is located in Appendix C

All together, this high-throughput RT-PCR screen has identified a number of novel potential splicing regulators of *PRMT1* exon 2 alternative splicing. We focused on two splicing factors which induced the greatest shift in the splicing pattern of *PRMT1* exon 2, namely SNW domain containing protein 1 (SNW1) and RNA-binding protein RALY (RALY). Interestingly, these findings identify the inclusion of *PRMT1* exon 2 as the first known RALY-regulated alternative splicing event.

Modulation of SNW1 and RALY regulate *PRMT1* exon 2 alternative splicing

To validate our RT-PCR screen and to further examine the idea that SNW1 and RALY regulate *PRMT1* exon 2 alternative splicing we modulated the levels of these splicing factors and examined any changes in exon 2 inclusion. MCF7 cells were transiently transfected with either a control non-targeting siRNA (CTRL) or siRNAs specifically targeting *SNW1* or *RALY* mRNAs. SNW1 depletion was accompanied by a significant, 11.5% increase in the inclusion of *PRMT1* exon 2, whereas the depletion of RALY resulted in a 9.5% decrease in exon 2 inclusion (Fig 29A). The changes in exon 2 splicing were also observed in a second breast cancer cell line, MDA-MB-231, whereupon depletion of RALY, exon 2 inclusion decreased 15.2% (Fig 30A). Depletion of SNW1 and RALY protein levels were confirmed in MCF7s by Western blotting and, after normalization, revealed efficient depletions of 60.2% and 66.7%, respectively, compared to CTRL (Fig 29B and C). The change in *PRMT1* exon 2 splicing induced through SNW1 and RALY depletion was also sufficient to result in a 62% increase and 48.9% decrease in PRMT1v2 protein expression, respectively, as compared to CTRL levels (Fig 29B and C). The decrease of PRMT1v2 protein level was further confirmed using a C-terminal specific PRMT1 antibody which detects both the v1 and v2 isoforms, a result which also revealed no change in PRMT1v1 protein levels upon RALY depletion (Fig 29B and C). Taken together, these results

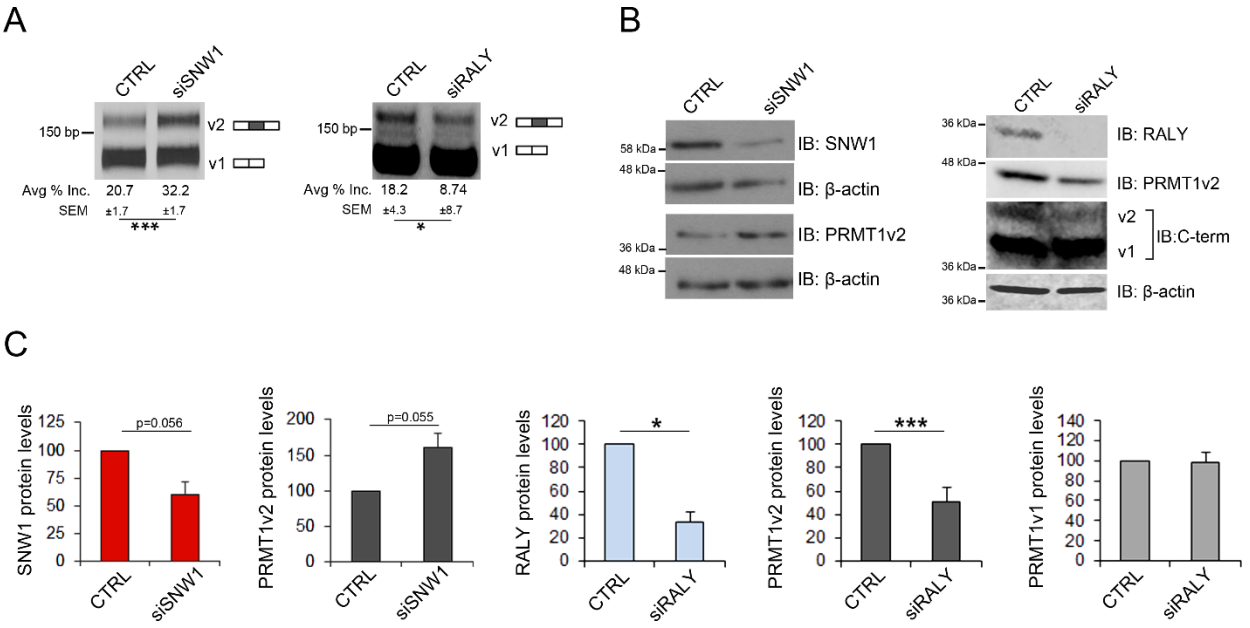

Figure 29. Splicing factors regulate PRMT1 exon 2 splicing.

(A) A scramble (CTRL) or an siRNA against *SNW1* mRNA or *RALY* mRNA were transfected into MCF7 cell lines and RNA and protein were collected after 48 hours. RT-PCR was used to detect and analyses *PRMT1v1* and *PRMT1v2* spliced isoforms. Average percent exon 2 inclusion was determined as described in formula (1) in materials and methods. (B) Protein levels were assessed by western blotting and using β-actin as a loading control. (C) Quantification of mRNA and protein expression levels. Data are means +/- SEM ($n \geq 3$) * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

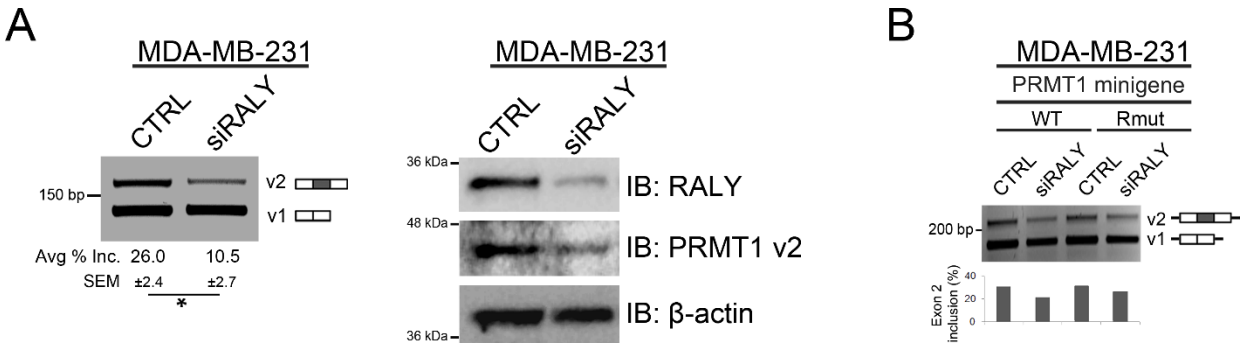

Figure 30. Splicing factors regulate PRMT1 exon 2 splicing in a second breast cancer cell line.

(A) A scramble siRNA (CTRL) or an siRNA against *RALY* mRNA was transfected into MDA-MB-231 cell lines and RNA and protein were collected after 48 hours. RT-PCR was used to detect and analyses *PRMT1v1* and *PRMT1v2* spliced isoforms. Average percent exon 2 inclusion was determined as described in formula (1) in materials and methods. Protein levels were assessed by western blotting and using β-actin as a loading control. (B) *PRMT1* WT minigene exon 2 splicing patterns in CTRL (siScramble) or siRALY siRNA transfection. MDA-MB-231 cells were first transfected with 1µg of each siRNA and minigenes were transfected 24 hours after. Following another 24 hours, cells were harvested. RT-PCR was used to detect and analyses *PRMT1v1* and *PRMT1v2* spliced isoforms. Percent exon 2 inclusion was determined as described in formula (1) in materials and methods.

validated our RT-PCR screen findings and suggest that SNW1 normally acts as a repressor of *PRMT1* exon 2 inclusion, whereas RALY would promote the inclusion of exon 2.

Mechanistic insights into the splicing regulation of *PRMT1* exon 2

In order to gain some insight into the mechanism by which SNW1 and RALY could regulate *PRMT1* exon 2 splicing, we examined whether these proteins associated with *PRMT1* pre-mRNA. RIPs were performed on MCF7 cell protein lysates using antibodies against SNW1 and RALY to purify endogenous protein and interacting components. Immunoprecipitation for SNW1 and RALY showed purification of each protein in the IP lanes (Fig 31A). RT-PCR, using primers to amplify a sequence within intron 2 of *PRMT1* pre-mRNA, revealed the presence of *PRMT1* pre-mRNA in both the IP for SNW1 and RALY. By contrast, *GAPDH* mRNA was not enriched in either IP (Fig 31A). These findings show that SNW1 and RALY associate with the endogenous *PRMT1* pre-mRNA in breast cancer cells, strongly suggesting they may directly impact PRMT1 splicing profile.

To further investigate SNW1 and RALY's role in splicing regulation of exon 2, we developed a novel *PRMT1* minigene system. We constructed a *PRMT1* WT minigene made up of the 157 nts of exon 1, the 2,556 nts of intron 1, the 54 nts of exon 2, the 205 nts of intron 2, the 108 nts of exon 3, the 248 nts of intron 3, the 104 nts of exon 4, and finally 12 nts of intron 4 (Fig 31B). Transfection in MCF7s showed that the minigene fully recapitulates the differential alternative splicing of this event observed with the endogenous *PRMT1* pre-mRNA, producing both *PRMT1v1* and *v2* isoforms with similar splicing ratios (Fig 31C). Moreover, the *PRMT1* minigene showed a decrease in exon 2 inclusion upon RALY depletion, similar to the endogenous gene (Fig 31D). These results confirm the validity of our test system in breast cancer

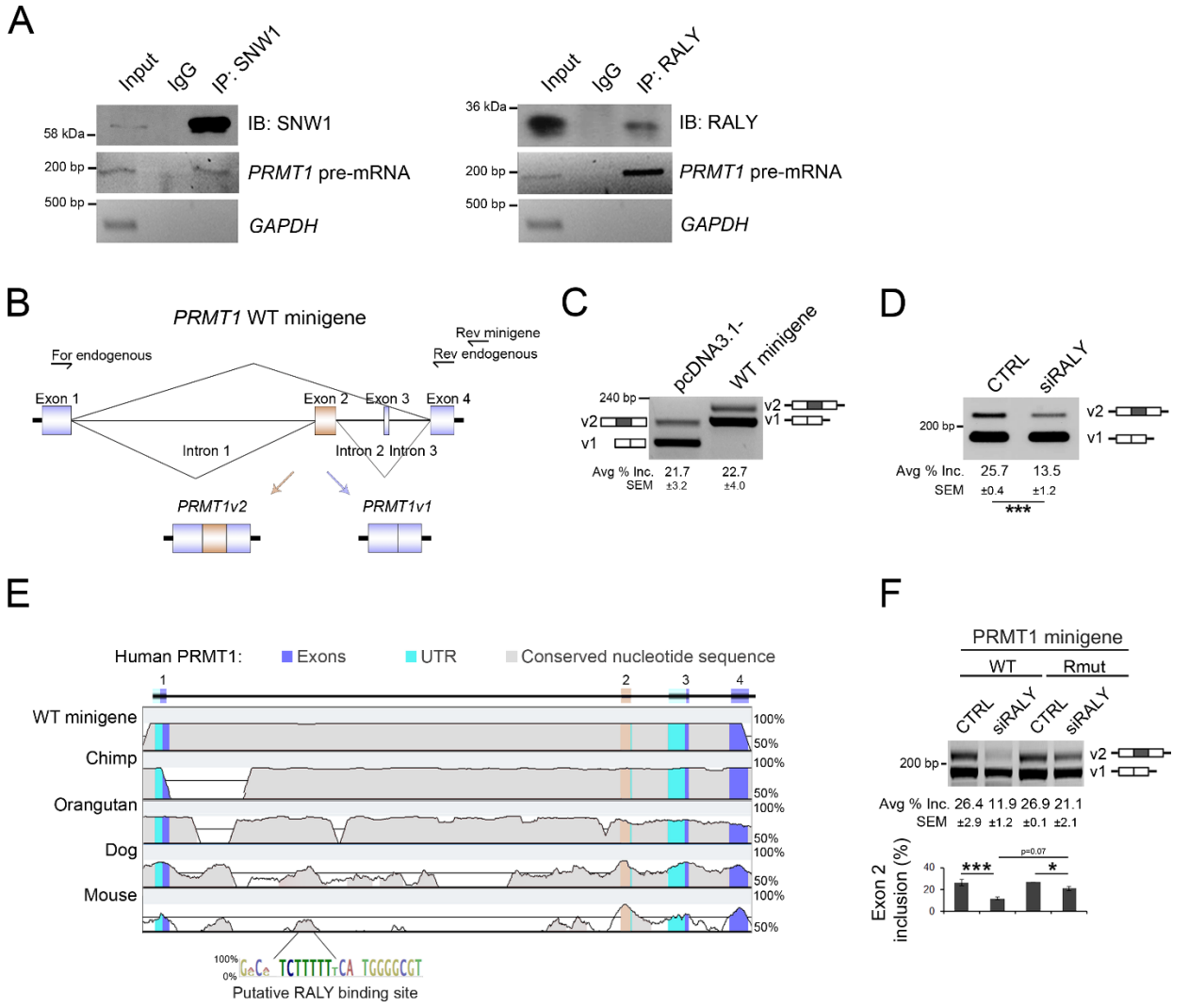

Figure 31. PRMT1 pre-mRNA binding to splicing factors and PRMT1 minigene experiments.

(A) *PRMT1* pre-mRNA binds to splicing factors SNW1 and RALY. Representative figure of the RNA immunoprecipitation (RIP) approach to investigate splicing protein binding to *PRMT1* pre-mRNA. Primers located in intron 2 of the *PRMT1* pre-mRNA were used as shown. RNA was DNase-treated prior to RT-PCR. IP of splicing factors was assessed by western blot. *PRMT1* pre-mRNA bound to SNW1 and RALY was assessed by RT-PCR and *GAPDH* was used as a control. (B) *PRMT1* minigene diagram as described in detail in text. Black boxes next to exons 1 and 4

represent minigene-specific primer sets that were designed to only amplify PRMT1 minigenes (C) *PRMT1* exon 2 inclusion splicing pattern from endogenous *PRMT1* mRNA and Wild-Type (WT) minigene. 0.25 µg of WT minigene was transfected and cells were harvested 24 hours later (D) *PRMT1* WT minigene exon 2 splicing patterns in CTRL (siScramble) or siRALY siRNA transfection. MCF7 cells were first transfected with 1µg of each siRNA and minigenes were transfected 24 hours after. Following another 24 hours, cells were harvested. RT-PCR was used to detect and analyses *PRMT1v1* and *PRMT1v2* spliced isoforms. (E) VISTA alignment plot of PRMT1 minigene. Putative RALY binding site shown here as identified by RBPmap (described in text). (F) *PRMT1* exon 2 inclusion splicing pattern from endogenous *PRMT1* mRNA and WT or RALYmutant (Rmut) minigene with putative RALY binding site deleted. *PRMT1* WT or Rmut minigene exon 2 splicing patterns in CTRL (siScramble) or siRALY siRNA transfection. MCF7 cells were first transfected with 1µg of each siRNA and minigenes were transfected 24 hours after. Following another 24 hours, cells were harvested. RT-PCR was used to detect and analyses *PRMT1v1* and *PRMT1v2* spliced isoforms. Average percent exon 2 inclusion was determined as described in formula (1) in materials and methods. Data are means +/- SEM ($n \geq 3$) * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

cells. With the minigene established, we next used it to identify segments within intronic regions that may contribute to exon 2 splicing regulation. A VISTA alignment plot identified regions of high conservation within the introns surrounding exon 2, comparing across five mammalian species (Fig 31E). Bioinformatics analysis of these conserved regions with RBPmap, a tool that identifies potential binding sites of numerous RBPs (Paz *et al.*, 2014; Ray *et al.*, 2013), revealed a putative RALY binding site, TCTTTTT, in the intron upstream of exon 2, this being within intron 1 (Fig 31E). We hypothesized that this site may be required for RALY-regulated splicing of exon 2. To test this, we generated a *PRMT1* minigene deletion mutant in which this site was deleted (Rmut) and performed a series of RALY knockdown experiments in MCF7 cells using the WT and Rmut *PRMT1* minigenes. RALY depletion induced a 14.5% decrease in exon 2 inclusion in the WT minigene (Fig 31F). A similar decrease was seen in a second cell line, MDA-MB-231s (Fig 30B). However, this observed effect in the reduction in exon 2 inclusion was almost completely abrogated upon RALY depletion in the Rmut minigene (5.8%) (Fig 31F and Fig 30B). Notably though, the deletion of this putative RALY binding site itself was not sufficient to induce a shift in the exon 2 splicing profile. These results suggest that this putative RALY binding site may act as a *cis*-regulatory element in exon 2 splicing, however other RALY binding sites, in addition to other regulators, are likely to play a role.

We extended our investigation to search for other intronic regions that may harbour elements capable of regulating *PRMT1* exon 2 splicing. We generated a series of minigene deletion mutants by systematically deleting 10 segments (~300 bp) within both the upstream and downstream introns flanking exon 2 (Fig 32A), an approach we felt generated an unbiased set of deletion mutants for examination. Minigenes were transiently transfected into MCF7 cells and several regions were identified that, when deleted, appeared to induce altered *PRMT1* exon 2

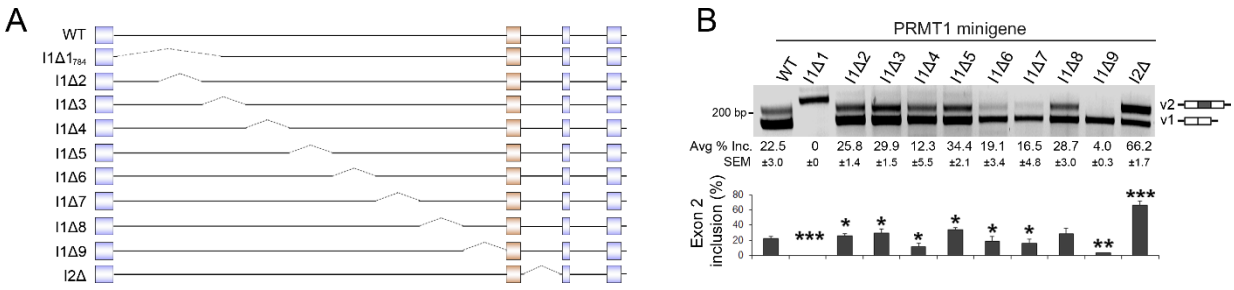

Figure 32. PRMT1 minigene deletion mutants.

PRMT1 intronic deletions were generated through systematic deletion of 300 bp segments within intron 1 and intron 2. Each deletion is represented as a segment number (1-9) with a Δ symbol to reflect deleted portion. (B) PRMT1 deletion mutant minigenes are transfected (0.25 µg) into MCF7s and expressed for 24 hours, at which time the cells were harvested. RT-PCR was used to detect and analyses *PRMT1v1* and *PRMT1v2* spliced isoforms. Average percent exon 2 inclusion was determined as described in formula (1) in materials and methods. Data are means +/- SEM ($n \geq 3$) * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

splicing patterns, as compared to the WT minigene (Fig 32B). Thus, these regions could contain Intronic Splicing Enhancers (ISEs) or Intronic Splicing Silencers (ISSs), as the deletion of these segments caused either exon 2 skipping or inclusion, respectively. The deletion of a segment beginning at the 5'Splice Site (SS) of intron 1 resulted in a complete loss both the *PRMT1v1* and *v2* spliced isoforms, likely due to the complete deletion of the intron 1 5'SS. Potential ISEs were identified in deleted segments 4, 6, 7 and 9, whereas potential ISSs were identified in deleted segments 3, 5, 8, and the deletion of intron 2 (Fig 32B). Importantly, there were deletions which did not affect exon 2 splicing, suggesting that the changes observed in inclusion of exon 2 upon specific segment deletions was not simply a consequence of shortening of intron 1 length (~300 bp less). Taken together, these results have identified several highly conserved intronic segments, that when deleted, appear to influence the splicing of *PRMT1* exon 2, consisted with our hypothesis that additional cis- and trans-regulators beyond RALY will likely contribute to PRMT1 alternative splicing. Those will need to be explored further in future mechanistic studies.

SNW1 and RALY have altered expression in breast cancer cell lines

The identification of SNW1 and RALY as regulators of *PRMT1* exon 2 alternative splicing in MCF7 cells, led us to examine the expression of these factors in normal and other breast cancer cell lines where we have previously observed distinct profiles of exon 2 splicing(Baldwin *et al.*, 2012; Goulet *et al.*, 2007). RT-qPCR and Western blotting analysis revealed altered expression at both the mRNA and protein levels for SNW1 and RALY in three breast cancer cell lines, Hs578T, MDA-MB-231, and MCF7, as compared to the non-tumorigenic breast cell line, MCF10A. Compared to MCF10A, *SNW1* mRNA, and protein levels were significantly decreased, by $\geq$64% and $\geq$76%, respectively, in all the breast cancer cell lines examined (Fig 33A and B). In contrast, *RALY* mRNA and protein

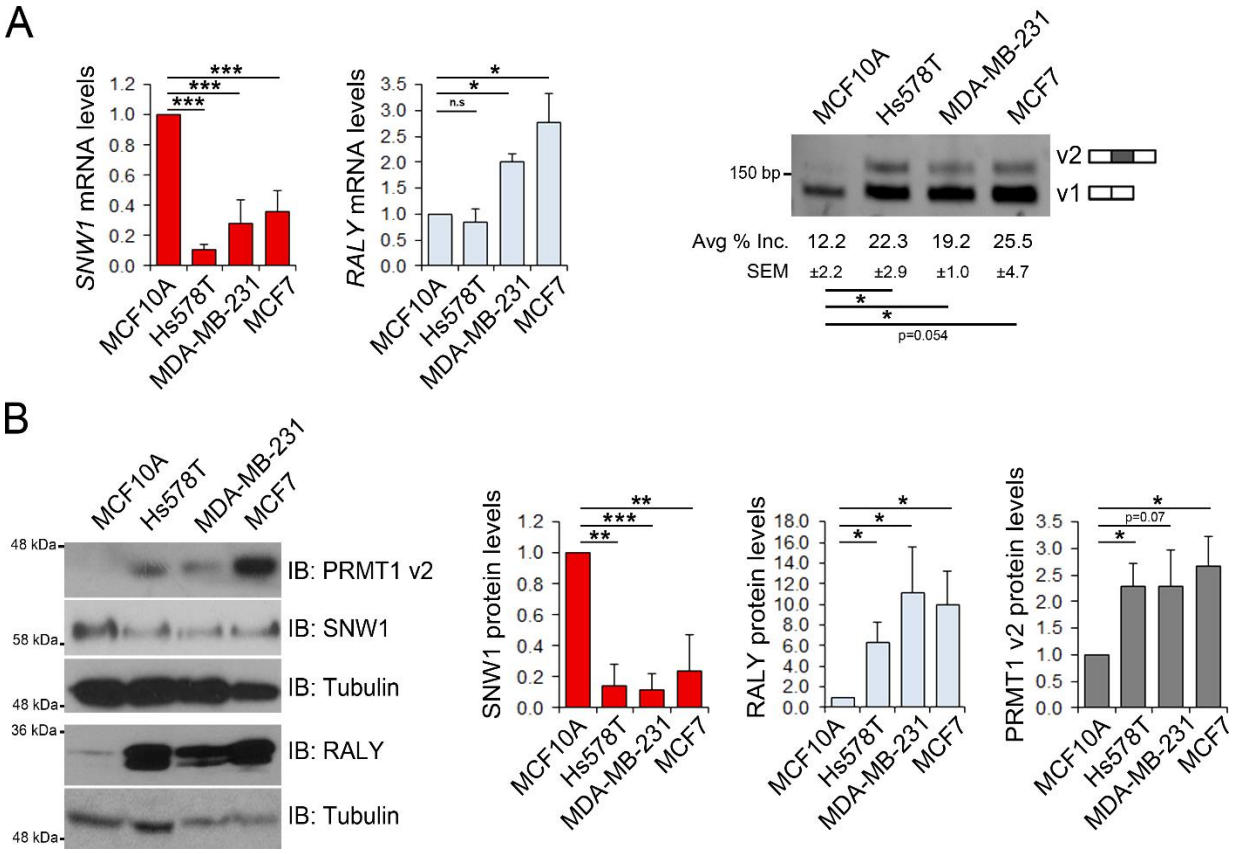

Figure 33. Endogenous levels of splicing factors in normal and breast cancer cell lines.

RNA and protein lysates were collected from normal breast cell lines (MCF10A), and three breast cancer cell lines (Hs578T, MDA-MB-231, MCF7). (A) RT-qPCR was used to measure *SNW1* and *RALY* mRNA expression levels and levels were normalized to 18s RNA. RT-PCR was used to detect and analyses *PRMT1v1* and *PRMT1v2* spliced isoforms. Average percent exon 2 inclusion was determined as described in formula (1) in materials and methods. (B) Quantification of protein expression levels were analyzed by western blotting and normalized to Tubulin. Data are means +/- SEM (n≥3) * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

levels were significantly increased by ~140% and ≥520% respectively, in the majority of the breast cancer cell lines compared to MCF10A (Fig 33A and B). One exception was seen in Hs578Ts, where *RALY* mRNA levels appeared unchanged when compared to MCF10A (Fig 33A). This result may suggest that perhaps the observed increase in RALY protein and not mRNA in this cell line is due to regulation of RALY mRNA translation or protein stability. Quantification of *PRMT1* exon 2 splicing pattern revealed an ~10.1% increase in the inclusion of exon 2 in all breast cancer cell lines (Fig 33A). PRMT1v2 protein levels were also increased by ~140% upregulation in all breast cancer cell lines, compared to MCF10A (Fig 33B). This upregulation of both *PRMT1v2* mRNA and protein levels in breast cancer cell lines are consistent with previous reports. Taken together, these results provide the first evidence to support the hypothesis that altered expression of SNW1 and RALY, at both the mRNA and protein levels, may be responsible for the observed aberrant splicing profile of *PRMT1* exon 2 in breast cancer cell lines.

Depletion of RALY significantly lowers breast cancer cell invasive potential

Previously, we showed that the depletion of PRMT1v2 levels significantly decreased both cancer cell survival and cell invasion of the aggressive breast cancer cell line, MDA-MB-231s(Baldwin *et al.*, 2012). Based on this data, in conjunction with our novel findings presented here, we performed a proof-of-concept experiment wherein we modulated RALY levels in order to modify *PRMT1* exon 2 alternative splicing, and examined the effect on the invasive potential of a highly aggressive breast cancer cell line MDA-MB-231. The knockdown of RALY caused a significant (15.6%) decrease in the number of cells that migrated through the Transwell chamber as compared to CTRL conditions (Fig 34A). Next, we assessed invasive potential of MDA-MB-

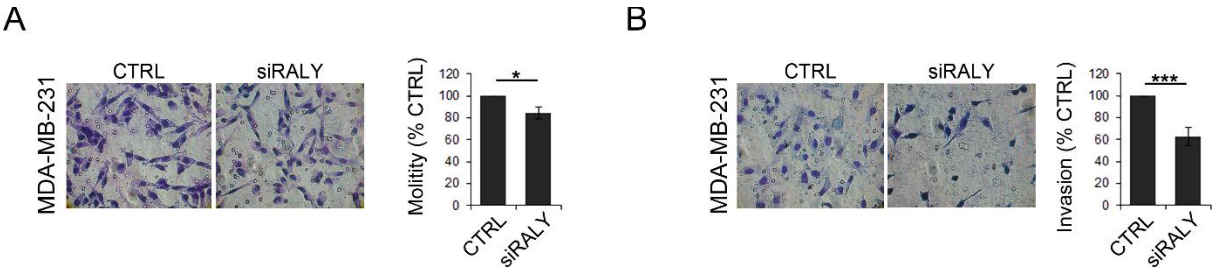

Figure 34. RALY levels affect motility and invasiveness of breast cancer cell lines.

(A and B) A scramble siRNA (CTRL) or an siRNA against RALY were transfected into MDA-MB-231 cell lines and assays were carried out as described in M.M and in ref (Baldwin *et al.*, 2012). Data are means +/- SEM (n=3) * $p<0.05$, ** $p<0.01$.

231 cells after knockdown of RALY using Transwell chambers coated with Matrigel. MDA-MB-231 CTRL cells effectively invaded through these chambers consistent with our previous findings (Baldwin *et al.*, 2012). Upon depletion of RALY, a significant reduction (37.5%) in the number of cells that invaded through the Matrigel-coated Transwell chamber was observed (Fig 34B), although to a lesser extent than previously observed upon reduction of PRMT1v2 alone (Baldwin *et al.*, 2012). The results from this *in vitro* assay, demonstrate that depletion of RALY is sufficient to modify *PRMT1* exon 2 alternative splicing, and in turn, significant affect the motility and invasive potential of breast cancer cells.

SNW1 and RALY have altered expression in breast cancer patients

Lastly, we extended our analysis of SNW1 and RALY expression in breast cancer from cell lines to breast cancer tissue in patients. First, the expression of these splicing factors was investigated using the Human Protein Atlas (HPA) portal (v.15), a web-based resource presented as a tissue-based map of the human proteome representing all the major human tissues and organs corresponding to an analysis of >16,975 protein-encoding genes (Uhlén *et al.*, 2015). This resource provides an unbiased approach to assessing the expression of splicing factors in normal breast tissues, (average of the annotated protein expression in adipocytes, glandular cells, and myoepithelial cells), and breast cancer tissues via IHC staining (Uhlén *et al.*, 2015). Analysis of seven ductal carcinoma breast tumour IHC staining for SNW1 showed that 71% of samples had a low-med SNW1 expression, as compared to high SNW1 levels reported in normal breast tissue (Fig 35A – top panels). Analysis of seven ductal carcinoma breast tumour IHC staining for RALY, using two distinct RALY antibodies, showed 71% of tumour samples with high RALY expression levels, as compared to the medium level expression observed in normal breast tissue (Fig 35A – bottom panels). Analysis of the IHC staining of all breast tumour samples available

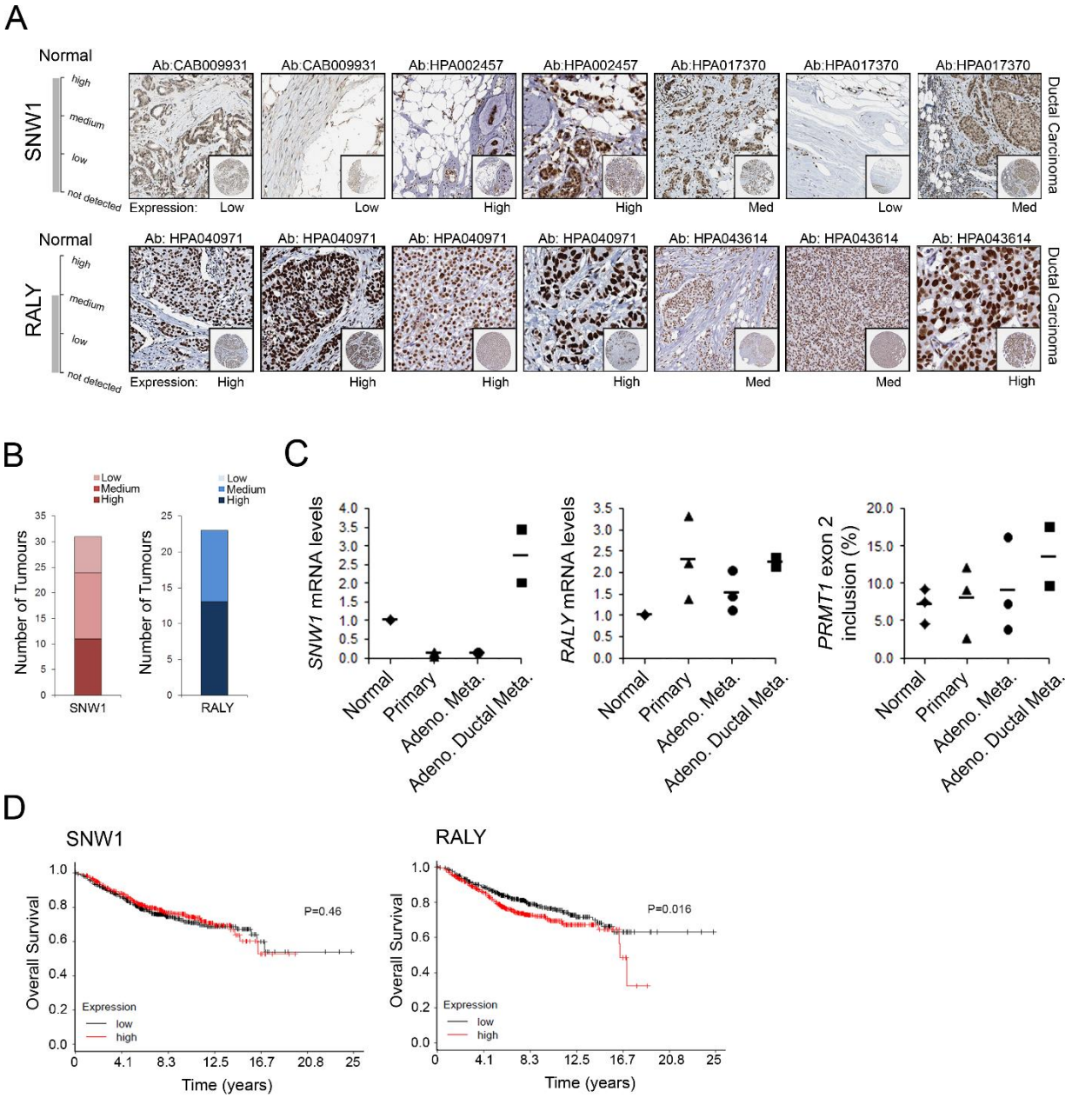

Figure 35. Splicing factor levels in breast cancer patient tumours.

(A) Immunohistochemistry for splicing factors obtained from the Human Protein Atlas. Specific tumour types and antibodies are described in detail in the results section. (B) SNW1 and RALY staining expression from HPA of all breast tumour samples in the database. (C) RT-qPCR was used to measure *SNW1* and *RALY* mRNA expression levels in breast cancer patient tumors obtained from Origene (described in detail in Materials and Methods). mRNA levels were

normalized to 18s RNA. RT-PCR was used to detect and analyses *PRMT1v1* and *PRMT1v2* spliced isoforms. Percent exon 2 inclusion was determined as described in formula (1) in materials and methods. (D) The genes for each splicing factor were inputted into the km plotter to analyze the prognostic value of each gene. To determine this value patient samples, in this case, n=1117, the km plotter first divides the patient samples into two groups, for example, high and low expression values, based on the median expressions of the specific splicing factor gene searched. Next, the two patient cohorts, including a 95% confidence interval and log rank P value, are compared and presented in the K-M survival plots shown here.

for these splicing factors in the HPA revealed that the majority of breast tumours had med-low SNW1 levels as compared to the high levels reported in normal breast tissue. Strikingly, a majority of tumour samples showed high RALY expression as compared to the med expression seen in normal breast tissue provided by the HPA (Fig 35B). These findings strongly support the notion that the misregulation of these splicing factors in breast cancer cells and patient tissues, could lead to the overexpression of the PRMT1v2 isoform previously reported in breast cancer cell lines (Goulet *et al.*, 2007).

We next investigated *SNW1* and *RALY* levels, in RNA isolated from 11 breast tissue samples, three within normal limits, and eight breast tumours. RT-qPCR analysis revealed *SNW1* mRNA levels were decreased by at least 90%, whereas *RALY* mRNA levels were increased, by 130%, in primary breast tumours as compared to normal breast tissue (Fig 35C). Similarly, metastatic breast tumours showed decreased *SNW1* mRNA (~86.5%) and increased *RALY* (~50%) mRNA levels as compared to normal breast tissue (Fig 35C). An exception to this trend was observed in two metastatic adenocarcinoma ductal tumour samples where an ~170% increase in *SNW1* mRNA was observed. Although the number of metastatic adenocarcinoma ductal tumour samples (2) is lower compared to our other sample types, this allowed us to correlate the splicing factor levels with *PRMT1* exon 2 inclusion in these tumour samples. Consistent with the hypothesis that the altered expression of these splicing factors correlate with *PRMT1* exon 2 splicing, the majority of breast tumour samples showed increased levels of *PRMT1* exon 2 inclusion, as compared to normal controls (5/8) (Fig 35C).

Lastly, we generated K-M plots in order to assess the potential correlation between SNW1 and RALY expression level and overall survival (OS) of breast cancer patients and to examine whether these genes could serve as potential biomarkers for probability of breast cancer patient

survival(Györffy *et al.*, 2010). K-M plots were generated with time (in years), starting at time=0 (time of diagnosis or mastectomy) until the time of death, on the x-axis and the probability of the patient's OS on the y-axis. SNW1 gene expression levels did not show a significant correlation between expression levels and OS (p=0.46) (Fig 35D). Interestingly, the K-M plot for RALY revealed a strong negative correlation (p=0.016) between the OS and the gene expression levels of RALY (Fig 35D). Taken together, this analysis provides additional evidence showing the altered expression of SNW1 and RALY in breast cancer and moreover, that RALY may serve as a useful biomarker when determining breast cancer patient's survival prognostic.

Taken together, this series of experiments demonstrate that key splicing factors of *PRMT1* exon 2 splicing, namely SNW1 and RALY, both have aberrant expression profiles in breast cancer samples correlative with the overexpression of the *PRMT1v2* isoform. Moreover, we show that the modulation of RALY expression alters *PRMT1* exon 2 splicing patterns and, thus, affects the invasive potential of breast cancer cells. Taken together, these findings in conjunction with our previous work, demonstrate the potential in specifically targeting *PRMT1* exon 2 alternative splicing as a therapeutic strategy for decreasing the metastatic potential of breast cancer cells.

4.5 Discussion

Identification of elements that regulate *PRMT1* exon 2 splicing

In the present study we identified several splicing factors, including more specifically SNW1 and RALY, that regulate the alternative splicing of the *PRMT1*v2 isoform, a variant we previously showed to be overexpressed in breast cancer cells and promote the survival and invasion of breast cancer cells (Baldwin *et al.*, 2012). This is the first identification of a splicing factor that can regulate *PRMT1* exon 2 splicing patterns. Moreover, to our knowledge, this is the first specific splicing event regulated by the modulation of RALY levels.

SNW1 (a.k.a. NcoA62/SKIP), is a conserved, multifunctional protein involved in the transcriptional co-regulatory activity of several transcription factors. Previously, SNW1 inhibition was found to block the splicing of a growth hormone minigene cassette (Zhang *et al.*, 2003a), although the mechanism by which SNW1 regulates splicing is not fully understood. SNW1 has also been found to be present in multiple stages of spliceosome assembly and interacts with several RNA splicing factors including components of the U5 snRNP (Albers *et al.*, 2003; Folk *et al.*, 2004; Jurica and Moore, 2003; Kim *et al.*, 2001; Makarov *et al.*, 2002; Zhang *et al.*, 2003a). RALY, a conserved member of the hnRNP family of RBPs, is expressed throughout development and in most adult tissues (Khrebtukova *et al.*, 1999; Michaud *et al.*, 1993). Although it has been suggested that RALY plays multiple roles in RNA metabolism, i.e. pre-mRNA splicing, its function remains elusive and poorly understood. To our knowledge no RALY-regulated splicing targets have been identified (Jurica and Moore, 2003). RALY's identification as a splicing factor stems from its association with the spliceosomal C complex (Jurica and Moore, 2003) and other prominent splicing factors, such as PABP1, ELAVL1, hnRNP-C, FMRP, and RBFOX1/2, although the RALY misregulation does not appear to influence RBFOX1/2 splicing targets (Sun *et al.*, 2012;

Tenzer *et al.*, 2013). Recent high-resolution mass spectrometry showed RALY in DROSHA RNA processing complexes, with numerous splicing factors, such as hnRNP U, H1, and M (Larsen *et al.*, 2016).

In this study, we uncovered results that are consistent with SNW1 and RALY acting as splicing factors. The *PRMT1* pre-mRNA associated with endogenous SNW1 and RALY protein by immunoprecipitation, however, whether these proteins are binding directly to the *PRMT1* pre-mRNA is still unknown. For RALY, this interaction may be through the conserved RNA recognition motif found in its protein sequence (Khrebtukova *et al.*, 1999; Michaud *et al.*, 1993; Tenzer *et al.*, 2013), although more work must be done to determine whether this domain plays a role in RALY's ability to regulate the association with *PRMT1* pre-mRNA. We identified a putative RALY-binding site located within intron 1 of *PRMT1*. Deletion of this site in our Rmut *PRMT1* minigene resulted in a partial reduction of the skipping of exon 2 upon RALY depletion, as compared to WT. As some degree of RALY-regulated splicing remained in the Rmut, this partial effect suggests there are additional RALY binding sites needed for RALY-regulated exon 2 splicing. It is possible that this site, and other RALY binding sites require additional factors for this regulatory pathway. For instance, hnRNP C2 was also observed to regulate exon 2 levels in our screen. Interestingly, hnRNP C2 was found to associate with RALY in an RNA-dependent manner (Tenzer *et al.*, 2013). It may be informative to examine interactions between RALY and these other splicing proteins to further define the mechanism by which RALY may regulate *PRMT1* exon 2 splicing.

PRMT1 **exon 2 splicing regulators, SNW1 and RALY have altered expression levels in breast cancer**

Recently, several comprehensive reviews have summarized both the well-studied and emerging novel events associated with altered splicing in several cancer types, including breast cancer (Anczuków and Krainer, 2016; Grosso *et al.*, 2008; Silipo *et al.*, 2015). Numerous RBPs are shown to have altered expression levels in cancer leading to significant misregulation and alterations in splicing patterns (Anczuków and Krainer, 2016; Anczuków *et al.*, 2012; Grosso *et al.*, 2008; Karni *et al.*, 2007; Twyffels *et al.*, 2011). Here we present evidence of two more examples of splicing proteins, SNW1 and RALY, that exhibit altered expression patterns in breast cancer. Investigating these altered expression patterns may be of considerable importance, as we hypothesize that the observed misregulation promotes the production of the *PRMT1v2* isoform, as depicted in our working model (Fig 36).

In addition to the work here, several other groups have examined SNW1 and suggested it may play a role in cancer (Chen *et al.*, 2011b; Liu *et al.*, 2014; Olson *et al.*, 2010; Sato *et al.*, 2015; Tolde and Folk, 2011). SNW1 was first identified, through a yeast two-hybrid system, to bind with the avian retrovirus oncogene v-ski protein, Ski, within a region required for Ski's transforming activity (Dahl *et al.*, 1998). Olson *et al*. reported that SNPs found within the SNW1 gene were associated with a risk of breast cancer (Olson *et al.*, 2010). Lui *et al*., reported high expression levels of SNW1 in 37 out of 85 breast carcinoma patient tumours using IHC data and Western blotting analysis (Liu *et al.*, 2014). As this study has reported results that differ from ours (and the low SNW1 expression in normal breast tissue reported in the HPA database (Liu *et al.*, 2014; Uhlén *et al.*, 2015)) it is important to revisit the expression levels of SNW1 with different antibodies and increased sample numbers. Also, taking into account the stratifying expression based on primary vs. metastatic tumour, recurrent tumours, grade, and stage of disease would uncover valuable information with respect to the expression of these splicing

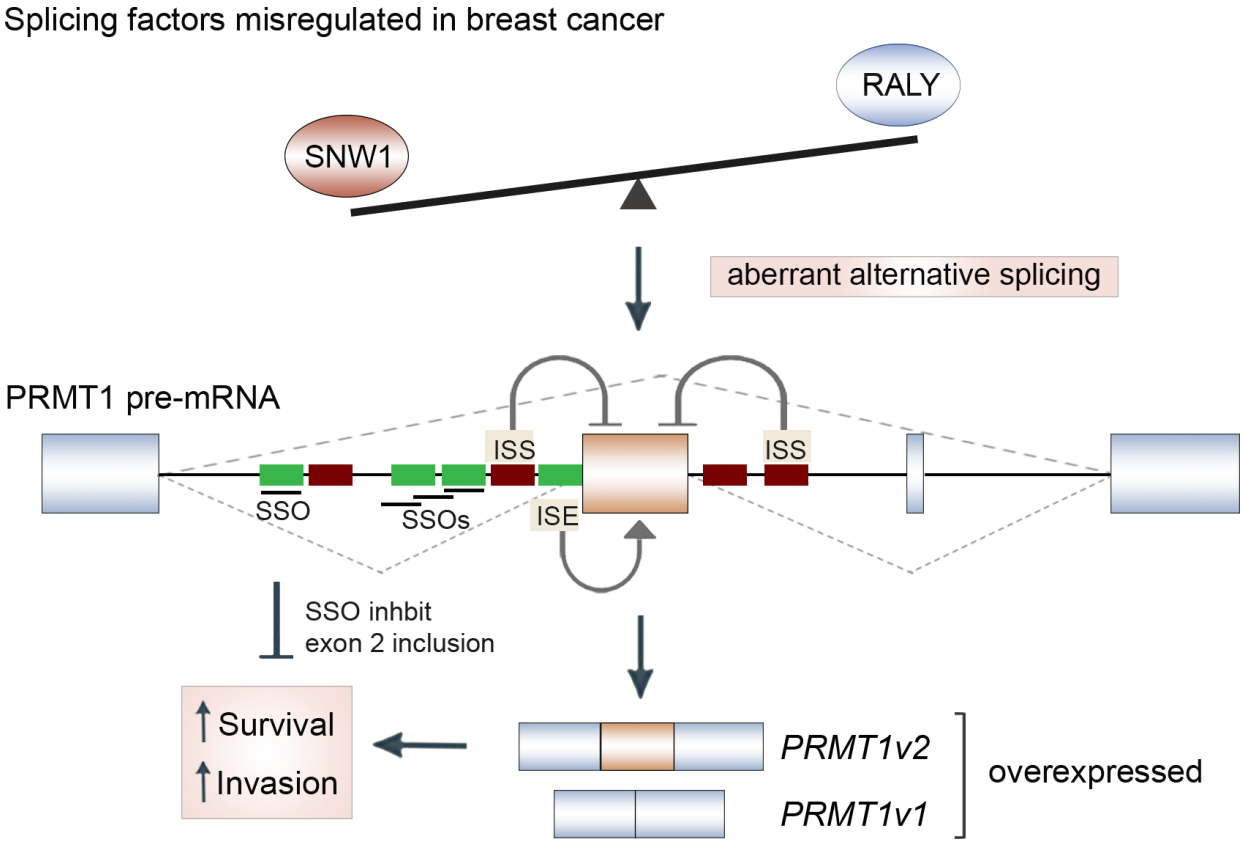

Figure 36. Model showing the effect of the altered expression of splicing factors SNW1 and RALY in breast cancer.

The up- and down-regulation of SNW1 and RALY, respectively, are shown to alter the alternative splicing of PRMT1 exon 2 inclusion and thus, affect survival and invasion of breast cancer cells. Potential SSOs are placed within regions that were found to promote exon 2 inclusion splicing patterns (intronic splicing enhancers). Constitutively or alternatively expressed exons are shown in blue or orange, respectively. Introns are shown as thin, solid black line, with the alternative splicing pattern of exon 2 as dashed lines. ISEs and ISSs are shown in filled in green or dark red boxes, respectively.

factors. These data suggest SNW1 expression is altered in breast cancer, although more work must be completed to determine the degree of alteration and the associated consequence of breast cancer onset and/or disease progression.

Providing a proof-of-concept demonstration, we show that RALY knockdown caused decreased production of the *PRMT1v2* isoform and partial decrease in the invasiveness of the aggressive breast cancer cell line, MDA-MB-231. Whether RALY is influencing the invasive potential of these cells exclusively through the modulation of *PRMT1v2* levels, is unknown at this time. In fact, RALY depletion was not sufficient to induce complete inhibition of invasion, suggesting that modulation of RALY is affecting additional invasion-regulated pathways in this cell line. It is possible that RALY is involved in other cancer pathways, perhaps independently of its roles in the splicing regulation of *PRMT1* exon 2. Tsofack and colleagues, uncovered that RALY was required for resistance to oxaliplatin in a subset of colorectal cancer cells (Tsofack *et al.*, 2011). The identification of RALY-regulated splicing mRNA targets would be an essential step to elucidating the pathways, specifically those relating to cancer biology, in which RALY plays a crucial role.

Investigating altered splicing pathways caused by the changed expression patterns of splicing factors is essential to understanding their contribution to cancer biology. Moreover, the idea of targeting splicing, using Splice-Switching Oligonucleotides (SSO) therapeutics to reverse pro-cancer splicing events has been proposed as a potential avenue in cancer therapies (Bonnal *et al.*, 2012; Kotake *et al.*, 2007; Mercatante *et al.*, 2001; Salton and Misteli, 2016). In our work here, we aimed to uncover splicing factors that regulated the *PRMT1v2* isoform and through proof-of-concept experiments, modulated those factors in order to decrease the invasive potential of breast cancer cells, by targeting the regulation of the alternative splicing of PRMT1 exon 2. We identified

several intronic regions which, upon deletion, resulted in a decreased inclusion of exon 2. As shown in our working model presented in figure 7, future efforts could focus on designing SSOs in order to specifically target these regions to decrease the production of *PRMT1v2* in breast cancer cells. Understanding these splicing pathways to identify potential therapeutic targets is quickly emerging as a powerful tool to help develop anti-cancer treatments.

4.6 Acknowledgments

The authors would like to thank Philippe Thibault, Dr. Alan Morettin, Genevieve Paris, Marie-Lou Lemieux, Christine Peladeau, and Dr. Tara E. Crawford Parks for general discussion and assistance.

Funding

EBC and AD were supported by The University of Ottawa Brain and Mind Research Institute Centre for Neuromuscular Disease—Scholarships in Translational Research Award. The work in the laboratory of JC was supported by The Cancer Research Society. The work in the laboratory of BC was supported by CIHR grant MOP-136948. BC is the Pierre C. Fournier Chair in Functional Genomics. JC was the recipient of a Canada Research Chair (Tier II) in RNA Metabolism funded through CIHR.

Chapter 5 - General Discussion

This thesis is comprised of three manuscripts forming a collection of work that investigated the misregulation of post-transcriptional mechanisms in three human diseases, SMA, DM1, and breast cancer. One general overarching connection between these pathologies is that defects in pre-mRNA splicing pathways can be observed in each case, although the causes of the splicing dysfunction differ significantly in each disease (Fig 37A-C). Importantly though, as was outlined in detail in the introduction of this thesis, the molecular mechanisms responsible for the disease pathologies go beyond just pre-mRNA splicing, and include defects in other post-transcriptional pathways. For instance, our work in Sanchez, Bondy-Chorney *et al*., 2015 discovered that another post-transcription mechanism, NMD, was misregulated in SMA and we propose that this might contribute to the SMA phenotype (Chapter 3). These findings add to work done by many other groups to support the hypothesis that these complex disease pathologies are caused by a combination of numerous misregulated mechanisms, such as defects in NMD, microRNA processing, RAN translation, and general RNP complex dysfunction (including neuronal RNP transport), in addition to pre-mRNA splicing.

Despite the differences underlying the misregulation of post-transcriptional mechanisms between these diseases, it is obvious that the outcome of defective RNA regulation is often detrimental to the cell (Fig 37). This idea should highlight the importance of two things: 1) investigating the emerging role of RNA and post-transcriptional mechanisms in disease; and 2) understanding these mechanisms in order to exploit them for the development of effective and powerful RNA-related therapeutics. The articles presented in this thesis join many others in the continued effort to understand the molecular mechanisms underlying these complex human diseases.

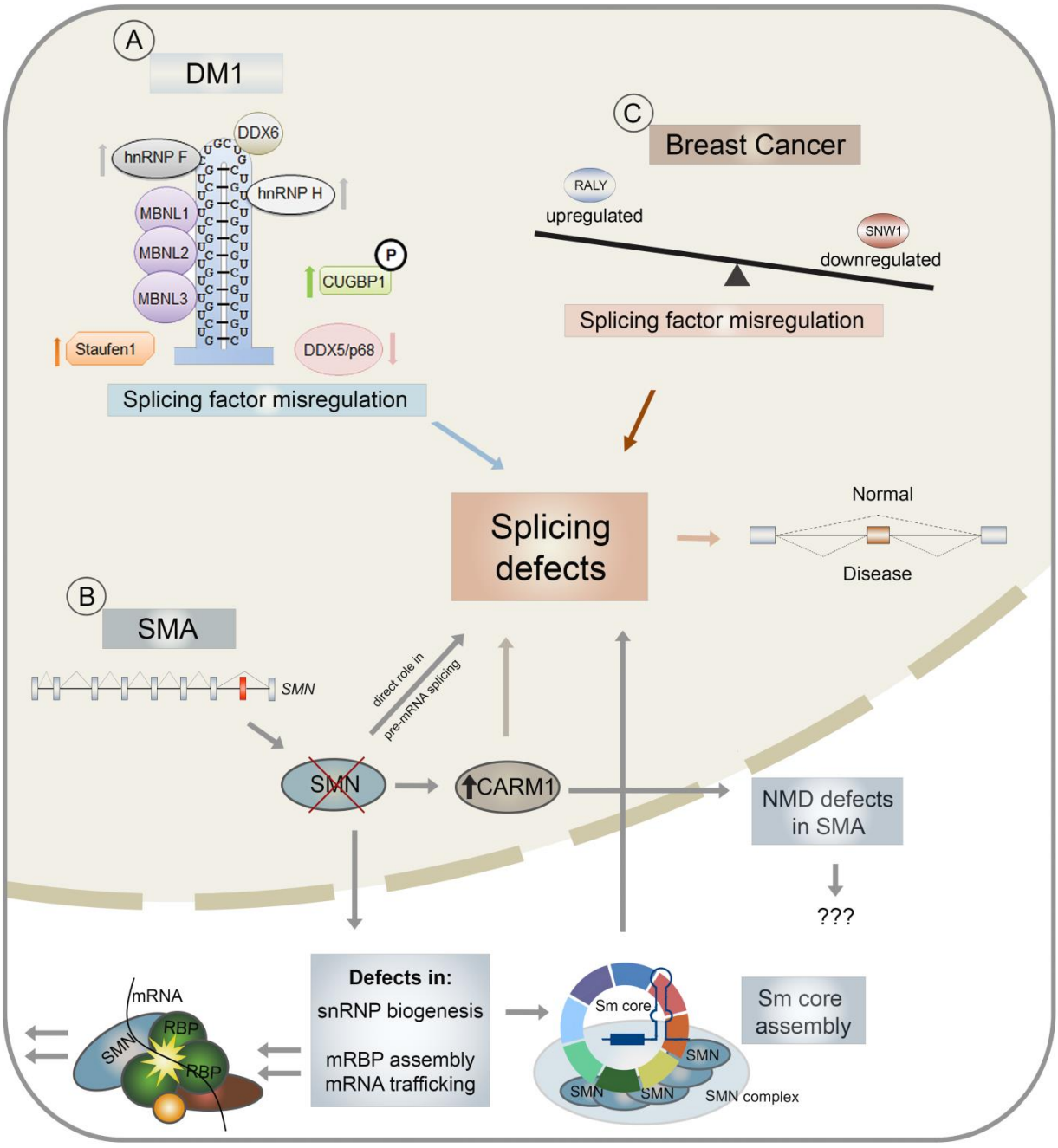

Figure 37. Pre-mRNA splicing defects in DM1, SMA, and breast cancer.

Model of the effects and consequences of the misregulation of RNA in neurogenerative disorders and cancer, with a focus on pre-mRNA splicing defects. (A) DM1 gain-of-function RNA toxic model shows misregulation of RBPs cause missplicing events (middle red box). (B) Several

consequences of SMN depletion on RNA pathways. For example, decrease in SMN protein levels can affect splicing defects through impaired snRNP biogenesis, defective mRNA localization and trafficking, and from direct involvement in the splicing reaction within the nucleus (gray arrows). Moreover, CARM1 (gray circle) proteins levels are increased as a result of SMN protein decrease can cause misregulation of the NMD pathway in SMA. (C) Misregulation of the protein levels of splicing factors (shown here as changes in expression levels of SNW1 and RALY – blue and red circles) in breast cancer can result in aberrant splicing events to occur ("pro-cancer") (red box).

5.1 Stau1's role in pre-mRNA splicing in DM1

Investigating the proteins that are misregulated in disease can lead to understanding their roles in the pathology and, in some cases reveal novel functions of those proteins. Over the past several decades studies in DM1 have focused primarily on only a few key RBPs in the pathology, such as MBNL1 and CUGBP1, and their roles in pre-mRNA splicing (Miller *et al.*, 2000; Philips *et al.*, 1998; Timchenko *et al.*, 2004). Although these RBPs undoubtedly contribute to many of the pre-mRNA splicing defects observed in DM1, it is important to remember that DM1 mouse models that are generated through the modulation of only one or two RBPs, such as MBNL1 and CUGBP1, are not able to recapitulate all aspects of the pathology, nor are they able to rescue the pathological defects (Kanadia *et al.*, 2003; Suenaga *et al.*, 2012). These findings raise several questions, including: 1) what are the other RBPs that are misregulated in DM1? 2) to what extent do these misregulated RBPs contribute to DM1-assiocated pre-mRNA splicing defects? 3) are the pre-mRNA splicing defects the primary contributor to the disease as often hypothesized? 4) what are the other functions of the misregulated proteins and how could those contribute to the pathology?

Recently, our group and others have carried out work to address some of these questions. Additional other RBPs have now been shown to be misregulated in DM1, including Stau1, hnRNP

H, DDX5, DDX6, TBPH, and BSF, some of which are suggested to contribute to the pathology (Jones *et al.*, 2015; Llamusi *et al.*, 2013; Paul *et al.*, 2006; Pettersson *et al.*, 2014; Ravel-Chapuis *et al.*, 2012). Our work on Stau1 in DM1 led us to discover a novel role for Stau1 as a splicing regulator, as we observed that the overexpression of Stau1 in DM1 conditions modulated the splicing patterns of several pre-mRNAs, including the *INSR* (Ravel-Chapuis *et al.*, 2012). Data supporting this novel function of Stau1 has since been reported by several other groups (Laver *et al.*, 2013; Milev *et al.*, 2012). Furthermore, in Bondy-Chorney *et al*., 2016a (Chapter 2) we investigated the extent to which Stau1 contributes to pre-mRNA splicing regulation, shown by using a high-throughput splicing screen, which demonstrated that modulation of Stau1 levels alters the splicing patterns of numerous ASEs. In future work, it could be highly informative to apply these types of high-throughput splicing technologies to the other newly identified misregulated RBPs in DM1. The results gathered from investigating the targets of misregulated hnRNPs in DM1, for example, could identify splicing defects that were previously undiscovered with only a focus on select RBPs, such as MBNL1. With regard to the role of Stau1 in DM1, it would be highly informative to carry out RNA-sequencing in various tissues of a mouse model in which the levels of Stau1 are modulated, for example in the recently described Stau1-overexpression transgenic mouse (Crawford-Parks *et al*., under revision). The results from this experiment would provide details about Stau1-regulated mRNA targets *in vivo*.

Furthermore, the importance of other tissues that are affected in DM1 is now being recognized and these findings are supporting the notion of DM1 being a multi-systemic disorder. An increased effort should be taken to complete large-scale splicing arrays not only in skeletal muscle of DM1 patients but also other tissues, such as the heart and brain (Charizanis *et al.*, 2012; Freyermuth *et al.*, 2016; Leroy *et al.*, 2006).

As mentioned above it is essential to remember that many of the RBPs misregulated in DM1 serve multiple functions and are involved in numerous RNA-related pathways, in addition to splicing regulation. For instance, MBNL1 was found to regulate the biogenesis of micro-RNA-1, a miRNA that plays important roles in myogenesis and normal heart function (Nasser *et al.*, 2008; Rau *et al.*, 2011). The misregulation of MBNL1 in DM1 has now been associated with the altered processing of micro-RNA-1 and consequently, the increased expression of its targets Gap Junction Protein Alpha 1 (GJA1) and Calcium Voltage-Gated Channel Subunit Alpha1 C (CACNA1C), in DM1 hearts (Rau *et al.*, 2011). Stau1 is also well-recognized for its multi-functional nature, as it plays roles in many cellular pathways including neuronal transport of RNA, translation efficiency, the stability of specific target mRNAs, stress granule assembly, and long-term memory formation in *Drosophila* (Dubnau *et al.*, 2003; Dugré-Brisson *et al.*, 2005; Kim Y K, 2007; Ravel-Chapuis *et al.*, 2012; Sugimoto *et al.*, 2015). Stau1's involvement in these multiple pathways suggests that the aberrant upregulation of Stau1 in DM1 our group reported in 2012, likely contributes to the DM1 pathology in several ways, in addition to its role as a splicing regulator. Indeed, several lines of evidence support this hypothesis. First, similar to MBNL1, Stau1 has also been suggested to be involved in miRNA pathways, as a recent study uncovered the Stau1 RNP complexes contained both RNA silencing elements and numerous miRNAs, including miR-124 (Peredo *et al.*, 2014). It would be informative to examine whether the aberrant upregulation of Stau1 in DM1, contributes to the altered expression profiles of miRNAs in the disease. Second, DM1 patients have been found to have an impaired differentiation program in their skeletal muscle (Amack and Mahadevan, 2004). In 2014, our lab showed that Stau1 negatively regulates myogenesis, thus suggesting that Stau1's role in this pathway may contribute to the impairment observed in DM1 (Ravel-Chapuis *et al.*, 2014). Third, the involvement of Stau1 in stress granule formation and assembly was

reported in 2009 (Thomas *et al.*, 2009). Recent work from our lab strongly suggests that this role may have important consequences in DM1, as we demonstrated that Stau1 impairs stress granule formation in DM1 myoblasts (Ravel-Chapuis *et al.*, 2016). Taken together, these findings support the notion that multi-functional RBPs, such as Stau1, act as disease modifiers in the DM1 pathology by affecting many additional post-transcriptional mechanisms beyond pre-mRNA splicing regulation.

Lastly, in Bondy-Chorney *et al*., 2016a (Chapter 2), we investigated the mechanism behind Stau1's ability to regulate pre-mRNA splicing not only to understand the basic mechanisms underlying this pathway but also to examine whether modulating Stau1 could be a potential therapeutic approach in DM1. As mentioned above, the idea of RBP modulation in DM1 is a promising strategy since the overexpression of Mbnl1 in the HSA^{LR} DM1 mouse model, via a recombinant adeno-associated viral vector, corrected several missplicing events associated with the disease including the observed myotonia (Kanadia *et al.*, 2006). Similarly, our work revealed that the overexpression of Stau1 under DM1 conditions rescued key hallmarks of the DM1 pathology, such as increased export and translation of CUG-expanded mRNA, and the modulation of two key missplicing events, the *INSR* and *CLCN1* (Ravel-Chapuis *et al.*, 2012). However, as was shown in the article Bondy-Chorney *et al*., 2016a, the overexpression of Stau1 as a potential therapy in DM1 may not be feasible since we observed that the Stau1-regulated alternative splicing was affecting ASEs in ways predicted to be both beneficial and detrimental for the pathology. These findings further support the hypothesis that Stau1 acts as a disease modifier in DM1. As was discussed in a follow-up perspective piece to this work, Bondy-Chorney *et al*., 2016b, a possible strategy moving forward, would be to create a balance of the misregulated RBPs in DM1, in order to return these proteins to their basal levels (Bondy-Chorney *et al.*, 2016a).

Perhaps the best therapeutic strategy to treat DM1 is not with the correction or restoration of the individual misregulated RBPs and/or their target pre-mRNAs, but with the specific targeting of the RNA-toxicity at its source, the CUG repeat expansion. As outlined in the introduction of this thesis, this idea has been investigated using ASO-based therapies by researchers out of the Thornton lab (Nakamori *et al.*, 2011; Wheeler *et al.*, 2007) and others (Mulders *et al.*, 2009), with promising results. Furthermore, the potential of additional RNA-silencing techniques, such as siRNAs, has also been examined. The injection of an siRNA directed against CUG repeats resulted in a 70-80% downregulation of the expanded CUG-repeat RNA and a restoration of several DM1-assiocated missplicing events towards wild-type splicing patterns (Sobczak *et al.*, 2013). Although this strategy appears effective, one major caveat of this method is that the siRNAs may have numerous off-target effects, i.e. target other CUG-containing transcripts. Indeed, in a limited survey of endogenous CUG-repeat containing mouse transcripts, Sobczak *et al*. found that although most were not affected, at least one, the *Txlnb* transcript, was significantly reduced upon siRNA treatment (Sobczak *et al.*, 2013). Moreover, only the correction of misregulated MBNL1 localization upon siRNA treatment was examined in this work. It would be very interesting to investigate whether reduction of the CUG-expanded RNA effectively corrects all RBP misregulation, such as the aberrant upregulation of Stau1. This would be of particular interest to our work, as we do not yet know what causes the upregulation of Stau1 in DM1.

5.2 CARM1's role as an NMD factor and in SMA

Understanding the post-transcriptional mechanisms that are misregulated in complex disorders such as DM1 and SMA is crucial to understand the disease. In Sanchez, Bondy-Chorney *et al*., 2015 (Chapter 3), we uncovered a novel misregulated RNA pathway in SMA. In this work, we investigated the impact of CARM1 upregulation in SMA and uncovered a novel function of

CARM1 in NMD (Sanchez, Bondy-Chorney *et al*., 2015). Since it appeared that CARM1 was upregulated in SMA and has a role in the regulation of the NMD pathway, we hypothesized that NMD is misregulated in SMA and thus, contributes to the pathology. In this study, we showed that a specific subset of CARM1 dependent NMD targets was indeed misregulated in SMA, therefore, supporting our hypothesis. This was the first study to demonstrate that NMD is misregulated in SMA and we propose that this misregulation contributes to the SMA pathology (Fig 37).

There are numerous avenues in which future work can focus on to further elucidate the findings of Sanchez, Bondy-Chorney *et al*., 2015. For example, we do not know the full extent of the CARM1-assiocated misregulation of the NMD pathway in SMA. Moreover, how widespread are these effects, i.e. what downstream pathways could be affected? In our work presented in this thesis, we used a targeted approach to uncover several mRNAs that were subject to changes in the NMD pathway via CARM1 modulation. This now raises the question of how many other mRNAs are CARM1-assiocated NMD targets? Future work should implement the recently described large-scale NMD microarray technology in order identify the impact of NMD misregulation in SMA, in both SMA control and CARM1 knockdown cells (Huusko *et al.*, 2004). Using this technology, we could begin to get an idea of both the specific mRNAs that are regulated by CARM1-assiocated NMD and, importantly, a more global view of NMD misregulation in SMA. The results obtained from these experiments would also allow us to start identifying the types or classes of mRNAs that are sensitive to CARM1-dependent NMD. Moreover, once identified we could search for the presence of a specific motif, defined through sequence or perhaps secondary structure, that predicts for CARM1 sensitivity.

As discussed in the introduction of this thesis, a very promising treatment for SMA has already emerged with the use of ASOs. This example demonstrates the immense benefits that can come

from understanding missplicing in a disease to such a high degree that we can then exploit the mechanism to develop effective therapies for complex diseases that have no effective therapies. With regard to the work presented in Sanchez, Bondy-Chorney *et al*., 2015 (Chapter 3), it would be interesting to investigate whether upon treatment of the previously mentioned ASO-10-27, the ASO used to promote the inclusion of *SMN2* exon 7 and increase the production of functional SMN, whether the SMA-related NMD defects we observed would be rescued. Presumably, since it increases the SMN levels, a factor we previously showed regulated CARM1 levels, this treatment should also restore CARM1-regulated NMD defects in SMA conditions.

Importantly, although results from clinical trials using ASOs, particularly in SMA treatments appear very positive, it is important to remember that several caveats exist in ASO therapeutics, including the development of effective delivery systems and optimization of the long-term stability of ASO treatments. Similar to other therapeutics, ASOs require extensive and time-consuming troubleshooting, such as the detection of off-target effects. The presence of unexpected off-target effects, i.e. changes in mRNA transcription or in non-targeted splicing events, could reveal significant problems for a promising ASO.

5.3 PRMT1v2 splicing in breast cancer

Elucidating the details of pre-mRNA splicing can be inherently complex, as splicing reactions and pathways contain numerous players that contribute to a wide range of outcomes. Nevertheless, studies aimed at understanding post-transcriptional mechanisms such as pre-mRNA splicing, yield highly relevant findings and raise many questions about the fate of a pre-mRNA after the splicing reaction is complete. For instance, what is the function of this spliced mRNA? Does this mRNA contain PTCs, and if so, is the message appropriately degraded? Is this mRNA recruited for storage? Is it translated into protein? These types of questions are important to understanding the

full consequences of post-transcriptional mechanisms, such as pre-mRNA splicing, and in many cases, they can be difficult to answer.

For instance, to investigate whether a specific alternatively spliced isoform is translated into a functioning protein may require isoform-specific tools. This can be difficult, especially if the splice variant of interest differs from predominantly expressed transcripts by only the inclusion/exclusion of a single exon. Generating an isoform-specific antibody, for example, can be difficult, however, it allows for detection and investigation of a specific alternatively spliced protein product. An example of this can be seen in the PRMT1v2 antibody our lab developed in order to study the protein product produced from the inclusion of *PRMT1* exon 2 (Baldwin *et al.*, 2012). This isoform-specific antibody was generated using a synthetic PRMT1v2 peptide translated from the 54 bp sequence of exon 2 and successfully detects one band corresponding to PRMT1v2 (Baldwin *et al.*, 2012). This antibody allows us to investigate specific features of the endogenous PRMT1v2 protein, such as localization in tumour tissues via immunohistochemical techniques. Additional tools used to study the effects of a specific spliced isoform are custom siRNAs. Using a highly specific siRNA allows us to target only the splicing isoform of interest and to investigate the effects of reducing that alternative event. Using tools such as specific siRNAs and antibodies allowed our group to uncover that *PRMT1* exon 2 inclusion was increased in breast cancer and, furthermore, that it contributed to the survival and invasive potential of breast cancer cells (Baldwin *et al.*, 2012).

Our recent study demonstrates that the overexpression of the *PRMT1v2* isoform plays an important role in breast cancer and supports a common role for PRMTs in cancer. Indeed, several recent high-impact studies, including extensive reviews, have shown the role of PRMTs in cancer, highlighting both the consequences of aberrant expression and the presence of spliced isoforms

that may play critical roles in the disease (Yang and Bedford, 2013; Zhong *et al.*, 2012). The article Bondy-Chorney *et al*., (in preparation, Chapter 4) included here, extends our initial observation that the *PRMT1v2* isoform is overexpressed in breast cancer and explores the splicing mechanisms that promotes exon 2 inclusion in the pathology. To investigate this splicing event, our study uses splicing-specific tools previously developed and novel minigene constructs to complement data obtained from a high-throughput RNAi RT-PCR screen. This type of work not only builds upon our previous studies but also provides key information regarding the details of the PRMT1v2 splicing mechanism into the bigger picture of breast cancer research.

The future directions that should be taken with regard to this work are twofold; 1) investigate the impact of RALY upregulation in breast cancer and, 2) inhibit the production of *PRMT1v2* with ASOs to decrease the invasive potential of breast cancer cells. As the manuscript of Bondy-Chorney *et al*., (in preparation, Chapter 4) is the first to report that not only are RALY mRNA and protein levels altered in breast cancer but, also that the depletion of RALY decreases the invasive potential of breast cancer cells, there is much to be investigated. What is the extent of RALY-regulated pre-mRNA splicing in breast cancer? To answer that question we would first need to identify RALY mRNA targets. High-throughput RNA-sequencing experiments in breast cancer cells, using either RALY IPs or conditions where RALY is depleted or overexpressed, could be used to identify RALY targets.

Concerning inhibiting the production of *PRMT1v2* with ASOs to decrease the invasive potential of breast cancer cells there are several aspects that must be considered. The initial stages of ASO design can provide numerous challenges. For example, testing whether any specific sequence of ASO redirects the splicing pattern of a pre-mRNA may require the use of numerous ASOs placed in a tiled format that span variable regions of the pre-mRNA. This can prove both time-consuming

and costly. Therefore, a mandatory first step that must be taken to determine what regions are required for the splicing regulation of the target exon. Our work in Bondy-Chorney *et al*., in prep, (Chapter 4), demonstrates the preliminary steps that were taken to determine these types of features with regards to *PRMT1* exon 2 splicing. As shown in this article, we uncovered several intronic regions that may harbour sequences that act as *cis*-regulatory elements to control the inclusion of *PRMT1* exon 2. Future experimental work will include designing ASOs that target these key sections and investigate which ASOs yield the highest amount of exon 2 skipping and effect on the invasive potential of breast cancer cells. The work done in this article is an example of the initial steps that need to be taken in designing and using RNA-based therapeutics, like ASOs, in disease treatment. Furthermore, this manuscript provided the important proof-of-concept principle experiments that demonstrated that the modulation of *PRMT1v2* pre-mRNA splicing regulation impacted on PRMT1's downstream functions, specifically regulation of the metastatic potential in aggressive breast cancer cells. It is this proof-of-concept principle, which demonstrates the potential of developing ASOs to target this specific pre-mRNA splicing event in breast cancer.

5.4 The important role of misregulation of post-transcriptional mechanisms in disease

Although within the last two decades the role of pre-mRNA splicing and other post-transcriptional mechanisms have emerged as crucial players in disease pathologies, the influence or impact of these RNA-related pathways are not always considered. Specifically, as shown by the articles included in this thesis, defects in pre-mRNA splicing is a common disease-causing mechanism in numerous human diseases, with a high prevalence in neurodegenerative disorders and various cancers. As recent high-throughput deep sequencing studies show, the extent of alternative splicing is much greater than previously predicted (Pan *et al.*, 2008; Wang *et al.*, 2008). Moreover, it has

been hypothesized that over half of reported disease-causing mutations disrupt pre-mRNA splicing (López-Bigas *et al.,* 2005).

The impact of alternative splicing, both in normal biological functions and in disease, makes it somewhat surprising that in some work this critical post-transcriptional regulation step can be overlooked. For example, there is an overwhelming amount of data now available from online datasets that are related to disease (Barrett *et al.,* 2007), however several important considerations must be addressed when using them. For example, the majority of these are transcriptional profiling data that do not take into account isoforms generated by alternative splicing. Moreover, much of the transcriptional profiling data may not represent changes at the protein level, since transcript expression is not always correlated to protein expression (Gandin *et al.*, 2014; Kendrick, 2014). Future analyses should consider all isoforms, major and minor, produced by alternative splicing when investigating the expression changes in a transcript. At the very least, the potential variety and complexity of isoforms that may arise from the transcript must be considered. Not discussing alternative splicing can cause important results to be overlooked. For instance, it is not common to pay close attention to potential protein isoforms that are detected by a specific antibody. It may be that changes in banding patterns, i.e. presence or absence of additional protein bands on a Western blot, that are dismissed as background actually represent changes in specific splicing patterns which could have important biological relevance.

Numerous additional aspects should also be considered when investigating post-transcriptional mechanisms such as pre-mRNA splicing include cell type, tissue type, disease state, and even the individual organism itself. In 2008, Wang *et al*. used deep sequencing to investigate alternative splicing in the cerebellum of six individuals and found that >30% of ASEs differed between individuals (Wang *et al.*, 2008). This variability was thought to occur because of the different

splicing environments within each person, i.e. different *cis*- and *trans*-acting regulatory elements (Wang *et al.,* 2008). These individual splicing environments may prove to be highly significant when designing personalized therapeutics, as these differences could impact a specific person's disease severity, susceptibility, and their responses to treatments.

Work presented in Bondy-Chorney *et al*. (in preparation, Chapter 4) and Bondy-Chorney *et al*., 2016a (Chapter 2), show the necessity of using multiple cell lines to investigate splicing mechanisms. Between different cancer cell lines, even those reported to be similar in phenotype, a high degree of variability in *trans*-acting splicing factors, such as expression level, can be observed. In 2008, Venables *et al*. knocked down 14 of the major hnRNPs and studied the effect on 56 splicing events in several different cancer cell lines (Venables *et al.*, 2008). From this work, they found the presence of cell-type specific differences in these *trans*-acting factors that could impact the splicing environment of that cell type (Venables *et al.*, 2008). Examples of these differences in *trans*-acting factors are shown here in the expression levels of both RALY and SNW1, among the three different breast cancer cell lines as compared to a normal breast epithelial cells. These types of differences can make it very difficult to predict the effects of a splicing mechanism between different cellular environments. This issue should be considered when we design broad-range therapeutics that target an individual splicing factor or a specific alternative splicing event. On the other hand, the findings from examining sub-type specifics of cancer cells can provide much-needed selectivity for targeted, personalized therapies. Importantly, these differences can also serve as descriptive and highly informative biomarkers in cancer type classification and diagnosis.

Informative biomarkers can help detect cancers at the earliest stage possible with as low of a false-positive rate as possible (Hartwell *et al.,* 2006). In many cancer types early detection, i.e.

localized cancer versus distant disease (metastasis) significantly increases the patient's survival. For example, when detected early the survival rate of patients with lung cancer is 50%, versus the 2% survival rate for those with metastatic disease (Hartwell *et al.*, 2006). The significance of biomarkers in disease is also reflected in the impressive technologies that have emerged in this field. For example, the recent development of a portable multichannel spectrometer and high-throughput optical biosensor system that works with a smartphone, allows for easy quantification of specific protein concentrations of cancer biomarkers in human serum (Wang *et al.*, 2017). Wang *et al*. demonstrated the effectiveness of their system by measuring the protein concentration in human serum of Interleukin-6 (IL-6), a cancer biomarker for several cancer types including lung and breast cancer (Brichory *et al.*, 2001; Knüpfer and Preiß, 2007). However, although markers like IL-6 may serve as a good general cancer marker, it should be recognized that cancer is a highly diverse disease and it is unlikely that a single biomarker will be enough. Moreover, novel biomarkers that are present in specific cancer types and sub-types will be useful to help develop personal therapeutics for individual patients. Nevertheless, this recent study demonstrates the role of biomarkers in cancer diagnostics and research and demonstrate the importance of the continued effort to uncover new biomarkers, such as the splicing regulator RALY we examined in Chapter 4.

Overall, much can be gained by understanding post-transcriptional mechanisms, such as pre-mRNA splicing and NMD, as is shown here in the three articles presented in this thesis. Not only can we uncover the details of basic biological regulatory pathways but we can also examine them to identify novel therapeutic avenues. As discussed here, there have been major recent advances in the field of cellular and molecular medicine that show immense promise for use treatment of both neuromuscular disorders and cancers. The three articles included in this thesis describe

several key findings including mechanistic insights into Stau1's involvement in splicing and its involvement in the DM1 pathology, the novel role of CARM1 in the NMD, a pathway found to be misregulated in SMA, and finally the identification of key regulatory factors that control the alternative splicing of *PRMT1v2*, a player in breast cancer metastasis. Taken together, this work contributes greatly to the emerging field of post-transcriptional mechanisms in human disease and uncovers several potential targets that may be used in disease treatment.

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7.0 Appendix A.

Splicing Protein in Disease

Splicing Protein	Disease	Type of alteration	Related splicing defects	Selected References
CUBGP1	DM1/DM2	Upregulation due to increased stability	*INSR*	(Pettersson *et al.*, 2015; Savkur *et al.*, 2001; Timchenko *et al.*, 1996)
DDX5/ RNA helicase p68	DM1	Misregulated	*TNNT2*	(Jones *et al.*, 2015; Paul *et al.*, 2011)
DDX6	DM1	Misregulated – due to sequestration	*INSR*	(Pettersson *et al.*, 2014)
DDX17	DM1	Upregulated	*?*	(Paul *et al.*, 2011)
DDX9	DM1	Upregulated	*?*	(Paul *et al.*, 2011)
ESPR1 (RBM35A)	Cancer – multiple types including breast	Upregulated and downregulated	*CD44*, *FGFR2*	(Warzecha *et al.*, 2009)
hnRNPA1	Cancer – multiple types including breast, lung, and colon	Upregulated	*CD44*	(Loh *et al.*, 2015)
hnRNPA2/B1	Cancer – glioblastoma, breast, lung colon	Upregulated	*BIN1*, *WWOX*, *MST1r*, *CASP9*, *INSR*	(Golan-Gerstl *et al.*, 2011)
hnRNPE2	Cancer - Oral	Downregulated	*?*	(Roychoudhury *et al.*, 2007)
hnRNPF	Cancer - colon	Upregulated	*?*	(Balasubramani *et al.*, 2006)
	DM1	Upregulated	*?*	(Paul *et al.*, 2011)
hnRNP H	DM1	Increased	*INSR*	(Paul *et al.*, 2011)

	Cancer – glioblastoma	Upregulation	*MADD, MST1R*	(LeFave *et al.*, 2011)
hnRNPK	Cancer – Acute myeloid leukemia	Downregulated And upregulated	*CFLAR, VEGF*	(Gao *et al.*, 2013)
	DM1	Upregulated	*?*	(Paul *et al.*, 2011)
hnRNPL	Cancer – Oesophageal	Upregulated	*?*	(Qi *et al.*, 2008)
hnRNPM	Cancer – Breast	Upregulated	*CD44*	(Xu *et al.*, 2014)
HuD	Cancer – T-cell acute lymphoblastic	Upregulated	*IK*	(Bellavia *et al.*, 2007)
HuR	Cancer – Breast and ovary	Upregulated	*FAS*	(Izquierdo, 2008)
MBNL1	DM1/DM2	Decreased activity due to sequestration	*INSR*, etc	(Mankodi *et al.*, 2001; Miller *et al.*, 2000; Pettersson *et al.*, 2015)
PELP1	Cancer - Breast	Deregulated	*CD44, SRSF12*	(Mann *et al.*, 2014)
PRMT6	Cancer - Breast	?	*CD44, VEGF*	(Dowhan *et al.*, 2012; Harrison *et al.*, 2010)
PRPF31, PRPF8, and PRPF3	Retinitis Pigmentosa (RP)	Mutations in the autosomal dominant RP genes	Formation an assembly of U4/U6.U5 tri-snRNP	(Mordes *et al.*, 2006)
PTB	Cancer – multiple types including breast, and colon	Upregulated	*FGFR1, MRP1*	(He *et al.*, 2004; Jin *et al.*, 2000)
QKI	Cancer – multiple types including prostate and lung adenocarcinoma	Downregulated	*NUMB*	(Zong *et al.*, 2014)
RBFOX1	DM1	Reduced activity as a result of splicing	*Multiple ASEs, tau*	(Klinck *et al.*, 2014)
RBM5	Cancer – lung and prostate	Downregulated	*Caspase-2, FAS*	(Fushimi *et al.*, 2008)
RBM9 (FOX2)	Cancer - breast	Upregulated	Numerous ASEs in breast cancer EMT	(Venables *et al.*, 2009)

RBM10	Cancer – breast	Upregulated	*NUMB*	(Bechara *et al.*, 2013)
RBM17 (SPF45)	Cancer – multiple types including breast, bladder and colon	Upregulated	*FAS*	(Corsini *et al.*, 2007)
RBM20	Dilated cardiomyopathy (DCM)	Loss of function due to mutation	*Titin (N2BA-G)*	(Guo *et al.*, 2012)
Sam68	Cancer - prostate	Upregulated	*?*	(Busa *et al.*, 2007)
	Fragile X–associated tremor/ataxia syndrome	Decreased activity due to sequestration by CGG-repeats	*Bcl-xL, ATP11B*	(Sellier *et al.*, 2010)
SF1	Cancer - Colorectal	Downregulated	*WISP1, FGFR3*	(Shitashige *et al.*, 2007)
SMN	SMA	Downregulation due to mutations/deletions in *SMN1*	Altered snRNP biogenesis	(Zhang *et al.*, 2008)
SRm160 (SRRM1)	Cancer – Stomach and kidney	Upregulated	*CD44*	(Cheng and Sharp, 2006)
	DM1	Upregulated	*?*	(Paul *et al.*, 2011)
	Facioscapulohumeral muscular dystrophy	Downregulated	*Calpain 3*	(Pistoni *et al.*, 2013)
SRPSK1	Cancer – multiple types including breast, pancreas and colon	Upregulated	*MAP2K2*	(Hayes *et al.*, 2006)
SRSF1	Cancer – multiple types including breast, lung, and colon	Upregulated	*MKNK2, S6K1, BIN1, BCL2L11, MCL1, CASP2, CASP9, CASC4*	(Anczuków and Krainer, 2016)
SRSF2	Cancer - Ovary	Upregulated		(Fischer *et al.*, 2004)
SRSF3	Cancer - multiple types including	Upregulated and downregulation	*HIPK2, PKM2, PKM1*	(Kurokawa *et al.*, 2014; Wang *et al.*, 2012c)

	breast, lung, liver, and colon			
SRSF6	Cancer – multiple types including breast, lung, and colon	Upregulated	*INSR, MKNK2*	(Cohen-Eliav *et al.*, 2013; Karni *et al.*, 2007)
SRSF10	Colorectal	Upregulated	*BCLAF1*	(Zhou *et al.*, 2014)
Stau1	DM1	Upregulated	*INSR, CLC1*	(Ravel-Chapuis *et al.*, 2012)
TBP43	ALS	Loss of function - Nuclear depletion due to aggregation	Changes in splicing patterns of *TDP-43* targets	(Yang *et al.*, 2014)
TBX3	Ulnar–mammary syndrome in humans	Disrupted function due to mutations in *TBX3*	*Dlg3, Nfkb1*	(Franklin *et al.*, 2014)
TRA2β	Cancer – Breast, Cervical, Ovarian, Colon	Upregulated	*CD44*	(Best *et al.*, 2013; Watermann *et al.*, 2006)
U170K	Alzheimer disease	Aggregrate formation	Accumulation of unspliced precursor RNAs	(Bai *et al.*, 2013)
U2AF35	Cancer - Pancreas	Downregulated	*CCK-B*	(Ding *et al.*)
YB-1	Cancer - Ovary	Upregulated	*CD44*	(Fischer *et al.*, 2004)

ASE: Alternative Splicing Events

8.0 Appendix B.

This table can be found at:

http://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1005827

9.0 Appendix C.

This table can be found at:

https://www.dropbox.com/s/hxzkhxwg8w1uev5/Supplemental%20Table%201.%20Sherbrooke%20PRMT1v2v1%20splicing%20data.xlsx?dl=0

10.0 Appendix D.

Ms. Emma Bondy-Chorney
Correspondence language: English
Date of Birth: 12/31
Canadian Residency Status: Canadian Citizen
Country of Citizenship: Canada

Contact Information

The primary information is denoted by (*)

Language Skills

Language	Read	Write	Speak	Understand	Peer Review
English	Yes	Yes	Yes	Yes	

Degrees

2013/1 (2016/12) Doctorate, Doctorate of Science, Cellular and Molecular Medicine, University of Ottawa
Degree Status: All But Degree

Supervisors: Adonis Skandalis; Cindy Mehlenbacher; Adonis Skandalis; Jocelyn Côté and Bernard Jasmin

2010/4 - 2012/10 Master's Thesis, Masters of Science, Cellular and Molecular Biology, Brock University
Degree Status: Completed

Supervisors: Adonis Skandalis

2006/9 - 2010/5 Bachelor's Honours, Bachelor of Science, Biology, Brock University
Degree Status: Completed

2004/9 - 2006/5 Diploma, Biotechnology Technician, Biotechnology, Mohawk College of Applied Arts & Technology
Degree Status: Completed

Recognitions

2017/1 - 2017/5 Graduate Dean's Scholarship - 3,000 (Canadian dollar)
University of Ottawa
Prize / Award

2015/10 University of Ottawa Cellular and Molecular Medicine/Neuroscience PhD Poster Prize - First place - 250 (Canadian dollar)
University of Ottawa
Prize / Award

2015/5 - 2016/5 Center for Neuromuscular Disease Scholarships in Translational Research (STaR Award) - 10,000 (Canadian dollar)
University of Ottawa
Prize / Award

2013/1 - 2017/1 Admission Scholarship - Doctorate – University of Ottawa - 36,000 (Canadian dollar)
University of Ottawa
Prize / Award

2012/6 Graduated in the top 10% of Class - Brock University Masters of Science
Brock University
Distinction

2012/6	Dr. M.S. Manocha Memorial Prize Brock University Prize / Award
2011/1 - 2011/12	Dean Math & Science Fellowship - 5,000 (Canadian dollar) Brock University Prize / Award
2010/1 - 2010/12	Dean Math & Science Fellowship - 5,000 (Canadian dollar) Brock University Prize / Award
2006/5	Graduated top 3% of class 2006 - Mohawk College Biotechnology Mohawk College of Applied Arts & Technology Distinction
2003/5	Achievement Award - Biotechnology Ancaster High School Prize / Award

User Profile

Fields of Application: Biomedical Aspects of Human Health

Disciplines Trained In: Molecular Biology, Cell Biology

Areas of Research: Neurodegenerative Diseases, Bioinformatics, Biological and Biochemical Mechanisms, Breast Cancer

Research Specialization Keywords: Alternative Splicing, Breast Cancer, Neuromuscular disorders, RNA-binding Proteins, RNA metabolism

Research Disciplines: Molecular Biology, Cell Biology, Microbiology

Employment

2013/1 - 2016/12	Graduate Student University of Ottawa
2010/9 - 2012/12	Teaching Assistant: Biology: A Human Perspective Brock University
2010/9 - 2012/9	Teaching Assistant: Molecular Biology Brock University
2010/5 - 2012/9	Graduate Student Brock University
2011/1 - 2012/1	Cellular and Molecular Biology Journal Club: Co-founder Brock University
2011/1 - 2011/5	Graduate Student Representative - Biology Brock University
2010/10 - 2011/2	Molecular Biology Tutor Private
2005/1 - 2006/5	Student Representative - Biotechnology Mohawk College of Applied Arts & Technology
2004/9 - 2005/5	Student Representative - Biotechnology Mohawk College of Applied Arts & Technology

2000/1 - 2000/6 Co-op Placement - Volunteer
Hamilton General Hospital

Affiliations

The primary affiliation is denoted by (*)

(*) 2013/1 - 2016/12 PhD Candidate, University of Ottawa

Research Funding History

Awarded [n=1]

2013/1 - 2017/1
Principal Applicant

Admission Scholarship-Doctorat

Funding Sources:

2013/1 - 2017/1 University of Ottawa
Total Funding - 36,000 (Canadian dollar)
Funding Competitive?: No

Principal Investigator : Jocelyn Côté

Completed [n=2]

2015/5 - 2016/5
Principal Applicant

The University of Ottawa Centre for Neuromuscular Disease Scholarships in Translational Research Award

Funding Sources:

2015/5 - 2016/5 University of Ottawa
Staufen1's role as a disease modifier in DM1
Total Funding - 10,000 (Canadian dollar)
Funding Competitive?: Yes

2010/9 - 2012/9
Principal Knowledge User

Dean of Math & Science Fellowship - Brock University

Funding Sources:

2010/9 - 2012/9 Brock University
Total Funding - 10,000 (Canadian dollar)
Funding Competitive?: No

Mentoring Activities

2015/5 - 2016/10 Mentor, University of Ottawa
Number of Mentorees: 1
Mentored Onkar Bhanushali, undergraduate student in lab safety, training, and experimental procedure.

2016/1 - 2016/6 Mentor, University of Ottawa
Number of Mentorees: 1
Mentored Marie-Lou Lemieux, undergraduate student in lab safety, training, and experimental procedure

2015/5 - 2015/11	Mentor, University of Ottawa Number of Mentorees: 1 Mentored Camille Tremblay Laganiere, undergraduate student in lab safety, training, and experimental procedure
2011/6 - 2012/4	Mentor, Brock University Number of Mentorees: 1 Mentored Carrie Ess, undergraduate student in lab safety, training, and experimental procedure

Community and Volunteer Activities

2016/6	Ottawa Walk for Muscular Dystrophy Volunteer, Muscular Dystrophy Canada
2015/7	Let's Talk Science: Deep River Science Academy Day Volunteer, Let's Talk Science
2015/5	Ottawa Walk for Muscular Dystrophy Volunteer, Muscular Dystrophy Canada
2011/12	Christmas Hamper Toy Drive, Neighbour to Neighbour Community Services Food Bank
2011/10	Volunteer - Brock University Theatre group, Brock University
2011/5	Food bank: General volunteer, Neighbour to Neighbour Community Services Food Bank
2014/9 - 2015/1	Let's Talk Science Volunteer, Let's Talk Science
2010/9 - 2012/9	Laboratory Student Volunteer, Brock University

International Collaboration Activities

2017/6	ResearcherUnited States Participate in ongoing collaboration with the Krogan lab at UCSF. I will spend time at UCSF to learn the latest Mass Spectrometry techniques as part of this ongoing collaboration.
2017/1	Researcher, United States Participate in a going partnership with BioLegend to develop custom antibodies to study non-histone protein acetylation.

Presentations

1. (2016). Staufen1's role as a splicing factor and a disease modifier in Myotonic Dystrophy Type I. The University of Ottawa Cell Biology: Work In Progress, Ottawa, Canada
   Main Audience: Researcher
   Invited?: Yes
2. (2015). RNA-binding Protein Staufen1 Regulates Multiple Alternative Splicing Events in Myotonic Dystrophy Type 1. University of Ottawa RNA Club 2015, Ottawa, Canada
   Main Audience: Researcher
   Invited?: Yes
3. (2015). RNA-binding protein Staufen1 Regulates Multiple Alternative Splicing Events in Myotonic Dystrophy Type 1. University of Sherbrooke: Riboclub Monthly Session, Sherbrooke, Canada
   Main Audience: Researcher
   Invited?: Yes

Online Resources

1. Bondy-Chorney,E.P.Larus glaucescens DNA polymerase beta (POLB) mRNA, partial cds. (2012).
2. Bondy-Chorney,E.P.Pogona vitticeps DNA polymerase beta (POLB) mRNA, partial cds. (2011).
3. Bondy-Chorney,E.P.Ailurus fulgens DNA polymerase beta (POLB) mRNA, partial cds. (2011).

Conference Publications

1. Bondy-Chorney, E., Crawford, T. E., Ravel-Chapuis, A., Klinck, R.2, Chabot, B., Jasmin, B. J., and Côté, J.(2015). Staufen1 Regulates Multiple Alternative SplicingEvents Through Intronic Alu Elements which Ameliorates or Exacerbates the Myotonic Dystrophy Type I Phenotype. The Third Ottawa International Conference on Neuromuscular Disease and Biology, ,
   Poster
   First Listed Author
   Published, Invited?: Yes
2. Emma Bondy-Chorney and Jocelyn Côté. (2015). Alternative Splicing Regulation of PRMT1 v2 Isoform Decreases Invasiveness of Breast Cancer Cell Lines. RiboClub 2015, ,
   Poster
   First Listed Author
   Published, Invited?: Yes
3. Bondy-Chorney E., Crawford T. E., Ravel-Chapuis A., Jasmin B. J., Cote J. (2014). Thepre-mRNA Splicing Regulation Mechanism of the RNA Binding Protein Staufen 1. The University of Ottawa Graduate Seminar Presentation Day 2014, ,
   Poster
   First Listed Author
   Published, Invited?: Yes
4. Gabriel Sanchez, Emma Bondy-Chorney, Janik Laframboise, Geneviève Paris, Jocelyn Côté. (2014). A Novel Role for the Arginine Methyltransferase CARM1 in Nonsense Mediated Decay: Implications for Spinal Muscular Atrophy. RNA Society Conference, ,
   Abstract
   Co-Author
   Published, Invited?: Yes
5. Emma Bondy-Chorney, Aymeric Ravel-Chapuis, Tara E. Crawford Parks,Bernard J. Jasmin, and Jocelyn Côté. (2013). Staufen1 as a Splicing Modulator: Implications in DM1 Therapy. IDMC-9: International Myotonic Dystrophy Consortium, ,
   Poster
   First Listed Author
   Published, Invited?: Yes
6. Emma Bondy-Chorney and Adonis Skandalis. (2012). Tissue specific alternative splicing of DNA Polymerase beta. Annual Buffalo DNA Replication and Repair Symposium, ,
   Poster
   First Listed Author
   Published, Invited?: Yes
7. Emma Bondy-Chorney and Adonis Skandalis. (2011). Alternative splicing of DNA polymerase beta and the transient receptor potential cation channel subfamily v member 1. Annual Buffalo DNA Replication and Repair Symposium, ,
   Poster
   First Listed Author
   Published, Invited?: Yes