

**Novel Functions for the RNA-binding Protein Staufen1 in Skeletal Muscle Biology
and Disease**

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Abstract

Over the past decade several converging lines of evidence have highlighted the importance of post-transcriptional events in skeletal muscle. This level of regulation is controlled by multi-functional RNA-binding proteins and trans-acting factors. In fact, several RNA-binding proteins are implicated in neuromuscular disorders including myotonic dystrophy type I, spinal muscular atrophy and amyotrophic lateral sclerosis. Therefore, it is necessary to examine the impact of RNA-binding proteins during skeletal muscle development and plasticity in order to understand the consequences linked to their misregulation in disease. Here, we focused on the RNA-binding protein Staufen1, which assumes multiple roles in both skeletal muscle and neurons. We previously demonstrated that Staufen1 is regulated during myogenic differentiation and that its expression is increased in denervated and in myotonic dystrophy type I skeletal muscles. The increased expression of Staufen1 initially appeared beneficial for DM1 since further elevating Staufen1 levels rescued key hallmarks of the disease. However, based on the multi-functional nature of Staufen1, we hypothesized that Staufen1 acts as a disease modifier in DM1. To test this, we investigated the roles of Staufen1 in skeletal muscle biology and their implications for disease.

Our data demonstrated that Staufen1 is required during the early stages of muscle development, however its expression must remain low in postnatal skeletal muscle. Interestingly, the overexpression of Staufen1 impaired myogenesis through the regulation of c-myc translation. Since the function of c-myc in oncogenesis is well described, we investigated the role of Staufen1 in cancer biology. In particular, we determined novel functions of Staufen1 in rhabdomyosarcoma tumorigenesis, thus providing the first direct

evidence for Staufen1's involvement in cancer. Moreover, based on Staufen1's role in myogenic differentiation and in myotonic dystrophy type I, we generated muscle-specific transgenic mice to examine the impact of sustained Staufen1 expression in postnatal skeletal muscle. Staufen1 transgenic mice developed a myopathy characterized by histological and functional abnormalities via atrogene induction and the regulation of *PTEN* mRNAs. In parallel, we further investigated Staufen1-regulated alternative splicing and our data demonstrated that Staufen1 regulates multiple alternative splicing events in normal and myotonic dystrophy type I skeletal muscles, both beneficial and detrimental for the pathology. Collectively, these findings uncover several novel functions of Staufen1 in skeletal muscle biology and highlight Staufen1's role as a disease modifier in DM1.

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List of Abbreviations

aa: Amino-acid

AAV: Adeno-associated virus

ACCN3: Amiloride-sensitive cation channel 3

AChR: Acetylcholine receptors

ACTA1: Human skeletal actin gene

Adf-1: Adh transcription factor 1

ADPGK: ADP-dependent glucokinase

AKT2: Protein kinase Akt-2

ALS: Amyotrophic lateral sclerosis

ANK1: Ankryin 1

APOBEC3C: Apolipoprotein B mRNA editing enzyme catalytic subunit 3C

APP: amyloid beta precursor protein

ARE: AU-rich elements

Arf1: ADP-ribosylation factor 1

ARFRP1: ADP ribosylation factor related protein 1

ARMS: Alveolar rhabdomyosarcoma

ASE: Alternative splicing event

ASO: Antisense oligonucleotide

ATM: Ataxia telangiectasia mutated

AUF1: AU-rich element RNA-binding protein

bHLH: Basic helix-loop-helix

bp: Base-pair

BrdU: bromodeoxyuridine

BSF: Bicoid stability factor; homolog of human LRPPRC

c-myc: proto-oncogene c-myc

Ca²⁺: Calcium

CaMKII: Calcium / calmodulin-dependent kinase II

cAMP: cyclic adenosine monophosphate

CDK4: Cyclin dependent kinase 4

CDS: coding sequence

Chrna1: nicotinic acetylcholine receptor α 1 subunit

cIAP1: cellular inhibitor of apoptosis 1

CLCN1: Chloride voltage-gated channel 1

CLCN2: Chloride voltage-gated channel 2

CLCN6: Chloride voltage-gated channel 6

CMS: Congenital myasthenic syndrome

CNS: Central nervous system

CSA: Cross-sectional Area

CTL: Control

cTNT: Cardiac troponin T

CTX: Cardiotoxin

CUGBP1: CUGBP, ELAV-like family protein 1

CUGexp: CUG-expanded mRNAs

dCREB: Cyclic adenosine monophosphate response element binding

protein

DDX: DEAD-box helicase

Dia: Diaphragm

DM: Myotonic dystrophy

DM1: Myotonic dystrophy type 1

DMD: Duchenne muscular dystrophy

DMPK: Dystrophin myotonic protein kinase

dsRBDs: Double-stranded RNA-binding domains

Dvl: Dishevelled

EDL: Extensor digitorum longus

EJC: Exon junction complex

ENSA: Endosulfine alpha

ERMS: Embryonal rhabdomyosarcoma

ESE: Exonic splicing enhancer

ESS: Exonic splicing silencer

EWS: Ewings sarcoma

FGFR1: Fibroblast growth factor receptor 1

FHL3: Skeletal muscle LIM protein 2

FLI-1: Friend leukemia integration 1

FMRP: Fragile X mental retardation protein

FN1: Fibronectin 1

FOXO1: Forkhead box O1

FSHD: Facioscapulohumeral muscular dystrophy

G6PC3: Glucose 6 phosphatase catalytic subunit 3

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GFP: Green fluorescent protein

GSK3 β : Glycogen synthase kinase β

H&E: Hematoxylin and eosin

HA: Hemagglutinin

HIF1 α : Hypoxia-inducible factor 1 alpha

HMGB1: High-mobility group box 1

hnRNP: Heterogeneous ribonucleoprotein

HSMM: Human skeletal muscle myoblasts

HuR: ELAV like protein 1

IGF2BP1: Insulin-like growth factor 2 mRNA-binding protein 1

IGF2BP2: Insulin-like growth factor 2 mRNA-binding protein 2

INSR: Insulin Receptor

IP: Immunoprecipitation

IRAlus: Inverted repeat Alus

ISE: Intronic splicing enhancer

ISS: Intronic splicing silencer

JAG2: Jagged 2

KSRP: KH-type splicing regulatory protein

LBD3: LIM binding domain 3

LGMD: Limb girdle muscular dystrophy

LRRC23: Leucine rich repeat containing 23

MAFbx: Muscle atrophy F-box protein

MAPK: Mitogen activated protein kinase

MAPT: Microtubule-associated protein tau

MBNL: Muscle-blind like splicing regulator

MCK: Muscle creatine kinase

MDCMD: Merosin-deficient muscular dystrophy

MDM2: MDM2 proto-oncogene

MEF2: Myocyte enhancer factor 2

Mg²⁺: Magnesium

MHC: Myosin heavy chain

MHCemb : Embryonic myosin heavy chain

miRNA: MicroRNA

MLPS: Myxoid liposarcoma

MRF4: Muscle-specific regulatory factor 4

mRNPs: Messenger ribonucleoproteins

MTJ: Myotendinous junction

MuRF1: Muscle-specific RING finger 1 protein

Myf-5: Myogenic factor 5

MyoD: Myogenic differentiation 1

NFIX: Nuclear factor IX

NHP2L1: Non-histone protein 2 like-1

NLS: Nuclear localization signal

NMD: Nonsense-mediated mRNA decay

NMDA: N-methyl-D-aspartate

NMDAR1: NMDA NR1 receptor

NMJ: Neuromuscular junction

NPM: nucleophosmin

NRG1: Neuregulin 1

NUTF2: Nuclear transport factor 2

OGDH: Oxoglutarate dehydrogenase

OPMD: Oculopharyngeal muscular dystrophy

p14ARF: Cyclin-dependent kinase inhibitor 2A

PABPN1: Poly-(A)-binding protein N1

PAX: Paired box

PI: Propidium iodide

PI3K: Phosphoinositide 3-kinase

pre-mRNA: Precursor mRNA

PSI: Percent splicing index

PTEN: Phosphatase tensin homolog

QKI: KH domain, RNA-binding protein

qRT-PCR: Quantitative reverse transcription-polymerase chain reaction

RAN-translation: Non-ATG translation

RBD: RNA-binding domain

RBFOX1: RNA-binding protein, fox 1 homolog 1

RBP: RNA-binding protein

RIP: RNA Immunoprecipitation

RMS: Rhabdomyosarcoma

RNP: Ribonucleoprotein

RyR1: Ryanodine receptor 1

SBS: Staufen1-binding site

SERCA1: sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase 1

SG: Stress granule

SHKBP1: SH3KBP1-binding protein 1

shRNA: Short hairpin RNA

SINEs: Short-interspersed elements

siRNA: Small interfering RNA

SkMC: Skeletal muscle cells

SMA: Spinal muscular atrophy

SMD: Staufen1-mediated mRNA decay

SMN: Survival motor neuron

SMN1: Survival motor neuron 1

Smyd1: SET and MYND domain containing 1

snRNPs: Small nuclear RNPs

SOD1: Superoxide dismutase 1

SOL: Soleus

SRSF1: Serine/arginine-rich splicing factor 1

SS: Splice site

T1/2 Relax: Time to half relaxation

TA: Tibialis Anterior

TBC1D12/13: TBC1 domain family member 12

TBD: Tubulin-binding domain

TBPH: TAR-binding DNA binding protein 43 homolog

TDP-43: TAR DNA-binding protein 43

THRA: Thyroid hormone receptor alpha

TLS/FUS: Translocated in sarcoma/fused in sarcoma

TMA: Tissue microarray

TNNT3: Fast skeletal muscle troponin-T3

TNNT2: Cardiac troponin-T2

TP53: Tumor protein p53

tPEAK: peak twitch force

TTN: Titin

UTR: Untranslated region

VC: Variance Coefficient

WT: Wild type

XBP1: X-box binding protein 1

YB-1: Y-box binding protein 1

α -BTX: α -Bungarotoxin

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Chapter 1: General Introduction

Understanding the pathways involved in the formation of skeletal muscle has been a prominent area of research for several years. Historically, the emphasis has focused on transcriptional events; however, over the past decade converging lines of evidence demonstrate that post-transcriptional regulation is also required for muscle formation and maintenance. RNA-binding proteins (RBPs) and trans-acting RNAs facilitate post-transcriptional regulatory mechanisms in complexes called ribonucleoproteins (RNPs). It has been proposed that eukaryotes contain several hundreds of RBPs in response to the evolutionary pressures of increased post-transcriptional processes and they are ultimately responsible for the fine-tuning of gene expression (Anantharaman et al. 2002; Glisovic et al. 2008). In the series of studies presented here, we will discuss the implications of the RBP Staufen1 in skeletal muscle biology and the consequences for its misregulation in the neuromuscular system.

1.1 Skeletal Muscle Formation

In vertebrates, myogenesis is the process where proliferating myoblasts exit the cell cycle and fuse to form multi-nucleated myotubes and mature myofibers. This process is characterized by the expression of four basic helix-loop-helix (bHLH) transcription factors: myogenic differentiation 1 (MyoD) (Davis et al. 1987; Edmondson & Olson 1989), myogenic factor 5 (Myf-5) (Braun et al. 1989), myogenin (Wright et al. 1989), and muscle-specific regulatory factor 4 (MRF4) (Rhodes & Konieczny 1989; Braun et al. 1990; Miner & Wold 1990). The MRFs form heterodimers with E-proteins and bind to E-box consensus sequences located in the promoters of several muscle-specific genes to regulate muscle differentiation (Cao et al. 2010).

1.1.1 Embryonic Muscle Development

Skeletal muscle is generated from the mesoderm layer of the embryo and is divided into three anatomic regions, the paraxial, intermediate and lateral mesoderm (Bentzinger et al. 2012). During vertebrate embryogenesis, segments called somites are formed by the paraxial mesoderm, which generate the vertebrae, skeletal muscle and dermis (Dequéant & Pourquié 2008). In this context, somite differentiation is dependent on oscillations of temporal gene expression and activated signaling pathways (Cooke & Zeeman 1976; Dequéant & Pourquié 2008; Maroto et al. 2012). The dorsal cells of early somites form the dermomyotome that develop into the skeletal muscles of the body and limbs. This process is highly dependent on molecular signals from neighbouring tissues such as the neural tube, notochord and ectoderm. Muscle precursor cells at the lips of the dermomyotome delaminate to form the myotome (Sasoon et al. 1989; Kiefer & Hauschka 2001; Cinnamon et al. 2001). These cells then differentiate along the myogenic lineage in subsequent waves to form primary and secondary myofibers. During this process, a population of muscle precursor cells also migrate from the ventrolateral lips of the dermomyotome to form the limb bud where MRFs are activated and the cells differentiate to form the limb muscles (summarized in **Figure 1**).

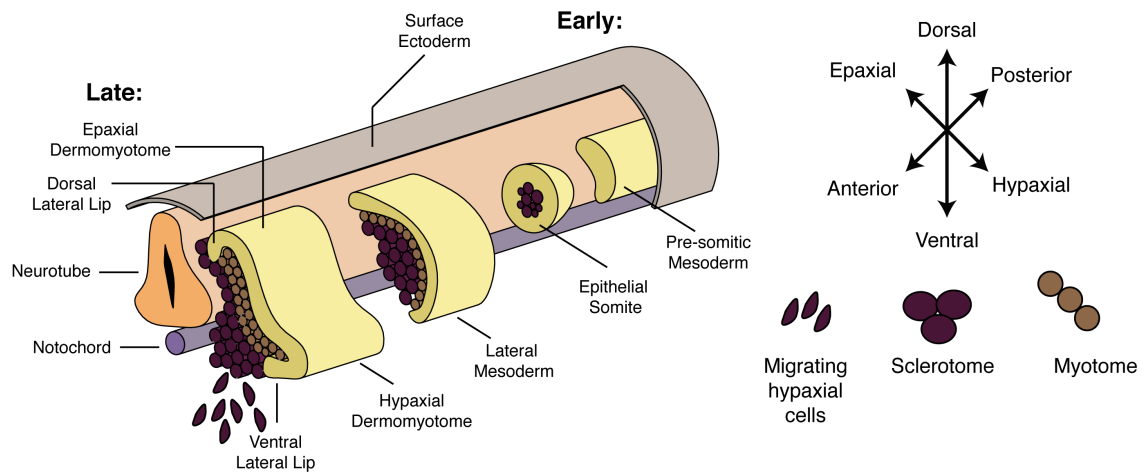


Figure 1. Formation of Embryonic Skeletal Muscle. Schematic representation of the developing embryo during somite formation. Molecular signals from the neural tube and notochord initiate the early ventral somites to de-epithelialize and to form the sclerotome, while the dorsal cells form the dermomyotome. Muscle precursor cells migrate from the lips of the dermomyotome to form the myotome, which then differentiate along the myogenic lineage in two waves. These processes form the epaxial and hypaxial muscles and some of the dermis. In addition, a population of hypaxial dermomyotomal cells migrates away from the ventrolateral lips to form the limb bud.

1.1.2 Sequential Activation of the Myogenic Regulatory Factors

Induction of the myogenic lineage is dependent on the sequential activation of MRF expression, which is regulated by muscle precursor cells that express the paired box (PAX) transcription factors, Pax3 and Pax7 (Kassar-Duchossoy et al. 2005; Relaix et al. 2005; Ben-Yair 2005; Gros et al. 2005). Several mouse models with targeted null mutations in the MRFs were generated to characterize their impact on skeletal muscle development (Braun et al. 1992; Rudnicki et al. 1992; Rudnicki et al. 1993; Hasty et al. 1993; Nabeshima et al. 1993; Venuti et al. 1995; Rawls et al. 1995). Based on phenotypic analyses of these mice, a model was developed to describe the roles of each MRF during myogenesis, with Myf-5 and MyoD expression required for proper myoblast formation and myogenin and MRF4 acting downstream to mediate terminal differentiation (Figure 2A).

In addition to the MRFs, the myocyte enhancer factor 2 (MEF2) proteins have key roles in communicating extracellular signals to the genome during myogenesis (Molkentin & Olson 1996). There are four *Mef2* genes – *Mef2A*, *Mef2B*, *Mef2C*, and *Mef2D* – in vertebrates and the MEF2 proteins are members of the MADS family of transcription factors (Black & Olson 1998). These proteins contain a MADS domain that mediates dimerization of MEF2 proteins with the MRFs and directs binding to A/T rich DNA consensus sequences in muscle specific genes (Pollock & Treisman 1991; Gossett et al. 1989). Together, sequential activation of the MRFs in combination with MEF2 transcriptional activity regulate key events during skeletal muscle formation and maintenance (Wang et al, 2001).

1.1.3 Postnatal Muscle Growth, Maintenance and Repair

The regulatory events mediating embryonic skeletal muscle development are highly refined processes that prepare the musculature system for adult life. Following birth, myogenic progenitor cells, referred to as satellite cells, exist in a quiescent state under the basal lamina of mature muscle fibers (Mauro 1961; Collins et al. 2005; Montarras et al. 2005). The postnatal satellite cell population is a heterogeneous group of committed muscle progenitor cells and non-committed stem cells that originate from Pax-expressing muscle precursor cells in the dermomyotome and are required for adult muscle growth, maintenance and repair (Gros et al. 2005; Schiendel et al. 2006).

The expression of Pax7 is necessary for proper satellite cell function, since Pax7-deficient mice display a complete loss of satellite cells (Seale et al. 2000). However, it was later demonstrated that juvenile Pax7 null mice only have a reduced number of satellite cells, which are decreased in adult muscle (Oustanina et al. 2004). Although Pax3 expression is important for embryonic muscle development and is downregulated before birth (Horst et al. 2006), it is also expressed in quiescent and activated satellite cells (Relaix et al. 2006). In fact, residual satellite cells identified in Pax7-deficient mice are attributed to Pax3 expression in interstitial cells that co-express MyoD during muscle regeneration (Kuang et al. 2006).

Satellite cells are responsible for regenerating skeletal muscle following injury or exercise (**Figure 2B**), and the satellite cell pool is maintained. The self-renewing capacity of satellite cells are preserved by either asymmetric division or stochastic differentiation (Kuang et al. 2008; Bentzinger et al. 2012). Asymmetric division occurs when satellite cells divide and produce one self-renewing satellite cell and one committed muscle

progenitor cell. In contrast, a stochastic model entails two daughter cells formed by symmetric division, which then randomly self-renew or differentiate along the myogenic lineage (Kuang et al. 2008). It is this equilibrium between self-renewal and differentiation that is crucial for postnatal muscle homeostasis.

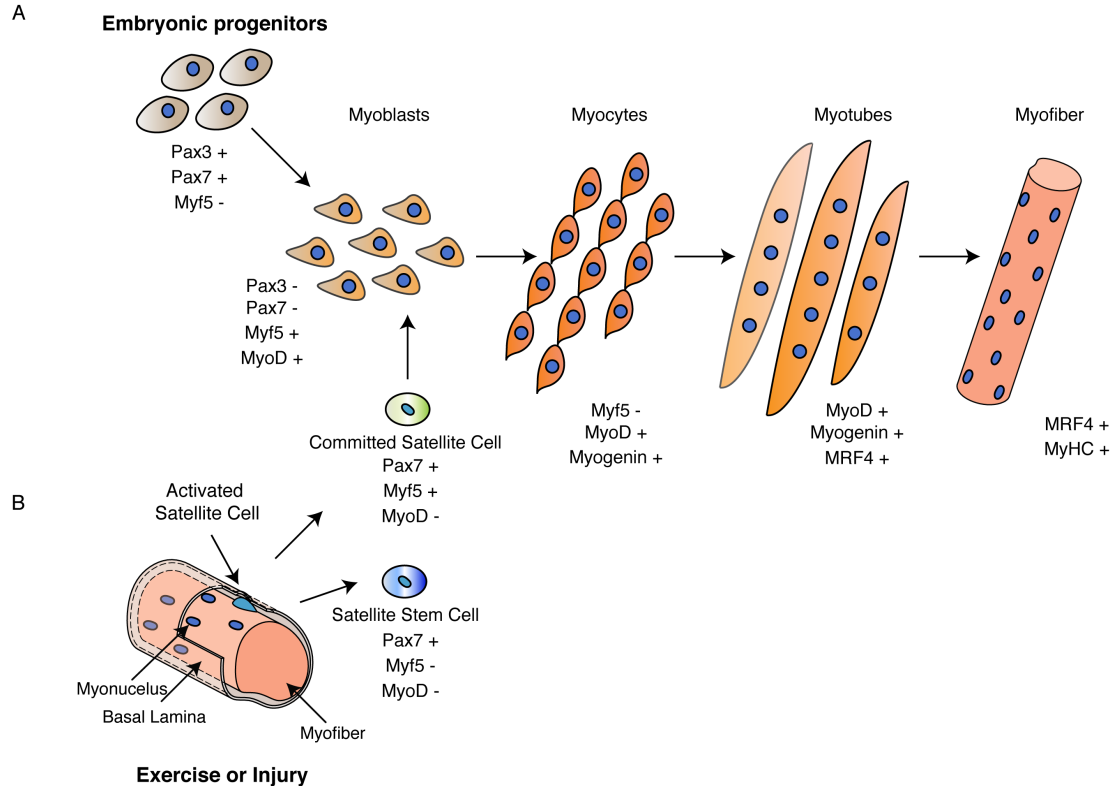


Figure 2. The induction of myogenic differentiation from embryonic and postnatal muscle progenitor cells. (A) Embryonic progenitor cells expressing Pax3 and Pax7 form myoblast cells expressing early myogenic regulatory factors, Myf5 and MyoD. These cells begin to align and express MyoD and myogenin, which then fuse to form multinucleated myotubes expressing late differentiation markers, myogenin and MRF4. Following innervation and vascularization, these cells form mature myofibers. **(B)** Upon exercise or injury, skeletal muscle satellite cells become active and divide to form a committed muscle progenitor cell expressing Pax7 and Myf5, which will enter into the myogenic differentiation program to form new myofibers. The active satellite cell will also form an identical daughter cell via asymmetric division to replenish the satellite cell pool.

1.2 The Importance of Post-transcriptional Regulation in the Neuromuscular System

1.2.1 Regulatory Roles for RNA-binding Proteins in Myogenesis

As described above, the events involved in skeletal muscle growth, maintenance and repair require the coordinated action of various transcription factors and signaling pathways. However, in addition to transcriptional activity, post-transcriptional events are crucial for skeletal muscle development and homeostasis (Apponi et al. 2011). In particular, several RBPs regulate the stability and translation of key myogenic transcripts. Thus, RBPs have recently emerged as mediators of skeletal muscle differentiation.

Remarkably, many post-transcriptional events are conserved from invertebrates to vertebrate systems. For example, the RBP Hoip, controls embryonic muscle development in *Drosophila*. Specifically, Hoip mediates both myotube elongation and sarcomeric protein expression via regulation of myosin heavy chain (MHC) pre-mRNA splicing (Johnson et al. 2013). Interestingly, impaired myogenesis in mutant *hoip* embryos was rescued by the expression of the human ortholog, Non-histone protein 2 like-1 (NHP2L1) (Johnson et al. 2013). Moreover, knockdown of the zebrafish homolog, *nh2l1b*, using two independent morpholinos inhibited the formation of MHC positive myotubes (Johnson et al. 2013). This study highlights the conservation of post-transcriptional regulation from invertebrates to vertebrates thus indicating its importance during muscle formation.

Several other RBPs are also key mediators of post-transcriptional events during myogenesis. In particular, RBPs that recognize and bind AU-rich elements (AREs) located in specific mRNAs are heavily implicated during myogenic differentiation

(Apponi et al. 2011). One such example is of the RBP, CUGBP ELAV-like family protein 1 (CUGBP1; also known as CELF1) that is regulated during embryonic muscle development with high expression in limb muscles that decreases to low levels in postnatal muscles (Ladd et al. 2001). CUGBP1 is also elevated during muscle regeneration (Orengo et al. 2011) and the overexpression of CUGBP1 impairs myogenic differentiation *in vitro* and *in vivo* via regulation of *Mef2A* and the cell cycle regulator *p21* mRNA translation (Timchenko, Lakova, et al. 2001; Timchenko et al. 2004). Together these studies demonstrate that CUGBP1 regulates mRNA targets essential during early muscle formation while its expression must remain low in postnatal and differentiated skeletal muscle.

In addition, the RBP, ELAV like protein 1 (HuR), binds AREs located in myogenic targets and controls their expression during skeletal muscle formation. HuR stabilizes several transcripts during myogenesis including, acetylcholinesterase, *p21*, *MyoD*, myogenin and high-mobility-group box 1 (*HMGB1*) mRNAs via their 3' untranslated regions (UTR) and in turn, promotes muscle differentiation (Van Der Giessen et al. 2003; Figueroa et al. 2003; Deschênes-Furry, Bélanger, et al. 2005; Deschênes-Furry, Angus, et al. 2005; Riuzzi et al. 2012; Dormoy-Raclet et al. 2013). Given HuR's role in stabilizing key myogenic transcripts, the loss of HuR in C2C12 myoblast cells impairs differentiation, since these targets such as *MyoD* are destabilized and degraded in the absence of HuR (Van Der Giessen et al. 2003). Interestingly, HuR collaborates with the RBP, KH-type splicing regulatory protein (KSRP) in a mutually exclusive relationship. In fact, HuR and KSRP interact during the early stages of differentiation to stabilize/destabilize the cell cycle promoter nucleophosmin (*NPM*) mRNAs (Cammass et

al. 2014). In line with this, KSRP destabilizes *MyoD*, *p21* and myogenin mRNAs in myoblasts and maintains the cells in a proliferative state (Briata et al. 2005). In order for differentiation to proceed, KSRP is phosphorylated by p38 mitogen activated protein kinase (MAPK), decreasing its binding capacity for target transcripts (Briata et al. 2005). Moreover, mice deficient of KSRP display impaired muscle regeneration (Briata et al. 2012). Therefore, some RBPs such as HuR and KSRP, act together to regulate post-transcriptional events during myogenesis and the misregulation of either component impairs the formation of skeletal muscle.

Finally, the RBP, AU-rich element RNA-binding protein (AUF1; also called hnRNP D) predominantly induces decay of target transcripts, however, it can also regulate mRNA translation. Specifically, AUF1 increases during myogenesis where it destabilizes the cell cycle regulator *c-myc* (Brewer 1991; Brewer & Ross 1989) and promotes the translation of *Mef2C* to mediate terminal differentiation (Panda et al. 2014). Collectively, these examples support the notion that post-transcriptional regulation of gene expression is crucial for myogenic differentiation and that RBPs play a central role in these processes.

In this context, we previously demonstrated that the RBP Staufen1 is regulated during myogenesis, that it accumulates at the postsynaptic sarcoplasm of muscle fibers, and that it is increased in denervated skeletal muscles (Bélanger et al. 2003). Others have subsequently confirmed the observation that Staufen1 is regulated in skeletal muscle (Gong et al. 2009; Chang et al. 2012; Yamaguchi et al. 2008; Yamaguchi et al. 2012). Briefly, Yamaguchi et al. demonstrated that Staufen1 is a negative regulator of myogenesis and that the knockdown of Staufen1 induces precocious differentiation

(Yamaguchi et al. 2008). They later showed that Staufen1 regulates the stability of Dishevelled (*Dvl*) mRNAs via the 3'UTR (Yamaguchi et al. 2012). In addition, Staufen1 has been implicated in an mRNA decay pathway called Staufen1 mediated mRNA decay (SMD), which has been proposed to compete with nonsense-mediated decay (NMD) to regulate myogenesis (Kim et al. 2007; Gong et al. 2009). Despite these findings, the expression pattern of Staufen1 during embryonic muscle development and during muscle regeneration is unknown.

1.2.2 Implications for RNA-binding Proteins in Neuromuscular Disease

Given the importance of RBPs in mediating post-transcriptional events during muscle differentiation, it is not surprising that altered expression of these RBPs is implicated in several neuromuscular disorders. A comprehensive list of RBPs that are mutated or misregulated in various muscle diseases is demonstrated in **Table 1**. For example, CUGBP1 is elevated in various neuromuscular disorders, including congenital merosin-deficient muscular dystrophy, limb girdle muscular dystrophy, and myotonic dystrophy type I (DM1) (Timchenko, Cai, et al. 2001; Roberts et al. 1997; Orengo, Ward, and Cooper 2011; Savkur, Philips, and Cooper 2001). Indeed, muscle-specific CUGBP1 transgenic mice display morphological impairments such as muscle atrophy and increased muscle regeneration, consistent with neuromuscular pathologies (Timchenko et al. 2004; Ho et al. 2005; Ward et al. 2010).

Furthermore, the presence of GCG trinucleotide repeats within exon 1 of the gene encoding Poly-(A)-binding protein N1 (PABPN1) causes oculopharyngeal muscular dystrophy (Brais et al. 1998). Two separate studies generated transgenic mice with mutant PABPN1 under the control of ubiquitous promoters (Dion et al. 2005; Hino et al.

2004). These models demonstrate that mutant PABPN1 accumulates in skeletal muscle and neuronal cell nuclei resulting in progressive muscle weakness, peripheral nerve alterations and motor coordination deficits (Dion et al. 2005; Hino et al. 2004). Similarly, mouse models of AUF1 recapitulate several phenotypes consistent with neuromuscular disorders. Although AUF1's role in neuromuscular disease is unknown, AUF1 knock-out mice display several features reminiscent of these diseases including premature aging, kyphosis and impaired skeletal muscle formation (Pont et al. 2012). In this context and given the emerging roles of Staufen1 in skeletal muscle, we recently demonstrated that Staufen1 is elevated in DM1 skeletal muscle from both mouse models and patient biopsies and that its expression may act as a compensatory mechanism of the cell (Ravel-Chapuis et al. 2012).

Remarkably, treatment of spinal muscular atrophy (SMA) neurons with activators of p38 MAPK increases cytoplasmic HuR, which stabilizes *SMN* mRNAs (Farooq et al. 2009; Farooq et al. 2013). SMA, which is characterized by degeneration of α -motor neurons causing progressive muscle atrophy (Ogino & Wilson 2004) occurs as a result of homozygous mutations in the survival motor neuron 1 gene (*SMN1*) (Lefebvre et al. 1995). The stabilization of *SMN* mRNAs promotes motor neuron survival and thus, activation of p38 MAPK is beneficial for the pathology (Farooq et al. 2009; Farooq et al. 2013). Recently, the consequences of the loss of *Smn* in mouse skeletal muscles was examined (Boyer et al. 2014). Proliferating primary myoblasts isolated from a SMA mouse model showed a decrease in Pax7 and MyoD levels, while differentiating myoblasts had decreased myogenin and MHC expression (Boyer et al. 2014). Several

MRFs were also misregulated in early postnatal hindlimb muscles, at least partially independent of the state of innervation (Boyer et al. 2014).

HuR is also involved in amyotrophic lateral sclerosis (ALS), which is characterized by the selective death of upper and lower motor neurons, muscle weakness, atrophy and progressive paralysis (Rowland & Shneider 2001). Specifically, in a form of sporadic ALS caused by mutations in the superoxide dismutase 1 (*SOD1*) gene (Deng et al. 1993; Rosen et al. 1993), HuR is elevated in the cytoplasm of neuronal cells where it regulates key hallmarks of the disorder (Milani et al. 2013). In addition, specific mutations in the C-terminal region of the TET family member, translocated in sarcoma/fused in sarcoma (TLS/FUS), are correlated with familial ALS (Kwiatkowski et al. 2009; Vance et al. 2009). Collectively, these examples highlight the critical roles of RBPs both in neurons and skeletal muscle and their implications in neuromuscular disease.

Table 1. RNA-binding proteins commonly implicated in neuromuscular disease and sarcomas. Several RNA-binding proteins (RBPs) are misregulated in neuromuscular disorders or sarcomas, including DM1, SMA, ALS, congenital merosin-deficient muscular dystrophy (MDCMD), limb girdle muscular dystrophy (LGMD), oculopharyngeal muscular dystrophy (OPMD), facioscapulohumeral muscular dystrophy (FSHD), congenital myasthenic syndrome (CMS), sarcomas and myxoid liposarcoma (MLPS).

RBP	Primary Function	Disease	Mutation/ Misregulation	Reference
CUGBP1	mRNA stability, alternative splicing	DM1, MDCMD, LGMD	Increased	(N. A. Timchenko, Cai, et al. 2001; Roberts et al. 1997; Orengo, Ward, and Cooper 2011; Savkur, Phillips, and Cooper 2001)
DDX5	RNA helicase	DM1	Unclear	(Paul et al. 2011; Jones et al. 2015)
DDX6	RNA helicase	DM1	Loss of function	(Pettersson et al. 2014)
DDX17	RNA helicase	DM1	Increased	(Paul et al. 2011)
EWS	Exact function unclear	Ewings Sarcoma	t(11;22)(q24;q12)	(Riggi et al. 2007)
hnRNP A1	Alternative splicing	ALS	Decreased	(Honda et al. 2015)
hnRNP A2/B1	Alternative splicing	DM1	Increased	(Paul et al. 2011)
hnRNP H	Alternative splicing	1) DM1 2) CMS 3) ALS	1) Increased 2) Disrupted splice sites 3) Loss of function	(Paul et al. 2006; D. H. Kim et al. 2005; Paul et al. 2011; Rahman et al. 2015; Masuda et al. 2008; Y. B. Lee et al. 2013)
hnRNP F	Alternative splicing	DM1	Increased	(Paul et al. 2011)
hnRNP K	Alternative splicing	DM1	Increased	(Paul et al. 2011)
HuR	mRNA stability	ALS	Increased cytoplasmic expression	(Milani et al. 2013).
MBNL1, MBNL2, MBNL3	Alternative splicing	DM1	Loss of function	(Miller et al. 2000; Jiang et al. 2004; A Mankodi et al. 2001)
PABPN1	Polyadenylation	OPMD	Loss of function	(Brais et al. 1998)
QKI	Alternative splicing	DM1	Aberrantly spliced	(Klinck et al. 2014)
RBFOX1	Alternative splicing	1) DM1 2) FSHD	1) Dominant negative mutation 2) Decreased	(Klinck et al. 2014; Pistoni et al. 2013)
SRSF1	Alternative splicing	1) SMA 2) ALS 3) Sarcoma	1) Disruption of binding site 2) Loss of function 3) Increased	(Karni et al. 2007; Cartegni and Krainer 2002; Y. B. Lee et al. 2013)
Staufen1	mRNA localization, translation, alternative splicing	DM1	Increased	(Ravel-Chapuis et al. 2012; 2016)
TDP-43	Transcriptional regulation, alternative splicing	ALS	Cytoplasmic aggregation	(Neumann et al. 2006)
TLS/FUS	Multi-functional	1) MLPS 2) ALS	1) t(12;16)(q13;p11) 2) 13 mutations identified	(Riggi et al. 2007; Kwiatkowski et al. 2009; Vance et al. 2009)

1.2.3 Implications for RNA-binding Proteins in Sarcomas

In addition to neuromuscular disorders, RBPs are emerging as key regulators in cancer. For example, sarcomas represent aggressive solid tumours with poor prognosis, for which the molecular mechanisms are not well understood (Riggi et al. 2007). The exact cellular origin of sarcomas remains unclear, however it is thought that genetic mutations in mesenchymal progenitor cells are involved (Arvand & Denny 2001). Interestingly, chromosomal translocations in genes encoding members of the TET family of RBPs are characteristic of sarcomas (Riggi et al. 2007). Specifically, mutations in the ewing sarcoma (*EWS*) and *TLS/FUS* genes are implicated in Ewings sarcomas and myxoid liposarcomas, respectively (Riggi et al. 2007). In fact, the formation of a fusion protein containing EWS and friend leukemia integration 1 (FLI-1) account for > 85% of Ewings sarcoma tumours (Riggi et al. 2007; Kiven E. Lukong, Kai-wei Chang et al. 2008). Therefore these studies demonstrate that the post-transcriptional events mediated by the TET family of RBPs are crucial for proper cellular processing and when disrupted may lead to the formation of cancer.

Similarly, rhabdomyosarcomas (RMS) are described as skeletal muscle-like tumours since they often form in muscle beds and express several MRFs. However, RMS cells remain in the proliferative phase and are unable to terminally differentiate (Hettmer & Wagers 2010; Tapscott et al. 1993). The two major subtypes of RMS are embryonal RMS (ERMS) that occurs in children less than 10 years of age and alveolar RMS (ARMS) that affects both children and young adults (Fletcher et al. 2013). Recently, insulin-like growth factor 2 mRNA-binding protein 1 and 2 (IGF2BP1 and IGF2BP2, respectively) have been implicated in ERMS pathogenesis (Li et al. 2013; Faye et al. 2015). Both

IGF2BP1 and IGF2BP2 are elevated in ERMS and the knockdown of either factor inhibits ERMS tumorigenesis (Li et al. 2013; Faye et al. 2015). Specifically, IGF2BP1 regulates cellular inhibitor of apoptosis 1 (cIAP1) and in turn, the apoptotic response in ERMS cells (Faye et al. 2015), while IGF2BP2 mediates the mRNA and protein stability of *NRAS*, a commonly mutated gene in ERMS and drives oncogenesis (Li et al. 2013). Despite these advances, little improvement has been made in therapeutic options for RMS in several decades and therefore it is necessary to continue to investigate the molecular mechanisms underlying RMS in order to develop novel and successful therapies.

1.3 The Double Stranded RNA-Binding Protein Staufen

1.3.1 Structure and Function of Staufen in Invertebrates

Staufen is a double stranded RBP initially identified for its role in oogenesis and central nervous system (CNS) development in *Drosophila* and is composed of five double stranded RNA-binding domains (dsRBDs) (St Johnston et al. 1992). In invertebrates, Staufen is required for accurate localization of maternal mRNAs to the anterior and posterior poles of oocytes (St Johnston et al. 1989; St Johnston et al. 1991; Breitwieser et al. 1996). Specifically, Staufen mediates the localization and/or translation of *oskar*, *bicoid*, and *nanos* mRNAs during pole plasm formation, a crucial stage in germ-line formation and abdomen development in *Drosophila* (St Johnston et al. 1989; St Johnston et al. 1991; Breitwieser et al. 1996; Ferrandon et al. 1994). Staufen is also required for the asymmetric localization of *prospero* mRNAs through binding its 3'UTR during neuroblast cell division, an event dependent on the Staufen-Miranda protein interaction

(Li et al. 1997; Schuldt et al. 1998). During this process, Staufen localizes *prospero* mRNAs from the dividing neuroblast to the future ganglion mother cell, thus promoting neuronal cell differentiation (Li et al. 1997).

Increasing evidence suggests that in addition to Staufen's role in developing neurogenesis, it is also important in the maintenance of the mature nervous system. In fact, acute Staufen expression is required for long-term memory formation in *Drosophila* (Dubnau et al. 2003), an effect regulated by genetic interactions with Fragile X mental retardation and Argonaute-1 proteins (Bolduc et al. 2008). In this context, Staufen expression is dependent on the transcriptional activation of the cyclic adenosine monophosphate (cAMP) response element binding protein (*dCREB*) and magnesium (Mg^{2+}) block-dependent suppression of N-methyl-D-aspartate (NMDA) receptor activity, which underlies long-term memory in *Drosophila* (Miyashita et al. 2012; Dubnau et al. 2003). Finally, *Staufen* is a downstream transcriptional target of Adh transcription factor 1 (Adf-1) and is involved in dendrite development and plasticity (Timmerman et al. 2013).

In addition to Staufen's role in *Drosophila*, it is also involved in neurogenesis of other invertebrates such as the *Aplysia* (Liu et al. 2006). Specifically, Staufen mediates mRNA accumulation at the axon hillock of stimulated sensory neurons as well as in unstimulated neurons at the opposite pole of the cell body (Liu et al. 2006). Increasing evidence demonstrates that the Staufen family of RBPs have conserved functions in mRNA transport, localization and translational control from invertebrates to vertebrates. For example, there are two Staufen homologs in zebrafish that are required for germline

specification as well as in learning and memory, thus indicating that Staufen protein function is conserved across species (Ramasamy et al. 2006).

1.3.2 Structure and Function of Staufen in Mammals

There are two mammalian homologs of Staufen; Staufen1 and Staufen2. There are three major protein isoforms of Staufen1, Stau1⁵⁵, Stau1⁶³ and Stau1ⁱ, as a result of alternative pre-mRNA splicing (Wickham et al. 1999; Duchaine et al. 2000; Brizard et al. 2000).

Molecular mapping of Staufen1 cDNAs identified four transcripts, T1, T2, T3 and T4. A 55 kDa isoform is generated from T1, T2 and T4, with T2 being the most abundant, whereas a 63kDa isoform is produced from T3 that contains a DNA insertion resulting in an upstream ATG initiation codon and a 81-amino-acid extension in its N-terminal region (**Figure 3A**) (Wickham et al. 1999). An additional Staufen1 isoform, Stau1ⁱ, is generated due to a 18-base pair (bp) insertion that generates alternative splicing acceptor sites in exon 5 and results in the inclusion of a 6-amino-acid fragment in the protein (**Figure 3A**) (Brizard et al. 2000; Duchaine et al. 2000). Staufen1 is comprised of four dsRBDs, a nuclear localization signal (NLS), and a C-terminal tubulin-binding domain (TBD) (**Figure 3B**). Specifically, Staufen1 contains the conserved dsRBD2, dsRBD3, dsRBD4 and dsRBD5 identified in *Drosophila* with dsRBD3 and dsRBD4 as the active dsRBDs, while dsRBD2 and dsRBD5 do not appear to bind dsRNA (**Figure 3B**) (Micklem et al. 2000; Martel et al. 2010). The NLS is located immediately after dsRBD3 allowing for Staufen1 to shuttle between the nucleus and cytoplasm (Martel et al. 2006). Finally, the 6-amino-acid insertion in Stau1ⁱ is located within dsRBD3 and severely inhibits its RNA-binding capacity (**Figure 3B**) (Duchaine et al. 2000).

Similarly, the Staufen2 pre-mRNA is alternatively spliced to form three Staufen2 protein isoforms, Stau2⁵², Stau2⁵⁹ and Stau2⁶² (Duchaine et al. 2002; Buchner et al. 1999). Unlike mammalian Staufen1, which lacks the *Drosophila* dsRBD1, Staufen2 contains the conserved dsRBD1 in addition to dsRBD2, dsRBD3 and dsRBD4 (Duchaine et al. 2002). As observed with Staufen1, Staufen2 contains a TBD downstream of dsRBD4, indicating that Staufen2 can also interact with microtubules (Duchaine et al. 2002). Finally, Staufen2 only contains a rearranged C-terminal segment of dsRBD5 (Duchaine et al. 2002), which in *Drosophila* is necessary for protein-protein interactions (Schuldt et al. 1998).

In neurons, Staufen1 and Staufen2 are somatodendritically localized and Staufen-associated messenger RNPs (mRNPs) regulate mRNA localization and activity-dependent translation by moving bidirectionally along dendritic microtubules (Köhrmann et al. 1999; Kiebler et al. 1999; Tang et al. 2001; Krichevsky & Kosik 2001). These events are crucial for synaptic strength and maintenance of altered neuronal connectivity, ultimately responsible for hippocampus-dependent learning and memory (Marión et al. 1999). Interestingly, Staufen1 and Staufen2 have similar functions in neurons but are located in distinct particles, indicating that they have individual roles within the cell (Kiebler et al. 1999; Duchaine et al. 2002). Together, these studies demonstrate the importance of Staufen proteins in the CNS and highlight their multi-functional nature within the cell.

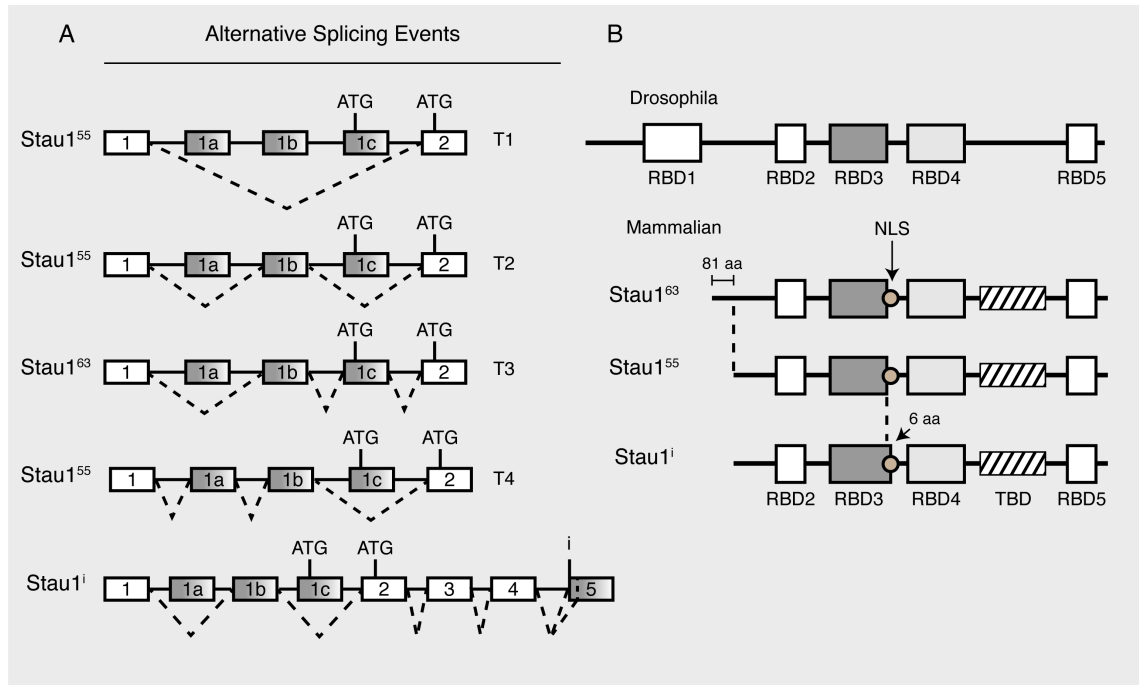


Figure 3. Molecular analysis of mammalian Staufen1 isoforms. (A) Schematic representation of alternative splicing events at the 5' end of the mammalian Staufen1 pre-mRNA. Stau1ⁱ is generated by differential 5'splicing acceptor sites in exon 5. The isoform name (corresponding to its respective weight in kDa) and transcript identification are depicted. Boxes represent exons, solid lines represent introns and dashed line represent alternative splicing events. (B) Comparison of the *Drosophila* Staufen protein structure with the mammalian Staufen1 protein isoforms. Staufen1 has four conserved RNA-binding domains (RBD) and a tubulin-binding domain (TBD). Staufen1 also has a nuclear localization signal (NLS) located in dsRBD3. The Stau1⁶³ isoform contains an 81-amino-acid (aa) insertion at the N-terminus and Stau1ⁱ has a 6 aa extension in dsRBD3. Diagrams are not drawn to scale.

1.3.3 The Multi-functional RNA-binding Protein Staufen1

Staufen1 has emerged as a multi-functional RBP and plays several key roles in RNA metabolism including mRNA localization (Tang et al. 2001), stability (Y. K. Kim et al. 2005; Kim et al. 2007; Gong et al. 2009), and translation (Dugré-Brisson et al. 2005; Ricci et al. 2013). Moreover, recent studies have demonstrated that Staufen1 is implicated in the cell cycle (Boulay et al. 2014), stress granule formation (Thomas et al. 2009; Thomas et al. 2005; Ravel-Chapuis et al. 2016) and alternative pre-mRNA splicing (Ravel-Chapuis et al. 2012; Sugimoto et al. 2015). Collectively, the multi-functionality of Staufen1 greatly impacts post-transcriptional regulation of gene expression across multiple tissues.

Similar to *Drosophila* Staufen, which regulates the translation of *oskar* mRNAs in oocytes (Kim-Ha et al. 1995; Micklem et al. 2000), mammalian Staufen1 also binds secondary structures within the 5'UTR of target transcripts to enhance translation (Dugré-Brisson et al. 2005). In addition, a recent large-scale screen showed that Staufen1 interacts with actively translating ribosomes and that Staufen1 regulates the translation of target mRNAs containing highly structured coding sequences (Ricci et al. 2013). As mentioned earlier, Staufen1 mediates an mRNA decay pathway, SMD, whereby Staufen1 binds the 3'UTR of target transcripts and recruits the NMD factor Upf1 to elicit mRNA decay (Y. K. Kim et al. 2005; Kim et al. 2007; Gong et al. 2009). Specifically, the ADP-ribosylation factor 1 (*Arf1*) is a canonical SMD target since it contains a 19-bp stem loop, deemed a Staufen1 binding site (SBS) located downstream of the normal termination codon in the 3'UTR (Y. K. Kim et al. 2005). Unlike some RBPs that bind specific sequences, Staufen1 has an affinity for short stem secondary structures similar to the

intramolecular SBS located in the *Arf1* 3'UTR. Additional SBSs include secondary structures containing Alu elements comprised of a single Alu paired with a long non-coding RNA or two Alu elements that are in opposite orientation that form an imperfectly paired inverted duplex, referred to as Inverted Repeat Alus (IR*Alus*) (Ricci et al. 2013; Gong & Maquat 2011; Elbarbary et al. 2013). The multitude of SBSs identified across the human genome and their various locations (i.e. 5' - and 3'UTR, introns, coding sequence, and intergenic regions) (Ricci et al. 2013) indicate the complexity of Staufen1-regulated events.

In addition to Staufen1's role in promoting mRNA translation and/or decay, Staufen1 is also regulated during the cell cycle. In this context, Staufen1 expression fluctuates throughout the cell cycle of transformed cell lines (HCT116 and U2OS cells) with high expression during the S phase to mitosis entry followed by a rapid decrease during mitosis (Boulay et al. 2014). The degradation of Staufen1 is regulated by the ubiquitin-proteasome and the E3 ubiquitin ligase anaphase promoting complex (Boulay et al. 2014). Staufen1 associates with ~275 mRNAs during the early prometaphase stage of mitosis prior to its degradation, however, sustained Staufen1 expression throughout mitosis impairs the proliferation of these cells (Boulay et al. 2014). This study highlights the importance of Staufen1-regulated post-transcriptional events during the cell cycle and suggests a potential role for Staufen1 in cancer cells characterized by uncontrolled proliferation.

Staufen1 is also implicated during stress granule (SG) formation. The cellular stress response involves global translation silencing via SGs, which are large cytoplasmic structures that contain translationally-stalled mRNAs, pre-initiation factors and RBPs

(Kedersha et al. 2013). Staufen1 is recruited to SGs and is involved in the localization of polysomal mRNAs to SGs in several cell types (Thomas et al. 2005; Thomas et al. 2009). Moreover, the knockdown of Staufen1 enhanced SG formation in NIH 3T3 and U2OS cell lines (Thomas et al. 2009) and recently, we identified a role for Staufen1 in the stress response of normal and DM1 skeletal muscles (Ravel-Chapuis et al. 2016). As observed in neuronal cell types, Staufen1 is recruited to SGs in mouse C2C12 myoblasts; however, SGs did not form in response to external stimuli in DM1 myoblasts (Ravel-Chapuis et al. 2016). Similar to previous reports in non-muscle cell lines (Thomas et al. 2009), the knockdown of Staufen1 enhanced SG formation in DM1 myoblasts (Ravel-Chapuis et al. 2016).

Finally, we demonstrated that Staufen1 regulates alternative pre-mRNA splicing of two key transcripts in DM1 skeletal muscle (Ravel-Chapuis et al. 2012). Specifically, Staufen1 promotes the insulin receptor (*INSR*) and chloride voltage-gated channel 1 (*CLCN1*) alternative splicing towards wild-type (WT) patterns (Ravel-Chapuis et al. 2012). A recent study confirmed Staufen1-regulated alternative splicing and interestingly demonstrated that Staufen1 regulates the cytoplasmic splicing of X-box binding protein 1 (*XBPI*) through binding a SBS within the 3'UTR (Sugimoto et al. 2015). Collectively, all of the examples provided here indicate that Staufen1's multi-functional role is conserved across numerous cell types and further highlights the importance of Staufen1 in the neuromuscular system.

1.4 Myotonic Dystrophy Type I

Myotonic Dystrophy (DM) is an autosomal dominant neuromuscular disorder and is the most common form of muscular dystrophy observed in adults. The classic form, DM1 is

a multi-systemic disease affecting several tissues. DM1 patients experience a wide range of symptoms that include muscle hyperexcitability (myotonia), progressive muscle wasting, cardiac conduction defects, ocular cataracts, gastrointestinal dysfunction, endocrine abnormalities such as insulin resistance, and cognitive impairments (Chau & Kalsotra 2014). DM1 affects 1 in 8000 individuals worldwide, with a notable increase in prevalence in certain regions of Québec, Canada and Basque Country, Spain (Mathieu & Prevost 2012; López de Munain et al. 1993). DM1 is caused by the expansion of CTG repeats in the 3'UTR of the dystrophin myotonia protein kinase (*DMPK*) gene (Brook et al. 1992; Mahadevan et al. 1992; Fu et al. 1992) and is categorized by the age at which symptoms are observed – congenital DM1, childhood-onset, adult-onset, and late-onset DM1 (Udd & Krahe 2012; Turner & Hilton-Jones 2014). Unaffected individuals have 5-37 CTG repeats, whereas affected individuals carry anywhere from >50 repeats and can reach lengths of 5000 repeats in the *DMPK* gene (**Figure 4A**) (Turner & Hilton-Jones 2014). Initial studies suggested a clear correlation between CTG repeat length, age of onset and disease severity (Harley et al. 1993); however, more recent work demonstrates that there is only moderate correlation, which occurs when CTG repeat length is ≤ 400 trinucleotides (Hamshere et al. 1999). The discrepancy between repeat length and disease severity is attributed to the unstable nature of the CTG expansion resulting in somatic mosaicism (Lavedan et al. 1993). This in part explains the varying age of onset observed between DM1 patients, and the differences in disease severity observed across different organs within an individual, thus making it difficult to classify the severity of DM1 based solely on repeat numbers (Morales et al. 2012). In fact, since repeat numbers vary across

tissues within a DM1 patient, the current standard of staging by blood tests, must only provide a range of CTG repeats rather than a concrete number.

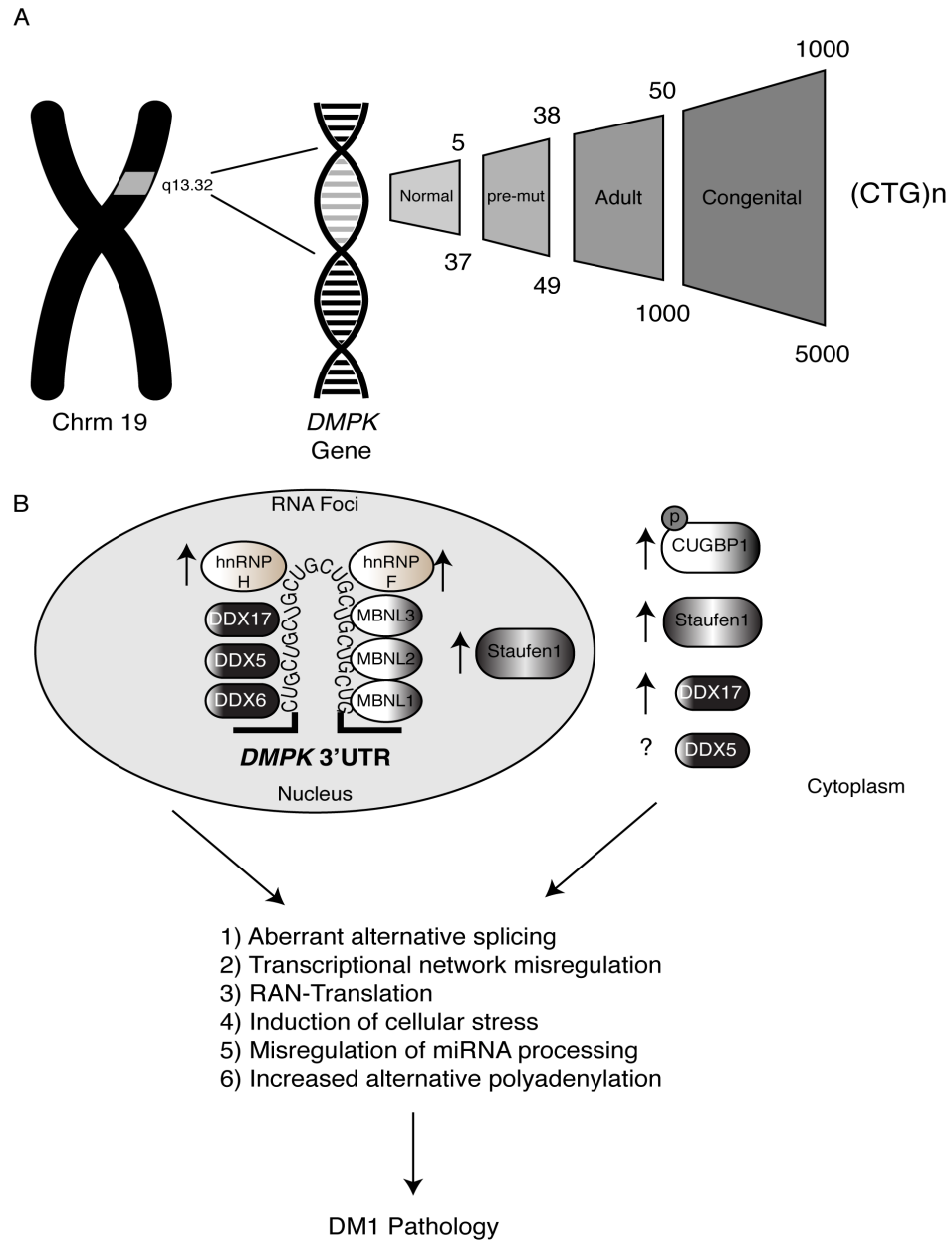


Figure 4. DM1 is caused by CTG repeats in the *DMPK* gene. (A) An unaffected individual has 5-37 CTG repeats whereas an affected DM1 patient can contain >50 up to 5000 CTG repeats in the *DMPK* gene. (B) The CTG repeats are transcribed to form CUG expansions in the 3'UTR of *DMPK* mRNAs that aggregate in the nucleus and form RNA foci. Consequently, several RNA-binding proteins (RBPs) are misregulated resulting in widespread cellular remodeling and the DM1 pathology.

Numerous mechanisms have been proposed to describe how this mutation, which does not modify the protein-coding region of the gene, leads to the multi-systemic symptoms characteristic of DM1. Early efforts investigated the impact of DMPK loss, and while *Dmpk* knockout mice had reduced skeletal muscle force generation (Reddy et al. 1996) and abnormal cardiac conduction (Berul et al. 1999), there was no evidence of myotonia, a key hallmark of the disease (Reddy et al. 1996). In addition, the identification of genes in close proximity to the CTG repeats such as *Six5* showed that the CTG expansion causes transcriptional repression of *Six5* (Boucher et al. 1995; Klesert et al. 1997; Thornton et al. 1997). Formation of cataracts in mice harbouring a disrupted *Six5* gene validates that transcriptional repression of neighbouring genes does impact the disease pathology; however, similar to *dmpk* knockout mice, key hallmarks such as myotonia were not observed (Klesert et al. 2000; Reddy et al. 1996). Moreover, there is considerable evidence indicating that a gain of function due to the expanded RNA is a primary contributor to the pathology (Charlet-B et al. 2002). Together, these studies demonstrate that DM1 symptoms likely arise through a combination of *DMPK* and *SIX5* haploinsufficiency paired with RNA-toxicity.

1.4.1 RNA Toxic Effect of CTG Repeats in the *DMPK* gene

The CTG repeats in the *DMPK* 3'UTR form CUG expanded (CUG^{exp}) mRNAs, which aggregate in the nucleus and form RNA foci (Taneja et al. 1995; Davis et al. 1997). The RNA foci cause the sequestration and the misregulation of various transcription factors and RBPs with Muscle-blind like splicing regulator 1 (MBNL1) and CUGBP1 being the most heavily studied (Lee & Cooper 2009). As a result, a toxic cellular effect is observed,

which directly affects the expression, metabolism and splicing of target mRNAs and contributes to the DM1 pathology (O'Rourke & Swanson 2009).

In support of the RNA toxic model, a muscle specific transgenic mouse containing the human skeletal actin (*ACTA1*) gene with CTG repeats in the 3'UTR was created (Mankodi et al. 2000). To recapitulate the mutant CUG^{exp} mRNAs, CTG repeats were inserted into the last exon of the *ACTA1* gene between the termination codon and the polyadenylation site to generate mice harboring a long expansion (250 CTG repeats) or a short expansion (5 CTG repeats), termed HSA^{LR} and HSA^{SR}, respectively (Mankodi et al. 2000). The HSA^{LR} model develops myotonia as early as 4 weeks of age and has increased central nucleation, ring fibers and variable muscle fiber size, which are consistent with the DM1 pathology (Mankodi et al. 2000). In addition, RNA foci are present in the nuclei of HSA^{LR} muscles confirming the hypothesis that CUG^{exp} mRNAs are toxic in skeletal muscle fibers (Mankodi et al. 2000).

Numerous mechanisms have been proposed to describe how CUG^{exp} mRNAs cause widespread multi-systemic symptoms. The accepted models include a combination of the following: 1) the aberrant alternative splicing of various key pre-mRNAs (Mankodi et al. 2001, 2005; Philips et al. 1998, Savkur et al. 2001), 2) misregulation of the transcriptional network (Ebralidze et al. 2004; Dansithong et al. 2011; Osborne et al. 2009; Kalsotra, Ravi K Singh, et al. 2014; Du et al. 2010), 3) the presence of repeat-associated non-ATG translation (RAN-translation) (Zu et al. 2010), 4) the induction of a cellular stress response (Huichalaf et al. 2010), 5) the misregulation of microRNA (miRNA) processing (Kalsotra, Ravi K Singh, et al. 2014; Rau et al. 2011; Krol et al. 2007) and 6) the increased use of alternative polyadenylation sites (Batra et al. 2014).

Collectively, these events contribute to the vast array of symptoms observed both within an individual and between affected DM1 patients (**Figure 4B**).

1.4.2 Impact of Aberrant Alternative pre-mRNA Splicing in DM1

The splicing of pre-mRNA is a dynamic process that involves the removal of introns and the ligation of the remaining exons, an event necessary for the formation of mature mRNAs and highly important for almost all aspects of eukaryotic cell biology (Braunschweig et al. 2013). This process is dependent on the action of large RNP machinery called the spliceosome. The major spliceosome is comprised of five small nuclear RNPs (snRNPs): U1, U2, U4/U6, whereas U5, U11, U12, and U4atac/U6atac form the minor spliceosome (Braunschweig et al. 2013). The major spliceosome is responsible for the removal of the majority of introns while the minor spliceosome regulates the excision of ~1 in 300 introns in the human genome (Steitz et al. 2008). The assembly of the major spliceosome is dependent on the stepwise recognition of highly conserved splicing signals: the 5' splice site (SS), the branch point and the 3' SS (Wahl et al. 2009; Hoskins & Moore 2012).

Alternative pre-mRNA splicing allows for a single gene to express multiple mRNAs, some of which encode proteins that have altered peptides or UTRs. This process occurs in >95% of human genes and contributes to the diversity of the human proteome (Lee & Rio 2015). Many RBPs can function as splicing factors that recognize and bind cis-acting regulatory elements to either positively or negatively regulate spliceosome assembly. These regulatory elements are short nucleotide sequences located within introns and exons forming intronic or exonic splicing enhancers (ISE and ESE, respectively) or silencers (ISS and ESS, respectively). Errors in alternative pre-mRNA splicing are linked

with several disease states including many cancers and neuromuscular disorders (Cooper et al. 2009; Padgett 2012).

The impact of CUG^{exp} mRNAs in DM1 on alternative pre-mRNA splicing contributes to several key hallmarks of the disease. Initial investigation into aberrant splicing of mRNAs in DM1 revealed that the alternative splicing of the cardiac troponin T (*cTNT*), *INSR* and *CLCN1* mRNAs were misregulated (Philips et al. 1998, Savkur et al. 2001, Mankodi et al. 2002). Reduced myocardial function and conduction abnormalities observed in DM1 has been linked to the aberrant inclusion of the embryonic exon 5 of *cTNT* (Mankodi et al. 2002; Phillips & Harper 1997). In addition, exon 11 of the human *INSR* pre-mRNA is preferentially skipped leading to increased expression of the IR-A isoform and ultimately a reduced cellular response to the metabolic effect of insulin (Savkur et al. 2001; Seino & Bell 1989; Moller et al. 1989). Finally, myotonia is caused by the aberrant inclusion of exon 7a in the *CLCN1* pre-mRNA, which forms a truncated protein in DM1 skeletal muscle (Mankodi et al. 2002). Together, these findings highlight the impact that aberrant alternative splicing has in DM1 and demonstrates that several key symptoms can be explained by these events.

Numerous other aberrant alternative splicing events (ASEs) in DM1 have now been well documented. Although the cause of insulin resistance and myotonia are attributed to the alternative splicing of the *INSR* and *CLCN1* pre-mRNAs, respectively, the molecular events responsible for progressive muscle wasting are less defined (Kimura et al. 2005). It is reported that DM1 myotubes have increased resting Calcium (Ca²⁺) concentrations (Jacobs et al. 1990). Intracellular Ca²⁺ homeostasis is dependent on two sarcoplasmic reticulum proteins in skeletal muscle, the Ryanodine Receptor 1 (RyR1) and

sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase 1 (SERCA1) (Kimura et al. 2005). Interestingly, both *RyR1* and *SERCA1* mRNAs are aberrantly spliced in DM1 skeletal muscle (Kimura et al. 2005). The *RyR1* fetal isoform ASI(-) that is formed by exon 70 exclusion, is increased in DM1, which causes decreased channel activity as compared to WT cells, indicating that it plays a key role in Ca^{2+} homeostasis (Kimura et al. 2005). In addition, the neonatal variant *SERCA1b* is expressed in DM1 skeletal muscle, which is characterized by an increase in exon 22 exclusion (Kimura et al. 2005). The fast skeletal muscle troponin T (*TNNT3*) contributes to the contractile properties of skeletal muscle and it contains a fetal (F) exon in DM1 and may play a key role in DM1 muscle weakness (Kanadia et al. 2003). Finally, enhanced exon 11 exclusion of the bridging integrator-1 (*BINI*) pre-mRNA impairs T-tubule biogenesis in DM1 skeletal muscle (Fugier et al. 2011). Collectively, these ASEs likely contribute to the muscle weakness observed in DM1 patients and mouse models.

Recent large-scale screens have identified additional ASEs that are present in human and mouse DM1 skeletal muscles (Yamashita et al. 2012; Du et al. 2010; Klinck et al. 2014). The ASEs include *LDB3* exon 4, *TTN* exon 45, *MBNL1* exons 6 and 10, *NFIX* exon 7 and 123, and *Smyd1* exon 39 (Yamashita et al. 2012; Du et al. 2010). For example, the alternative splicing of *LDB3* exon 4, codes for a structural protein (also called Cypher) that is located in the Z-line of skeletal muscles and interacts with α -actinin 2 and protein kinase C (PKC) regulating muscle contractility (Zhou et al. 1999; Zhou et al. 2001). The inclusion of *LDB3* exon 4 is necessary for proper cardiac muscle function with mutations in exon 4 causing dilated cardiomyopathy (Arimura et al. 2009). Thus, the increased inclusion of exon 4 in DM1 skeletal muscle may contribute to muscle atrophy

and muscle weakness characteristic of the DM1 pathology (Yamashita et al. 2012). The identification of these and numerous other ASEs show that alternative splicing is globally impacted by the presence of CUG^{exp} mRNAs, and further investigation into the functions of the proteins encoded by these pre-mRNAs and the proteins that regulate the splicing events may indicate new therapeutic targets for DM (**Figure 5**).

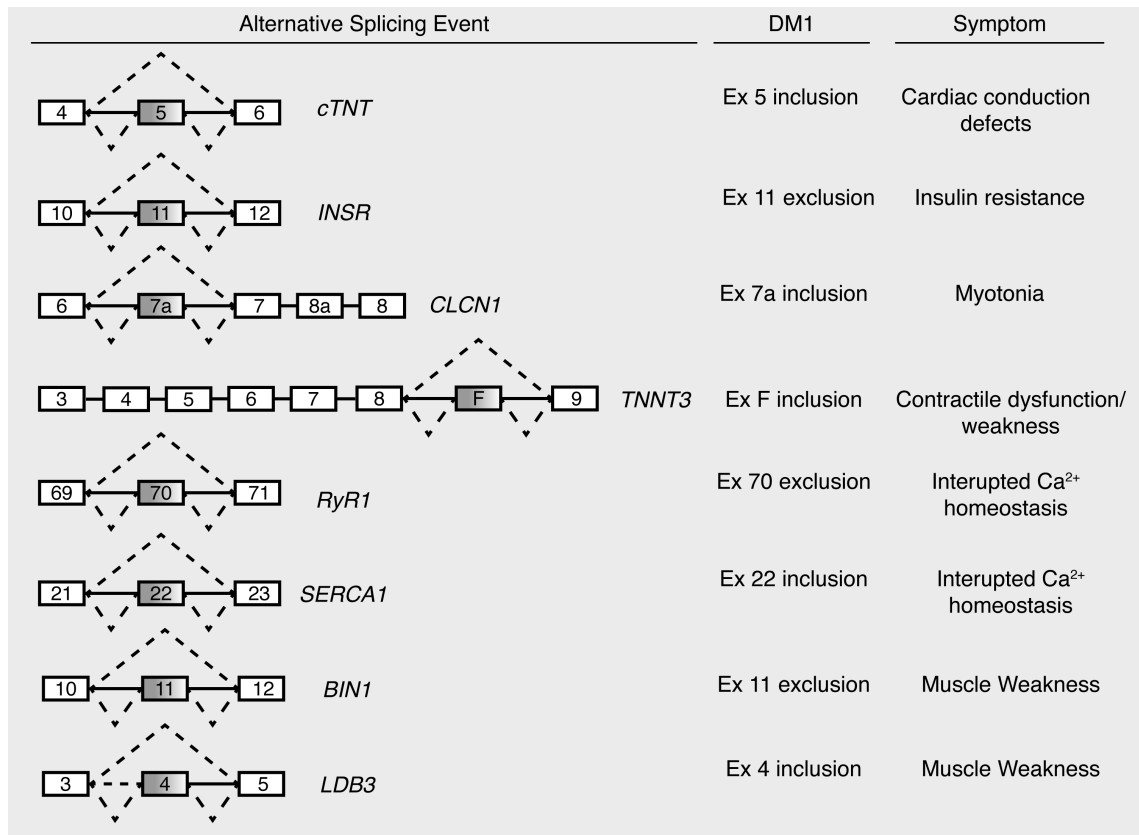


Figure 5. Key alternative splicing events that contribute to the DM1 pathology.

Schematic representation of alternative splicing events in DM1. Alternative exons are represented as grey boxes, while flanking exons are white boxes. Introns are represented as solid lines and the alternative splicing patterns as dashed lines.

1.4.3 Misregulation of RNA-binding Proteins in DM1

Evidence that DM1 is mediated through an RNA-based mechanism specific to the misregulation of RBPs has led to further investigation into their function in skeletal muscle. The MBNL family of RBPs (MBNL1, MBNL2, and MBNL3) are key mediators of DM1 and are sequestered by CUG^{exp} mRNAs leading to MBNL loss of function (Miller et al. 2000; Jiang et al. 2004; Mankodi et al. 2001). In contrast, CUGBP1 is up-regulated in DM1 heart and skeletal muscle (Dansithong et al. 2005; Savkur et al. 2001) as a result of protein stabilization via PKC phosphorylation (Kuyumcu-Martinez et al. 2007).

Multiple mouse models have been generated to study the function of these RBPs to gain a better understanding of their implications in DM1. Individual MBNL mutant mouse models were generated by targeted deletion of exon 3 in MBNL1, MBNL2, or MBNL3 (Charizanis et al. 2012; Poulos et al. 2013; Kanadia et al. 2003). Together these mice recapitulated several phenotypes relevant to DM1 including myotonia, central nucleation, split myofibers, and increased REM sleep (Charizanis et al. 2012; Poulos et al. 2013; Kanadia et al. 2003). Remarkably, distinctive ocular cataracts were present in MBNL1 mutant mice (Kanadia et al. 2003), MBNL2 mutant mice displayed learning and memory deficits (Charizanis et al. 2012), and MBNL3 mutant mice had impaired muscle regeneration (Poulos et al. 2013). More recently, a separate MBNL1 mutant model was created to analyze the consequence of MBNL1 sequestration on splicing defects in the DM1 brain (Suenaga et al. 2012). Unexpectedly, the loss of MBNL1 in the brain did not recapitulate many ASEs observed in the pathology (Suenaga et al. 2012). Together these mouse models demonstrate the impact of MBNL loss of function in mediating several

DM1 symptoms, however, they also highlight that other factors are involved since not all of the symptoms were reproduced.

In an alternative approach, localized adeno-associated virus (AAV)-mediated delivery of MBNL1 in HSA^{LR} mice was performed to test the safety and efficacy of multi-systemic MBNL1 overexpression for the treatment of DM1 (Kanadia et al. 2006).

Remarkably, the upregulation of MBNL1 reversed several ASEs characteristic of DM1, including *CLCN1*, thus rescuing myotonia in HSA^{LR} mice (Kanadia et al. 2006). This study was the first to show that increased expression of MBNL1 is tolerated and appears beneficial in reversing several DM1 symptoms. In addition, when MBNL1 transgenic mice were crossed with HSA^{LR} mice, the muscle myopathy was abolished, and central nucleation was significantly decreased (Chamberlain & Ranum 2012). Although these studies demonstrate that MBNL1 is a key regulator of several ASEs in DM1, they do not provide enough evidence that MBNL1 overexpression is an effective treatment plan. For example, these models are unable to indicate MBNL1's role in repairing muscle weakness, since HSA^{LR} mice do not develop severe myopathy (Mankodi et al. 2000). In addition, the dual transgenic mouse model reverts the homozygous nature of the HSA^{LR} transgene to a less severe hemizygous state, however, the authors compare all analyses to the homozygous HSA^{LR} model (Chamberlain & Ranum 2012). In this case, the improvement in several DM1 features is at least partially due to the loss of the allele containing the transgene rather than MBNL1 overexpression. Overall, these studies demonstrate that MBNL1 sequestration is a key contributor to DM1 and that MBNL1 overexpression may improve some hallmarks of the pathology, however, additional models are required to determine the impact of MBNL1 on other DM1 symptoms.

Several groups have identified a contributing role of CUGBP1 in DM1 symptoms such as delayed skeletal muscle differentiation (Timchenko et al., 2001), myotonia (Charlet-B et al. 2002), and insulin resistance (Savkur et al. 2001). To complement these findings, several muscle-specific CUGBP1 mouse models have been developed (Timchenko et al. 2004; Ho et al. 2005; Ward et al. 2010). In the most severe cases, an ~6-8-fold increase of CUGBP1 severely impaired muscle development and caused death, indicative of congenital DM1 (Timchenko et al. 2004; Ho et al. 2005). In contrast, transgenic mice with an ~2-3-fold increase in CUGBP1 developed mild muscle defects by ~1.5-2 years of age (Timchenko et al. 2004). Analyses of alternative splicing revealed that CUGBP1 overexpression reproduces several ASEs observed in DM1 including the *Clcn1* and *Tnnt2* in skeletal muscle and the heart, respectively (Ho et al., 2005). To further investigate the impact of CUGBP1 on alternative splicing and to avoid the neonatal lethality observed in the previous transgenic mice (Timchenko et al. 2004; Ho et al. 2005), a muscle-specific inducible system was developed (Ward et al., 2010). Transgenic mice with an ~8-fold increase in CUGBP1 expression have morphological and functional deficits (Ward et al., 2010). Extensive analyses of alternative splicing revealed that several ASEs observed in DM1 were recapitulated, including *Clcn1*, *RyR1* and *Serca1* (Ward et al., 2010). Collectively, these mouse models confirm that the misregulation of CUGBP1 in DM1 contributes to muscle defects observed in congenital DM1 and regulates key ASEs linked to the pathology.

Many other RBPs have now been identified as misregulated in human DM1 skeletal muscle and DM1 mouse models (**Figure 4B**). These include members of the heterogeneous ribonucleoproteins (hnRNPs) and the DEAD-box helicase (DDX) families

(Paul et al. 2006; D. H. Kim et al. 2005; Paul et al. 2011; Pettersson et al. 2014; Laurent et al. 2012; Jones et al. 2015). For example, hnRNP H, H2, H3, F, A2/B1, K, DDX5 and DDX17 are all elevated in DM1 myoblasts or cells containing CUG^{exp} mRNAs (Paul et al. 2011). In contrast, DDX5 expression is decreased in DM1 skeletal muscle from HSA^{LR} mice and patient muscle biopsies (Jones et al. 2015). The RBP hnRNP H inhibits the export of mutant *DMPK* mRNAs (D. H. Kim et al. 2005) whereas DDX6 interacts with CUG^{exp} mRNAs and its upregulation rescues several ASEs and decreases the presence of RNA foci by unwinding CUG^{exp} mRNAs *in vitro* (Pettersson et al. 2014). These findings demonstrate that in addition to MBNL1 and CUGBP1 misregulation, several other RBPs also contribute to the DM1 pathology and thus represent additional targets for therapeutic intervention.

As mentioned above, we recently identified that Stauf1 is misregulated in DM1 mouse and human skeletal muscles (Ravel-Chapuis et al. 2012). In attempts to determine the role of Stauf1 in DM1, we identified that Stauf1 interacts transiently with CUG^{exp} mRNAs in a length dependent manner, however it is not sequestered in the RNA foci of DM1 cells (Ravel-Chapuis et al. 2012). Remarkably, we showed that further increasing Stauf1 in DM1 rescues the nuclear export and translation of CUG^{exp} mRNAs (Ravel-Chapuis et al. 2012). In addition, Stauf1 overexpression regulates the alternative splicing of the *INSR* and *CLCN1* pre-mRNAs towards WT expression patterns (Ravel-Chapuis et al. 2012). These results highlight Stauf1 as a novel regulator of alternative pre-mRNA splicing and demonstrate that it is a key player in DM1. Therefore, it is now necessary to expand our knowledge of Stauf1's role in regulating alternative

splicing and the impact of elevated Staufen1 in skeletal muscle to determine its implications in DM1 and to investigate its potential as a therapeutic target.

1.5 Therapeutic Avenues for DM1

Several therapeutic strategies are aimed at disrupting the toxic RNA foci. In this context, the use of ribozymes to target the mutant mRNAs is being investigated. Treatment of DM1 cells with a group I intron ribozyme, DMPK-RZ1, catalyzed the cleavage of 12 CUG repeats and replaced them with 5 repeats in the *DMPK* mRNA providing a proof of principle for the use of ribozymes in DM1 (Phylactou et al. 1998). A subsequent study used a nuclear retained hammerhead ribozyme to target *DMPK* mRNAs (Langlois et al. 2003). Treatment of DM1 myoblasts with this ribozyme reduced both normal and mutant *DMPK* mRNAs and a partial rescue of the *INSR* splicing was observed (Langlois et al. 2003). Although much remains to be examined in terms of ribozyme target specificity and cellular localization, these studies highlight their potential in future DM1 therapeutics.

Antisense oligonucleotides (ASOs) are also being investigated for the treatment of DM1 (Wheeler et al. 2012; Wojtkowiak-Szlachcic et al. 2015). ASOs are synthetic nucleic acids that control gene expression through direct binding of specific sequences on the sense-strand of mRNAs (DeVos & Miller 2013). In the context of DM1, a morpholino ASO composed of CAG repeats (CAG25) was designed to interact with CUG^{exp} mRNAs and block MBNL sequestration (Wheeler et al. 2009). Intramuscular injection of the HSA^{LR} TA muscles reduced RNA foci and corrected MBNL-regulated ASEs, demonstrating their potential as a therapeutic approach for DM1 (Wheeler et al. 2009). Additional ASOs have since been designed to achieve systemic uptake and similar

effects were observed in treated DM1 mice (Wheeler et al. 2012). Based on the success of ASOs targeting CUG^{exp} mRNAs in pre-clinical studies (Lee et al. 2012; Wheeler et al. 2009; Wheeler et al. 2012; Wojtkowiak-Szlachcic et al. 2015), phase 2 clinical trials are ongoing with the ASO-based drug ISIS-DMPK_{rx} developed through Ionis pharmaceuticals.

In addition, the use of small molecules has been examined as a mode to disrupt MBNL binding to CUG^{exp} mRNAs (Warf et al. 2009; Childs-disney et al. 2007; Gareiss et al. 2008; Chakraborty et al. 2015; Childs-Disney, Hoskins, et al. 2012; Nakamori et al. 2016; Nguyen et al. 2015; Li et al. 2016; Childs-Disney, Parkesh, et al. 2012; Lee et al. 2009). For example, Disney and colleagues developed several small molecules including peptides containing Kanamycin A modules that have a high affinity for CUG expansions and treatment of DM1 cultured cells and mouse models rescued several ASEs that contribute to the pathology (Childs-Disney, Parkesh, et al. 2012; Childs-disney et al. 2007; Lee et al. 2009). Additional small molecules also show promise for the treatment of DM1, specifically, HeLa cells expressing 960 CUG repeats treated with pentamidine rescued the alternative splicing of the *INSR* and *cTNT* pre-mRNAs (Warf et al. 2009). Moreover, systemic treatment of HSA^{LR} mice partially rescued the alternative splicing of *Cln1* and *Serca1* pre-mRNAs (Warf et al. 2009). Interestingly, a global rescue of ASEs was not observed and rather only MBNL-regulated splicing events were altered in these studies indicating that the disruption of MBNL sequestration is not sufficient to treat all DM1 symptoms.

An alternative therapeutic approach for DM1 is centered on restoring the expression levels of RBPs that function as disease modifiers. In addition to MBNL1, which acts as a

major regulator of the DM1 pathology in skeletal muscle, several other RBPs including CUGBP1, hnRNP H, hnRNP F, DDX5, DDX6 and DDX17 have prominent roles (Paul et al. 2006; D. H. Kim et al. 2005; Paul et al. 2011; Pettersson et al. 2014; Laurent et al. 2012; Jones et al. 2015). Since RBPs are often multi-functional, it may not be feasible to drastically increase or decrease their expression, however, modulating the expression of these RBPs in attempts to fine-tune disease mechanisms is an attractive therapeutic strategy. For example, the treatment of DM1 myoblasts and HSA^{LR} mice with inhibitors of glycogen synthase kinase β (GSK3 β) restores cyclin D3/CDK4-mediated phosphorylation of CUGBP1, which rescues the expression of several CUGBP1-regulated translational targets such as *p21* and promotes muscle differentiation (Jones et al. 2012; Salisbury et al. 2008). Remarkably, this treatment is sufficient to improve morphological and functional deficits in HSA^{LR} mice including myotonia. These data support the notion that the modulation of disease modifiers in DM1 is sufficient to rescue key hallmarks of the disease.

1.6 Statement of Problem and Hypothesis

Despite significant advances over the past decade in our understanding of the mechanisms that mediate skeletal muscle formation, much remains to be determined. While the expression of MRFs is required for transcriptional activation of muscle genes, converging lines of evidence demonstrate the importance of post-transcriptional events in skeletal muscle formation, maintenance and repair. RBPs have emerged as key mediators of post-transcriptional regulation in skeletal muscle and importantly RBPs are often implicated in neuromuscular disease. Although the increased expression of the RBP, Staufen1 initially appeared beneficial for DM1, given its multi-functional nature, we hypothesize that Staufen1 functions as a disease modifier in the DM1 pathology. To test this hypothesis, we formulated the following objectives:

Objectives:

1. Characterize the impact of Staufen1 during myogenic differentiation.
2. Determine if Staufen1 also plays a role in cancer biology, specifically in Rhabdomyosarcoma.
3. Generate muscle-specific Staufen1 transgenic mouse lines and characterize the impact of sustained Staufen1 expression in postnatal skeletal muscle.
4. Determine if Staufen1 regulates a broad range of alternative splicing events in WT and DM1 skeletal muscle.
5. Investigate the impact of elevated Staufen1 expression in skeletal muscles of DM1 mouse models.

Chapter 2: Novel Roles of Staufen1 During Myogenic Differentiation

The RNA-binding protein Staufen1 impairs myogenic differentiation via a c-myc-dependent mechanism

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Author Contributions

In the following manuscript, the data presented in Figure 6, Figure 7 and Figure 11 was generated and analyzed by C.T.R, M.L.B.C and A.R.C. The data presented in Figure 8, Figure 9, Figure 10, Figure 12, Figure 13, and Figure 14 was generated and analyzed by T.E.C, M.L.B.C, and A.R.C. DNA constructs were developed by G.B., T.E.C and A.R.C. and the manuscript was written by A.R.C., T.E.C. and B.J.J.

Abstract

Recent work has shown that Staufen1 plays key roles in skeletal muscle, yet little is known about its pattern of expression during embryonic and postnatal development. Here, we first show that Staufen1 levels are abundant in mouse embryonic muscles and that its expression decreases thereafter, reaching low levels in mature muscles. A similar pattern of expression is seen as cultured myoblasts differentiate into myotubes. Muscle degeneration/regeneration experiments revealed that Staufen1 increases following cardiotoxin injection before returning to the low levels seen in mature muscles. We next prevented the decrease in Staufen1 during differentiation by generating stable C2C12 muscle cell lines overexpressing Staufen1. Cells overexpressing Staufen1 differentiated poorly as evidenced by reductions in the differentiation and fusion indices, and by decreases in MyoD, myogenin, MEF2A and MEF2C, independently of Staufen-Mediated mRNA Decay (SMD). However, levels of c-myc, a factor known to inhibit differentiation, were increased in C2C12 cells overexpressing Staufen1 through enhanced translation. By contrast, the knockdown of Staufen1 decreased c-myc levels in myoblasts. Collectively, our results show that Staufen1 is highly expressed during early stages of differentiation/development and that it can impair differentiation by regulating c-myc thereby highlighting the multifunctional role of Staufen1 in skeletal muscle cells.

Introduction

Skeletal muscle cell development, or myogenesis, is a tightly regulated process.

Progenitor cells originating from somites are determined for the myogenic lineage and become proliferating myoblasts. Upon receiving proper signals, myoblasts undergo terminal differentiation by withdrawing from the cell cycle and fusing to form multinucleated myotubes. This myogenic terminal differentiation step involves the orchestrated expression of myogenic regulatory factors such as MyoD, myogenin and MEF2, as well as cell cycle regulators including p21 and c-myc (Berkes & Tapscott 2005; Buckingham & Vincent 2009; Bentzinger et al. 2012; Bismuth & Relaix 2010). Although transcriptional control of myogenic events is now well recognized, post-transcriptional regulation has emerged over the years as another key level of regulation necessary for complete muscle differentiation. In particular, RNA-binding proteins such as K-homology Splicing Regulator Protein (KSRP), HuR, CUG-Binding Protein (CUGBP) and Muscleblind-like Protein (MBNL) have been reported to play a functional role in muscle cell proliferation and differentiation by affecting mRNA stability, decay and/or translation of key target transcripts encoding proteins essential for differentiation (Timchenko, Lakova, et al. 2001; Figueroa et al. 2003; Briata et al. 2005; Apponi et al. 2011; Amirouche et al. 2013; Deschênes-Furry, Angus, et al. 2005; Squillace et al. 2002). For example, it is well known that both HuR and KSRP bind to AU-rich elements (ARE) present in the 3'UTR of specific mRNAs yet, they regulate their stability in an opposite manner. While located predominately in the nucleus in myoblasts, HuR shuttles to the cytoplasm upon induction of differentiation where it binds and stabilizes p21, MyoD and myogenin mRNAs resulting in cell cycle arrest and promotion of myogenesis (Van Der

Giessen et al. 2003; Figueroa et al. 2003; Deschênes-Furry, Angus, et al. 2005; Beauchamp et al. 2010; von Roretz et al. 2011). In parallel, KSRP, which typically destabilizes target transcripts, dissociates from the common mRNA targets p21 and myogenin thereby also causing enhanced mRNA stability and hence, acting in a coordinated fashion with HuR (Briata et al. 2005).

As part of our efforts to identify RNA-binding proteins that play key roles in skeletal muscle, we became interested several years ago in Staufén (Belanger *et al.*, 2003). Originally discovered in *Drosophila*, Staufén is a RNA-binding protein that associates with extensive RNA secondary structures through one or more double-stranded RNA-binding domains (Marion *et al.*, 1999; Wickham *et al.*, 1999). Studies in mammals revealed the existence of two genes named *Staufen1* and *Staufen2*. The exact roles of *Staufen1* have yet to be defined in mammals, however, it is known to be a component of RNA granules. In neurons, *Staufen1*-containing granules are transported in dendrites in a microtubule-dependent manner, suggesting that *Staufen1* is also involved in mRNA transport (Kiebler et al. 1999; Köhrmann et al. 1999; Krichevsky & Kosik 2001; Mallardo et al. 2003; Brendel et al. 2004; Kanai et al. 2004). This is supported by the observation that cultures of hippocampal neurons isolated from mice expressing a mutant form of *Staufen1*, which lacks the third RNA-binding domain, display impairments in the dendritic delivery of mRNA-containing ribonucleoprotein particles, as well as a clear reduction in spine morphogenesis (Vessey et al. 2008). *Staufen1* assumes other functions in mammalian cells in addition to its conserved role in mRNA transport. For example, *Staufen1* regulates the translational efficiency of a sub-population of transcripts when bound to their 5'UTR (Dugré-Brisson et al. 2005). Furthermore, when *Staufen1* binds

downstream of a natural termination codon, it appears to elicit RNA degradation by a mechanism referred to as Staufen1-mediated mRNA decay (SMD) (Y. K. Kim et al. 2005). Finally, we recently discovered a novel function of Staufen1 in alternative pre-mRNA splicing (Ravel-Chapuis et al. 2012). Subsequent work has shown that Staufen1 interacts with splicing factors in 293T cells (Milev et al. 2012), and that it binds preferentially to transcripts that are alternatively spliced in *Drosophila* cells (Laver et al. 2013), thereby confirming our initial observation for a role of Staufen1 in pre-mRNA splicing. Altogether, it is therefore clear that Staufen1 is a multifunctional protein involved in the regulation of distinct cellular events.

In a previous study, we characterized the skeletal muscle expression of Staufen1 as well as its preferential accumulation within the postsynaptic sarcoplasm of neuromuscular junctions (Bélanger et al. 2003; see also Gardiol & St Johnston 2014). In fact, we were the first to report that Staufen1 levels are regulated during myogenic differentiation of cultured cells, and that its level of expression varies according to the state of innervation and the phenotype of muscle fibers. These initial observations suggest that Staufen1 is a key component of muscle fiber plasticity and maturation. Others have later confirmed the involvement of Staufen1 in myogenic differentiation, as the depletion of Staufen1 from C2C12 myoblasts achieved by siRNA promotes the spontaneous formation of myotubes in the absence of myogenesis induction (Yamaguchi et al. 2008). In this context, it was also proposed that Staufen1 regulates myogenic differentiation by participating in the balance between two competing mRNA decay pathways: SMD (see above) and nonsense-mediated mRNA decay (NMD). More specifically, it has been proposed that upon induction of differentiation, the efficiency of

SMD is increased while that of NMD is reduced. As a consequence, SMD targets such as Paired box 3 (*Pax3*) mRNAs are destabilized while classical NMD targets, i.e. myogenin transcripts, are stabilized (Gong et al. 2009). More recently, it was also reported that Staufen1-binding sites can be formed by intermolecular duplexing between short-interspersed elements (SINEs) found in the 3'UTR of target transcripts and those found in some long non-coding RNAs which, functionally, can expand the list of putative SMD targets (Wang et al. 2013).

Despite these advances, it remains unknown whether Staufen1 is also regulated during mouse skeletal muscle development *in vivo*. In addition, the contribution of Staufen1 to the regulation of major myogenic regulatory factors and transcription factors such as MyoD or MEF2 remains unclear. Here, we report the pattern of expression of Staufen1 during embryonic and postnatal skeletal muscle development. In addition, we complement these findings by also examining the expression profile of Staufen1 during cardiotoxin-induced muscle degeneration/regeneration, a well described *in vivo* model of myogenic differentiation. Finally, to better define mechanistically the role of Staufen1 in myogenic differentiation, we generated stable muscle cell lines overexpressing Staufen1 and assessed the functional consequences of this increased expression on several markers of the myogenic program. Collectively, our results show that Staufen1 is highly expressed during early stages of muscle differentiation/development and that it progressively decreases as differentiation proceeds reaching low levels in mature muscle. Moreover, we show that sustained expression of Staufen1 impairs differentiation by markedly reducing expression of key myogenic regulatory factors in a SMD-independent

manner while promoting translation of c-myc which causes undifferentiated myoblasts to remain in a proliferative state.

Results

Staufen1 Expression Decreases During Mouse Skeletal Muscle Development

To better understand the role of the RNA-binding protein Staufen1 in skeletal muscle *in vivo*, we sought to investigate its temporal pattern of expression during mouse skeletal muscle development. We performed Western blot analyses using protein extracts collected from wild-type mice at different stages of embryonic and postnatal development ranging from embryonic day 14.5 (E14.5) to adult (14 weeks). Given the small size of limbs of mouse embryos, the whole muscle mass of the leg was used for embryonic days E14.5, E18.5, and postnatal day PN1. Tibialis anterior (TA) muscles were used for adult mice.

As a control, we first examined expression of CUGBP1, a developmentally regulated RNA-binding protein in skeletal muscle (Ladd et al. 2001; Ladd et al. 2005; Lin et al. 2006). We show that its expression is significantly decreased during skeletal muscle development ($P < 0.001$) recapitulating expression profiles previously observed (**Figure 6A,B**). Then, we analyzed Staufen1 levels and show that Staufen1 is highly abundant in embryonic muscle limbs at E14.5 (**Figure 6A,C**). However, expression of Staufen1 decreases gradually ($P < 0.001$) throughout skeletal muscle mass development, resulting in a low level of expression in mature adult muscle. Since the whole muscle mass was used in these experiments, we cannot exclude that Staufen1 is decreased in several cell types contained within developing muscle tissues. By contrast, GAPDH and β -actin were used as loading controls and show an increased expression during muscle mass development (**Figure 6A**). Together, these results suggest that expression of the RNA-

binding protein Stauf1 is developmentally regulated during mouse skeletal muscle development.

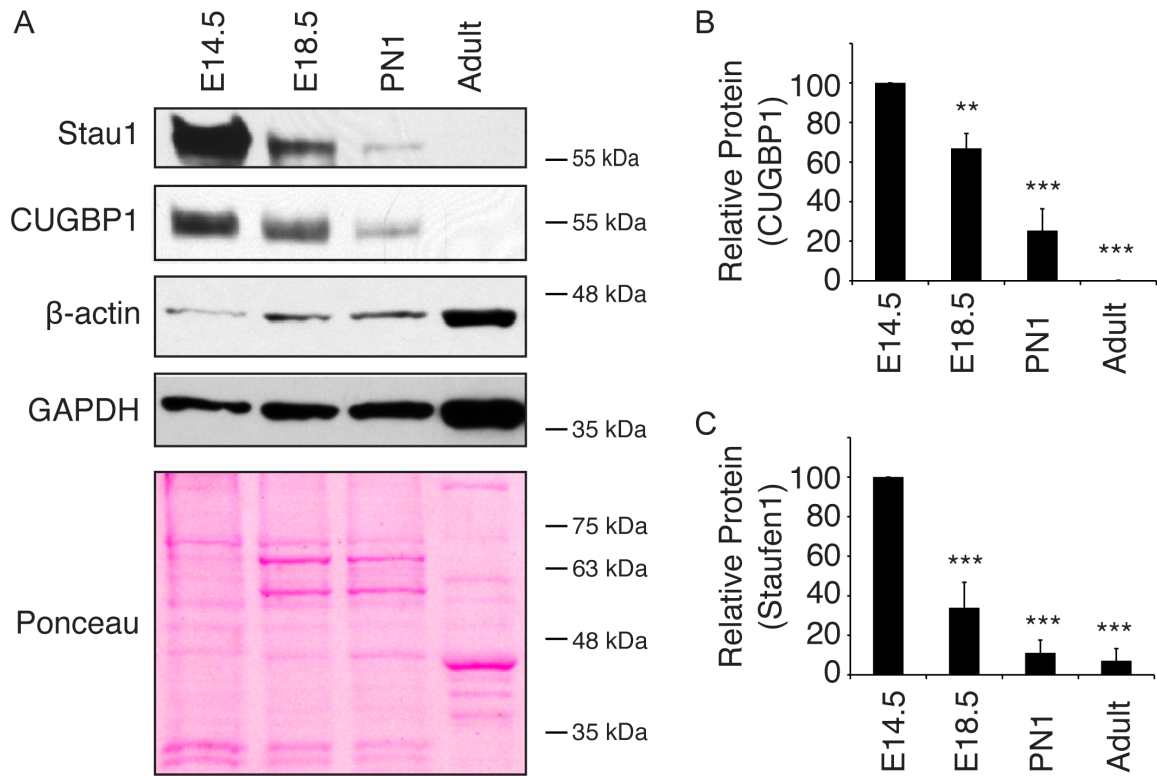


Figure 6. Stau1 decreases in developing wild-type muscle. (A) Representative Western blots showing Stau1, CUGBP1, β-actin and GAPDH protein levels during skeletal muscle development. Samples were from embryos (E14.5 and E18.5), new-born (PN1) and adult mice (14 weeks). Ponceau staining was used to show equal loading. (B) and (C) Relative quantification of Stau1 and CUGBP1 protein levels, respectively (n=3). Asterisks indicate significance (**P ≤ 0.01, and ***P ≤ 0.001).

Expression of Staufen1 is Modulated During Muscle Regeneration

To further show that expression of Staufen1 is regulated during muscle development *in vivo*, we also performed muscle regeneration experiments. Briefly, we injected cardiotoxin (CTX) in wild-type adult mouse TA muscles to induce muscle degeneration. Following the initial degeneration period, muscle stem cells become activated, fuse and differentiate to repair damaged fibers and create new ones, thereby partially recapitulating characteristics of myogenesis that occur during embryonic development (Bentzinger et al. 2012; Condrea 1974; Yin et al. 2013).

Western blots were performed using TA protein extracts obtained two, four, seven and 14 days post-cardiotoxin injection. Myogenin protein expression was initially analyzed. Note that in contrast to myotubes, adult muscles express low levels of myogenic regulatory factors including myogenin, because their expression is repressed by electrical-activity generated by motor neurons (Eftimie et al. 1991). Myogenin showed an increase ($P < 0.001$) in expression immediately after injury thereby confirming the induction of muscle regeneration. This was followed by a steady decrease ($P < 0.001$ and $P < 0.05$) in myogenin expression levels as the regeneration process advanced to completion fourteen days after cardiotoxin injection (**Figure 7A,B**). As a control, GAPDH expression was measured and showed a slight decrease in protein levels at day two and four post-injury as previously described (Orengo et al. 2011). In addition, Ponceau staining was used to confirm relatively even loading. Next, we measured Staufen1 protein expression and observed that Staufen1 gradually increased ($P < 0.001$) from two to seven days post-cardiotoxin treatment (**Figure 7A,C**). Staufen1 expression then returned to control levels fourteen days post-injury when muscle fibers are fully

regenerated. Variations of Staufen1 levels within different time-points reflect inter-individual variability of protein expression, which is commonly observed when using animal tissues. This induction of Staufen1 following cardiotoxin injection follows a similar pattern as the one observed with CUGBP1, also involved in the regulation of myogenic differentiation (Orengo et al. 2011).

To determine the cell type in which Staufen1 is increased during regeneration, immunofluorescence experiments were performed using Staufen1 antibodies on cryostat cross-sections of TA muscles obtained four and seven days post-cardiotoxin injections and control, saline-injected muscles. Laminin was used to delineate individual muscle fibers. In normal mature fibers, Staufen1 is expressed at low levels but faint cytoplasmic staining at the sub-sarcolemma can be distinguished (**Figure 7E,F**). In sharp contrast, Staufen1 levels are markedly increased in the cytoplasm of muscle fibers 4 days post-cardiotoxin injection, mostly in the perinuclear region of centrally-located nuclei (**Figure 7E**). Such relocalization of Staufen1 during muscle regeneration likely reflects a need for an enhanced nuclear function of Staufen1 at this regeneration step. Seven days post-injury, Staufen1 partially returns to the periphery of muscle fibers but remains strongly associated with nuclei and perinuclear regions (**Figure 7F**). Therefore, our results clearly show an increase of Staufen1 levels within regenerating skeletal muscle fibers thereby further indicating that Staufen1 is regulated during mouse skeletal myogenesis *in vivo*.

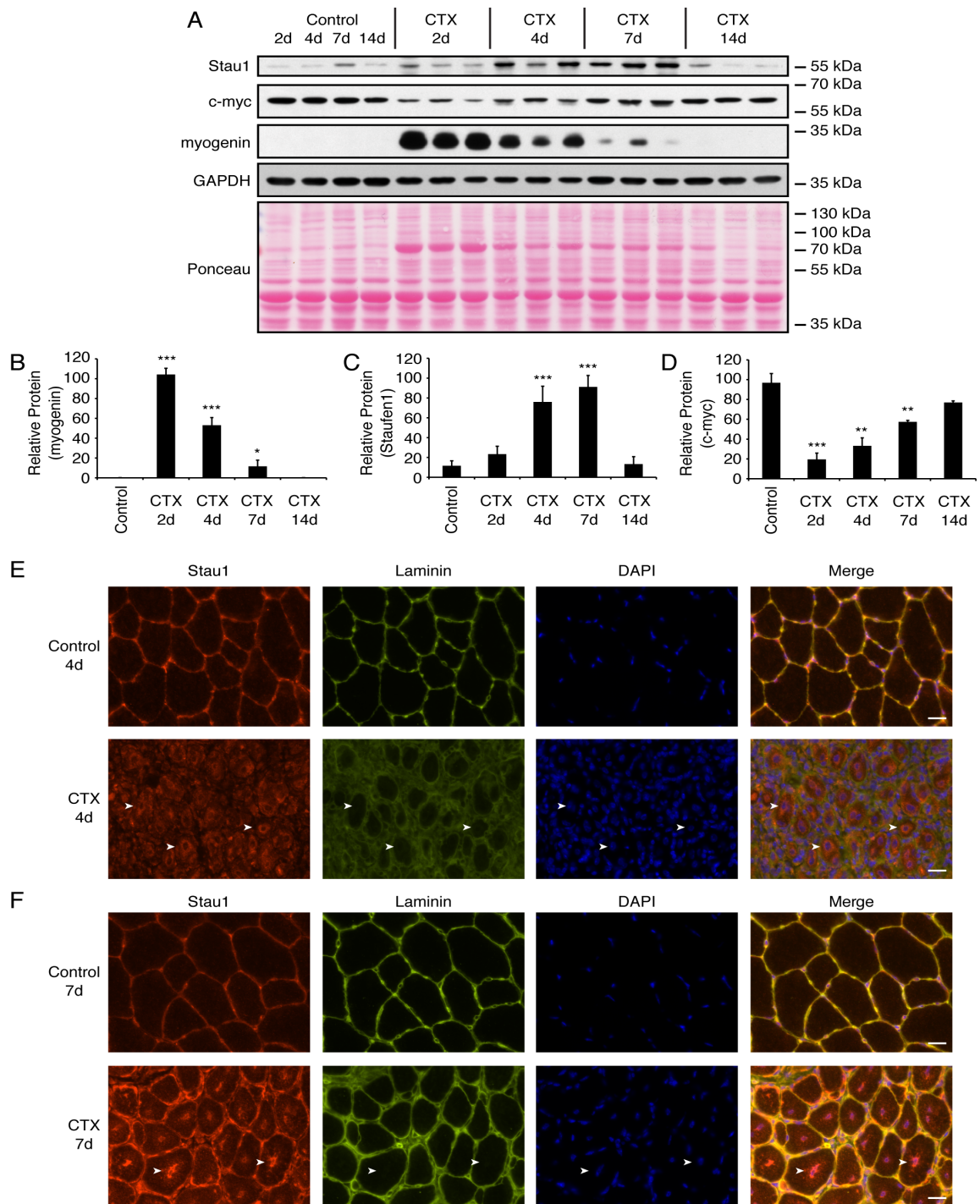


Figure 7. Staufen1 expression is modulated during skeletal muscle degeneration/regeneration. (A) Western blots showing Staufen1, c-myc and myogenin protein levels in regenerating tibialis anterior (TA) muscles. TA muscles were injected

with cardiotoxin (CTX) to induce muscle degeneration and harvested at 2, 4, 7 and 14 days post-injection. Saline-injected muscles were used as controls. GAPDH and Ponceau staining were used to show equal loading. **(B)**, **(C)** and **(D)** Relative quantification of myogenin, Staufen1, and c-myc expression levels, respectively. (n=3). **(E)** and **(F)** Immunofluorescence on cryostat cross-sections of control and CTX-injected TA muscles. Sections were stained with a Staufen1 (Red) and Laminin (Green) antibodies and nuclei with DAPI (Blue). Arrows point to marked staining of Staufen1. Same exposure parameters were used to allow the comparison of signal intensity. Using these parameters, a no primary antibody control shows no signal. Scale bars = 20 um. Asterisks indicate significance (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$).

Levels of Staufen1 Also Decrease During Myogenic Differentiation of Cultured Cells

Primary human skeletal muscle cells (SkMC) and myoblasts (HSMM) isolated from normal donors were also used to assess expression of Staufen1 during myogenic differentiation. The switch from high to low serum culture conditions induces myoblasts to exit the cell cycle and initiates differentiation by promoting the fusion of primary myoblasts into elongated, multinucleated myotubes. We thus measured the level of Staufen1 protein expression by Western blot during human primary cell differentiation and observed a progressive decrease in Staufen1 levels as cells differentiate (**Figure 8A,B**). In fact, Staufen1 levels steadily decreased within one to four days after the switch to low-serum, a time corresponding to the engagement of cells into the differentiation process as confirmed by the induction of myogenin (**Figure 8A**).

Mouse C2C12 myoblasts consist of a myogenic cell line derived from satellite cells, which have been extensively used over the years as a model system to study myogenic differentiation *ex-vivo* (Yaffe & Saxel 1977). We were the first to report that Staufen1 is expressed and regulated in skeletal muscles (Bélanger et al. 2003). However, following this initial discovery, discrepancies in the levels of Staufen1 protein and the role of Staufen1 during myogenic differentiation were reported (Kim et al. 2007; Yamaguchi et al. 2008; Gong et al. 2009; Yamaguchi et al. 2012). Conflicting data and conclusions over time, even within articles from the same laboratories led us to reanalyze the level of Staufen1 in these cells. We therefore also measured the levels of Staufen1 and myogenin proteins during C2C12 differentiation (**Figure 8C,D**) and observed a steady decrease of Staufen1 ($P < 0.05$) while myogenin is induced ($P < 0.001$). This decreasing pattern of Staufen1 expression as cells mature, parallels in pure myogenic cell populations derived

from both human and mouse, the patterns observed during embryonic muscle development and muscle regeneration. Together, these latter findings indicate that the patterns of Stauf1 expression observed *in vivo* reflect, at least partially, the modulation of Stauf1 levels within muscle cells.

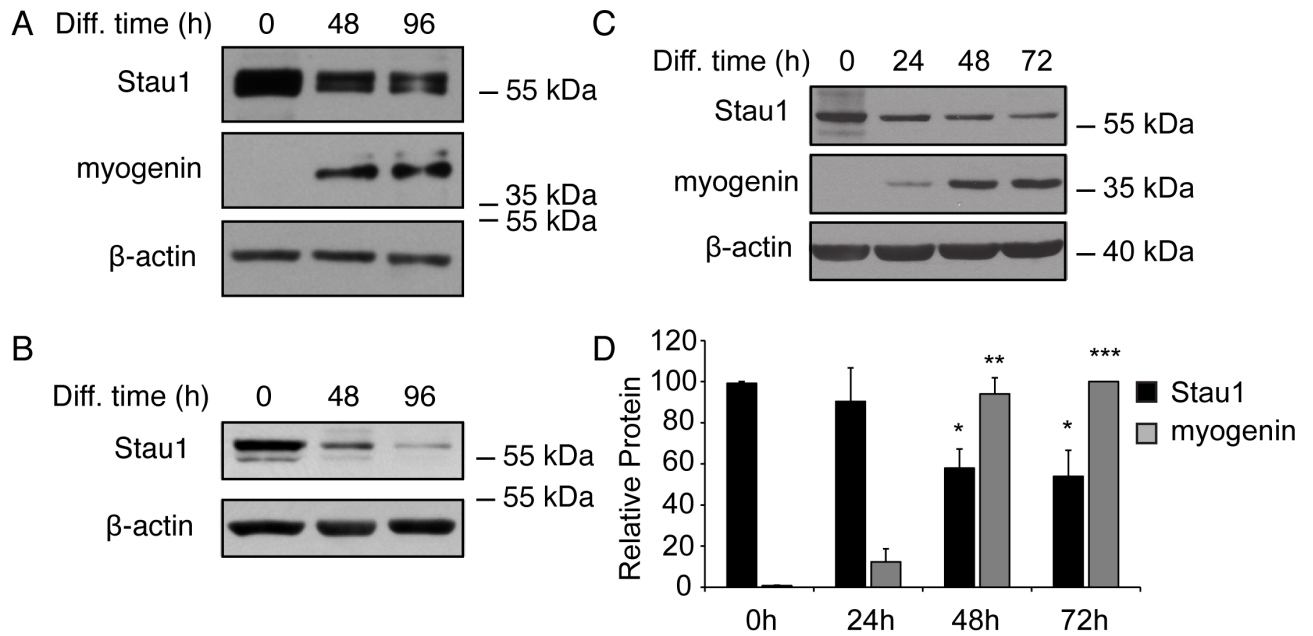


Figure 8. Stau1 decreases during myogenic differentiation. (A) and (B) Western blots showing Stau1 and myogenin protein levels in differentiating primary human skeletal muscle cells (SkMC) and myoblasts (HSMM), respectively. β-actin was used as a loading control. (C) Representative Western blots showing Stau1 and myogenin protein levels in differentiating C2C12 cells. β-actin was used to show equal loading. (D) Relative quantification of Stau1 and myogenin protein levels from differentiating C2C12 cells, respectively (n=3). Asterisks indicate significance (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$).

Increased Expression of Staufen1 Reduces the Efficiency of Myogenic

Differentiation

To specifically determine the impact of Staufen1 in skeletal muscle cell development and investigate its involvement in myogenic control, we sought to prevent the decrease of Staufen1 by establishing stable C2C12 myoblast clones that overexpress a HA-tagged Staufen1 construct. Stable C2C12 myoblasts were obtained by selecting for neomycin resistance with G418. Neomycin resistant clones were then screened for Staufen1-HA expression by Western blotting using an anti-HA antibody (**Figure 9A**). Two clones #15 and #25 were selected along with a control cell line established with a pcDNA3 control vector. Comparative analysis between control and Staufen1-HA #25 were performed by Western blot using anti-Staufen1 antibodies, and showed that Staufen1-HA is overexpressed by at least ~40% in clone #25 (data not shown).

To test the effect of Staufen1-HA overexpression, we initially performed morphological analyses to assess the kinetics and efficiency of differentiation of the stable cell lines. Fusion of myoblasts into myotubes in both clones #15 and #25 was substantially reduced at 96 hours of differentiation as shown by immunofluorescence using a pan-Myosin Heavy Chain (MyHC) antibody (**Figure 9B**). Decreased myoblast fusion likely results from a defect in the ability of cells to fuse but it can also be caused by a decrease in the differentiation potential of myoblasts. To further quantify this defect in differentiation, the fusion index (percentage of nuclei within myotubes having ≥ 3 nuclei) and differentiation index (percentage of nuclei within myotubes plus MyHC-positive mononucleated cells) were determined. Both fusion and differentiation indices were significantly ($P < 0.05$) decreased in clones #15 and #25 at 96 hours of

differentiation (**Figure 9C,D**). In addition, the decrease in fusion and differentiation indices was accompanied by a reduction ($P < 0.05$) in the overall myotube surface area (**Figure 9E**).

To complement these morphological analyses and further characterize the differentiation defects, we analyzed expression of two key muscle genes expressed during the early and later stages of muscle differentiation. Protein and mRNA expression were determined via Western blot and quantitative RT-PCR (qRT-PCR), respectively. Our results show that overexpression of Stauf1-HA induced a dramatic reduction ($P < 0.001$) in the expression of myogenin and MyHC in clones #15 and #25 as assessed by Western blotting (**Figure 10A,B,C**). The decrease in myogenin expression was also confirmed ($P < 0.05$) at the transcript level by qRT-PCR (**Figure 10D**).

C2C12 myoblasts can spontaneously lose the ability to differentiate and fuse into myotubes due to selection through successive passages. In addition, selection of individual stable clones can result in the selection of myoblasts with altered intrinsic differentiation potential. Therefore, to ensure that phenotypic and molecular changes observed in clones #15 and #25 are due to the expression of exogenous Stauf1-HA, transient transfections were also performed (**Figure 10E**). The results obtained from transient transfections also showed a pronounced reduction in myogenin expression in Stauf1-HA-overexpressing cells, which importantly supports and reproduces findings obtained with stable cell lines thereby ruling out a clonal effect.

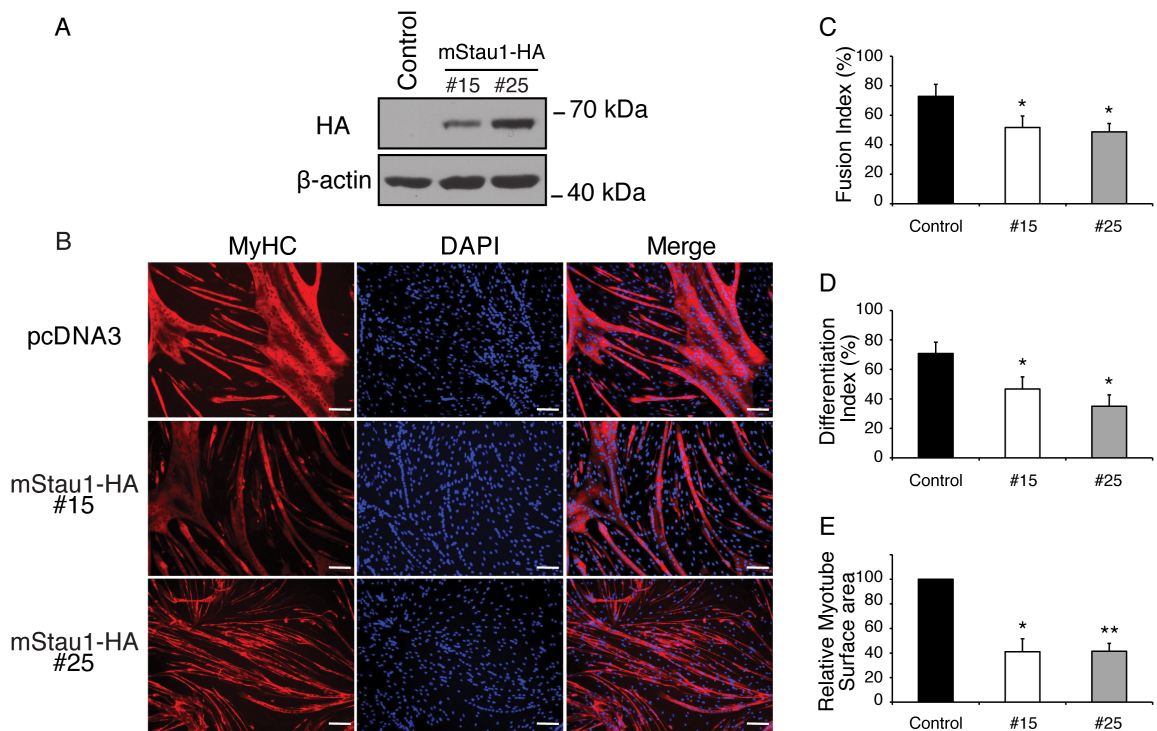


Figure 9. Stau1 overexpression inhibits myogenic differentiation. (A) Representative Western blots showing expression of Stau1-HA in stable C2C12 cell lines. Two clones displaying transgene expression were chosen (#15 and #25) along with a control stable cell line. (B) Immunofluorescence of stable cell lines after 96 hours of differentiation. Cells were stained with a pan-MyHC antibody (Red) and nuclei with DAPI (Blue). Scale bars = 100 μ m. (C) Fusion Index (n = 5), (D) Differentiation Index (n = 5), and (E) Myotube Surface Area (n = 3) of stable cell lines after 96 hours of differentiation. Asterisks indicate significance (* $P \leq 0.05$, and ** $P \leq 0.01$).

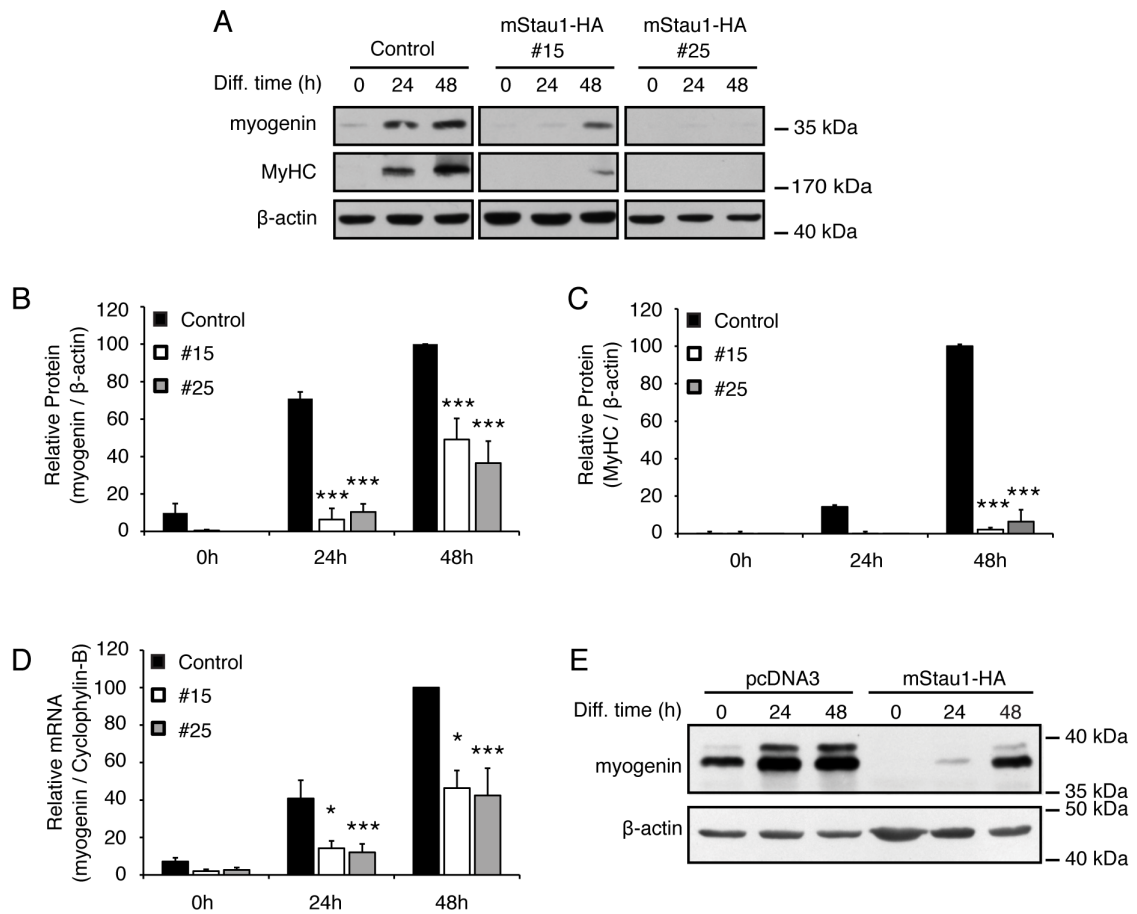


Figure 10. Staufen1 decreases the expression of the myogenic markers myogenin and Myosin Heavy Chain (MyHC). (A) Representative Western blots showing myogenin and MyHC expression during differentiation of Staufen1-HA stable C2C12 cells. β -actin was used to show equal loading. (B) and (C) Relative quantification of myogenin and MyHC protein levels, respectively ($n = 3$). (D) Relative quantification of myogenin mRNA levels as determined by qRT-PCR ($n=4$). Levels were normalized to Cyclophilin-B. (E) C2C12 cells were transiently transfected with mouse Staufen1-HA or a control empty vector (pcDNA3). 24 hours following transfection, cells were switched to differentiation medium and differentiated for the indicated time. Western blots showing

myogenin expression in transfected cells. β -actin was used as a loading control. Asterisks indicate significance (* $P \leq 0.05$, and *** $P \leq 0.001$).

The Decrease in Differentiation Potential is MyoD-independent

We then sought to investigate whether Staufen1 regulates expression of the myogenic regulatory factor MyoD, a crucial regulator of myogenesis (Berkes & Tapscott 2005; Buckingham & Vincent 2009; Bismuth & Relaix 2010; Bentzinger et al. 2012). We found that during proliferation and differentiation, expression of MyoD was significantly reduced ($P < 0.05$) in both Staufen1-HA-overexpressing clones compared to control cells (**Figure 11A,B,C**). To assess whether the addition of exogenous MyoD could rescue the differentiation defect, we performed MyoD add-back experiments. Briefly, stable cell lines were transfected with MyoD-Flag-myc or control vectors. Following transfection, cells were allowed to differentiate for three days. First, the increase in MyoD expression was confirmed by Western blot using MyoD antibodies. In these blots, the lower band corresponds to endogenous MyoD and the upper band to ectopic MyoD-Flag-myc (**Figure 11D**). Note that we were able to restore normal MyoD levels in Staufen1-HA stable cell lines (compare CTL+pcDNA3 and #25+MyoD). Then, the efficiency of differentiation was assessed by measuring expression of myogenin and by immunofluorescence using a pan-MyHC antibody. As illustrated in **Figure 11E, F** and **G**, no rescue of differentiation was observed in the Staufen1-HA stable cell line upon addition of MyoD-Flag-myc (compare both Staufen1-HA cells transfected with MyoD-Flag-myc to control cells transfected with MyoD-Flag-myc, and to Staufen1-HA cells without MyoD-Flag-myc). Taken together, these findings show that the inhibition of differentiation induced by Staufen1 is MyoD-independent.

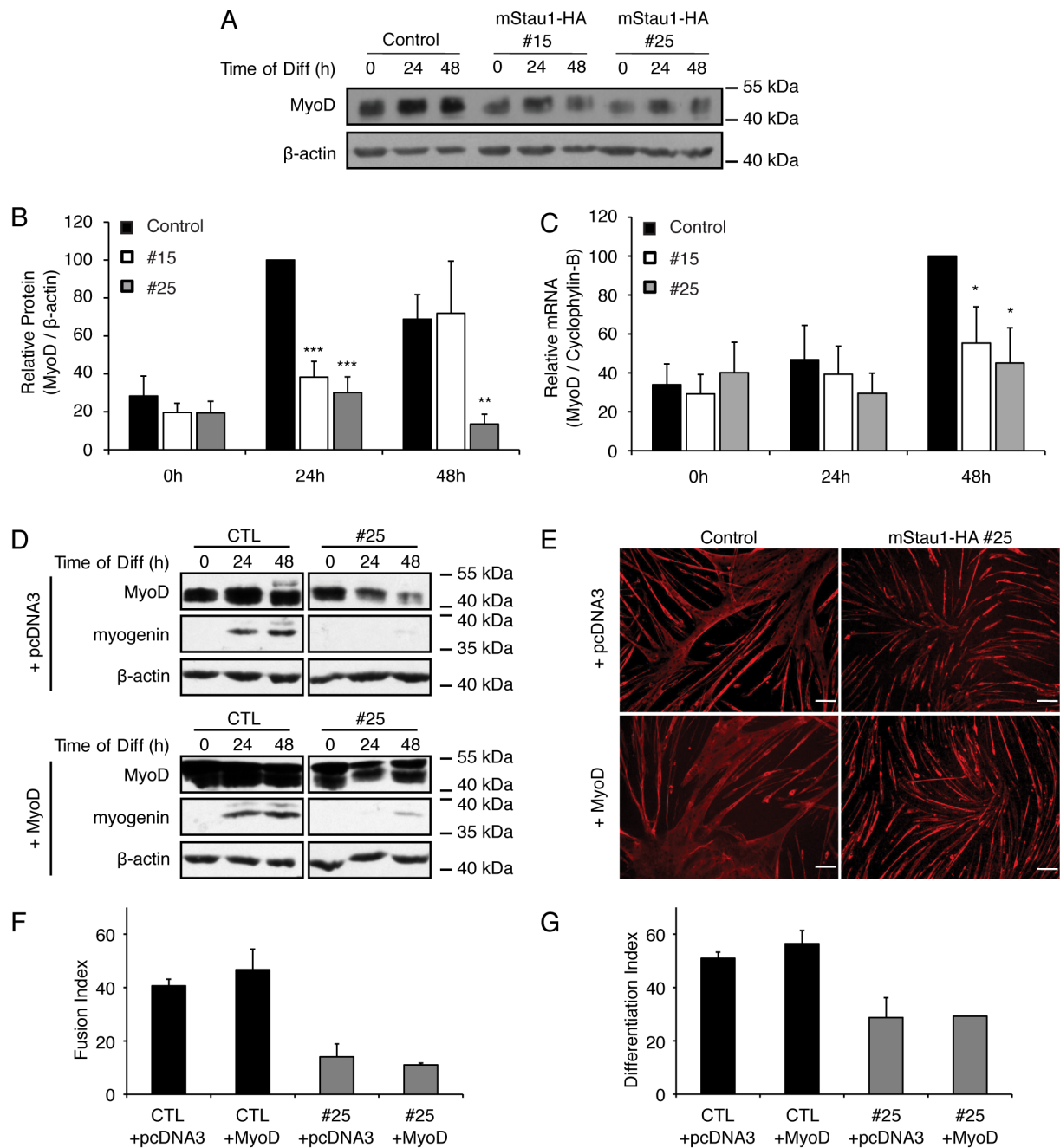


Figure 11. MyoD does not rescue the differentiation defect. (A) Representative Western blots showing MyoD protein level during myogenic differentiation of Staufen1-HA stable cell lines. β-actin was used as a loading control. (B) Relative quantification of MyoD protein levels normalized to β-actin (n = 4). (C) Relative quantification of MyoD mRNA levels as determined by qRT-PCR. Levels were normalized to Cyclophilin-B (n

= 4). **(D)** Stable cell lines were transfected with control or MyoD expression vectors. 24 hours following transfection, cells were allowed to differentiate for the indicated time period. Western blots showing MyoD (lower band, endogenous MyoD; upper band, ectopic MyoD-Flag-myc protein) and myogenin expression levels. β -actin was used to show equal loading. **(E)** shows immunofluorescence using a pan-MyHC antibody. **(F)** and **(G)** correspond to fusion and differentiation indices of C2C12 cells differentiated for 72 hours. Scale bars = 100 μ m. Asterisks indicate significance (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$).

Overexpression of Staufen1 Decreases Levels of MEF2 mRNAs in a SMD-independent Manner

It is well known that the transcription factor Myocyte Enhancer Factor-2 (MEF2) synergizes with MyoD to control the myogenic program (Molkentin et al. 1995; Berkes & Tapscott 2005; Buckingham & Vincent 2009; Bismuth & Relaix 2010; Bentzinger et al. 2012; Lilly et al. 1994). We therefore examined the mRNA levels of MEF2A and MEF2C by qRT-PCR in the Staufen1-HA-overexpressing clones. MEF2A and MEF2C mRNA levels were both decreased ($P < 0.05$) in Staufen1-HA-overexpressing clones compared to those seen in control cells (**Figure 12A,B**). In these experiments, we observed a slightly greater variability in MEF2C mRNA levels in-between conditions. This likely explains that MEF2C mRNA levels are significantly decreased only at 48 hours in clone #15. As mentioned in the Introduction, Staufen1 is thought to be involved in the control of mRNA stability by a mechanism referred to as Staufen1-mediated mRNA decay (SMD), which involves direct binding of Staufen1 to secondary structures present in the 3'UTR of target mRNAs, and subsequent mRNA decay (Y. K. Kim et al. 2005). This degradation mechanism has been shown to target a specific subset of mRNAs including Pax3, while other transcripts such as myogenin, are unaffected (Gong et al. 2009).

We thus wondered whether Staufen1 induces decay of other mRNAs involved in myogenic differentiation in Staufen1-HA-overexpressing stable cell lines. In this context, MEF2A mRNA appeared as a good candidate as it was found to be present in Staufen1-containing RNPs as revealed by microarray analysis (Furic et al. 2008), suggesting that Staufen1 may directly bind to and regulate MEF2A mRNA. Thus, we examined whether

MEF2A and MEF2C are targets of SMD. To test this, we used a similar assay to the one previously described in the initial description of SMD (Y. K. Kim et al. 2005). Briefly, Luciferase constructs containing the 3'UTR of potential targets were generated (**Figure 12C**) and transfected in HeLa cells. If these transcripts are SMD targets, co-transfection of these 3'UTR-Luciferase constructs together with Staufen1-HA should result in a decrease in reporter mRNA expression. Our results demonstrate that Staufen1-HA overexpression did not induce decay of MEF2A, MEF2C and MyoD reporter constructs, thereby ruling out a contribution of SMD to the lower levels of these myogenic regulatory factors observed in our stable cell lines (**Figure 12D**). It is important to note that similar results were also obtained in C2C12 myoblasts (data not shown). Given these negative results, we decided to also examine as a positive control whether indeed Staufen1 can decrease expression of luciferase-3'UTR constructs containing the Arf1 3'UTR as originally done in the work leading to the concept of SMD (Y. K. Kim et al. 2005; Kim et al. 2007). In our hands, Arf1 from both human (**Figure 12C**) and mouse (**Figure 12D**) did not appear subject to SMD (see Discussion for more details).

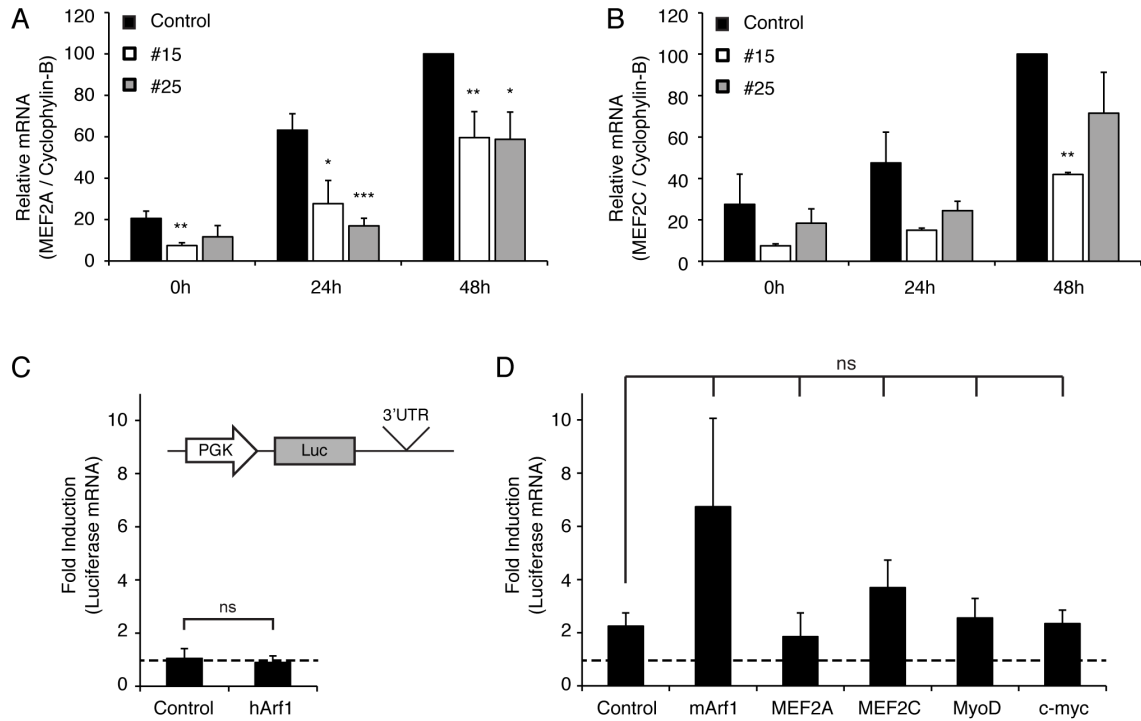


Figure 12. MEF2A, MEF2C, MyoD and c-myc are not SMD targets. (A) and (B) Relative quantification of MEF2A and MEF2C mRNA levels in stable C2C12 cells as determined by qRT-PCR. Levels were normalized to Cyclophilin-B (n = 4 and n = 3, respectively). (C) HeLa cells were co-transfected with luciferase vectors containing control or human Arf1 3'UTR together with human Staufen1. Following transfection, relative levels of luciferase mRNAs were determined by qRT-PCR and normalized to 18S. Data were also normalized to the level of luciferase mRNAs in absence of Staufen1 overexpression. (D) HeLa cells were co-transfected with luciferase vectors containing control or mouse Arf1, MEF2A, MEF2C, MyoD or c-myc 3'UTRs together with mouse Staufen1. Data were analyzed as in (C) (n = 3). Asterisks indicate significance (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$); and here ns indicate not significant.

Overexpression of Staufen1 Promotes c-myc Translation and Myoblast Proliferation

In parallel to the characterization of myogenic regulatory factors involved in muscle differentiation, we also performed a targeted approach to identify mRNAs regulated by Staufen1 and responsible for the impaired myogenesis. Although we examined the potential role of several candidates including p21, RalGDS, cdk5, p35, junD, c-jun, we were particularly interested in the transcription factor c-myc because it plays a role in the balance between proliferation/differentiation and is a known potent myogenic inhibitor (Miner & Wold 1991). Thus, we analyzed the levels of c-myc protein and mRNA by Western blot and qRT-PCR, respectively, in control versus Staufen1-HA-stable cell lines. In these experiments, we observed a significant increase ($P < 0.05$) in c-myc protein levels in Staufen1-overexpressing cells without a parallel increase in mRNA expression (**Figure 13A,B,C**). In addition, we next determined whether knocking down Staufen1 expression had a reverse effect on c-myc protein levels in muscle cells. Thus, C2C12 cells were transiently transfected with a shRNA targeting Staufen1 or a control. Western blot analyses showed a slight knockdown of Staufen1 in these experiments together with a modest decrease in c-myc protein expression (**Figure 13D**). To circumvent the limitations linked to the relatively low transfection efficiency of C2C12 cells and to improve the extent of the Staufen1 knockdown, we also transduced human primary HSMM cells with lentiviruses expressing one of two different shRNAs targeting Staufen1, or a control lentivirus. Western blots showed that both shRNAs induced a marked reduction in Staufen1 expression. As expected on the basis of our overexpression studies, downregulation of Staufen1 was accompanied by a decrease in c-myc protein levels (**Figure 13E**). Taken together, data obtained with these varied experimental

systems show that Staufen1 clearly regulates c-myc protein levels in skeletal muscle cells. Finally, to complement this work, we also assessed the levels of c-myc during muscle regeneration. Following an initial decrease in c-myc levels at two days post-cardiotoxin injection, the level of c-myc increased and essentially mirrored those of Staufen1 during early to mid-phases of muscle regeneration as expected based on our work with cells in culture (**Figure 7**).

Staufen1 is known to enhance the translation efficiency of a sub-population of transcripts by binding to their 5'UTR (Dugré-Brisson et al. 2005). In addition, Staufen1 knock-down in U2OS cells reduces c-myc protein without affecting mRNA levels (Weidensdorfer et al. 2009). Therefore, we examined whether Staufen1 overexpression promotes translation of c-myc mRNA. To test this, a Luciferase construct containing the 5'UTR of c-myc was first generated. The 5'UTR-Luciferase construct was then co-transfected with Staufen1-HA or control vectors. For these assays, HEK293T cells were used in order to reproduce conditions that led to the discovery of the translational role of Staufen1 (Dugré-Brisson et al. 2005). Following transfection, expression of Luciferase was monitored by a Luciferase assay, and the level of Luciferase mRNAs was measured by qRT-PCR. Overexpression of Staufen1-HA resulted in an increase in Luciferase activity (**Figure 13F**). Under these conditions, luciferase transcript levels were in fact decreased which, together with the increase in Luciferase activity, strongly suggests that Staufen1 promotes translation of c-myc mRNAs (**Figure 13F**). In this context, it is important to note that a decrease in c-myc mRNA expression was also observed in Staufen1-HA myoblasts and after 48 hours of differentiation (**Figure 13C**). This decrease in c-myc transcript level also occurs in a SMD-independent manner (**Figure 12C**).

In order to confirm whether overexpression of Staufen1-HA increases the translation of c-myc mRNA, we performed polysome fractionation experiments using proliferating stable cell lines. First, the optical density profiles of sucrose gradients were measured at 254 nm (**Figure 13G**). The top of the gradient contains free mRNAs and ribosomal subunits (40S, 60S and 80S) while the bottom contains mRNAs associated with polysomes. Then, we compared the expression profile of c-myc mRNAs along each gradient. As expected, qRT-PCR revealed enrichment in the levels of endogenous c-myc transcripts in polysomal fractions from Staufen1-HA stable cells (clone #25) in comparison to controls (**Figure 13G**). To confirm this effect, C2C12 cells were transiently transfected with a Staufen1-HA construct and analyzed as described above. First, we confirmed by Western blotting the Staufen1-HA overexpression (**Figure 14A**). Then, we determined by qRT-PCR the distribution of c-myc mRNAs in the sucrose gradient, and observed the same shift of c-myc mRNAs to the heavy polysome fraction upon Staufen1 overexpression (**Figure 14B**). This shift in distribution of c-myc mRNAs to the polysome fractions shows that more ribosomes are loaded onto c-myc transcripts thereby indicating that c-myc translation is enhanced by Staufen1-HA.

C-myc is known to repress expression of genes involved in cell cycle thereby inhibiting the irreversible proliferation arrest and promoting proliferation. Increased c-myc expression in Staufen1-HA stable cells should therefore result in increased cell proliferation. To test this, we performed proliferation assays by measuring Bromodeoxyuridine (BrdU) incorporation rate in control versus Staufen1-HA stable cell lines. Briefly, cells were cultured in the presence of the thymidine analogue BrdU for two hours. BrdU-positive cells were then visualized by immunofluorescence using anti-BrdU

antibodies. We noticed a significant increase ($P < 0.05$) in cell proliferation in Staufen1-HA-stable cell lines (**Figure 13H**). Therefore, the Staufen1-dependent increase in c-myc expression that we observed in terms of protein/mRNA steady-state measurements (**Figure 13A,B,C**), and in c-myc translation efficiency (**Figure 13F,G**), are both coherent with the dynamic change obtained in proliferation assays (**Figure 13H**). This increase in cell proliferation by Staufen1 observed after only 2 hours of BrdU labelling reflects the relatively large impact of Staufen1 over the switch between proliferation and differentiation.

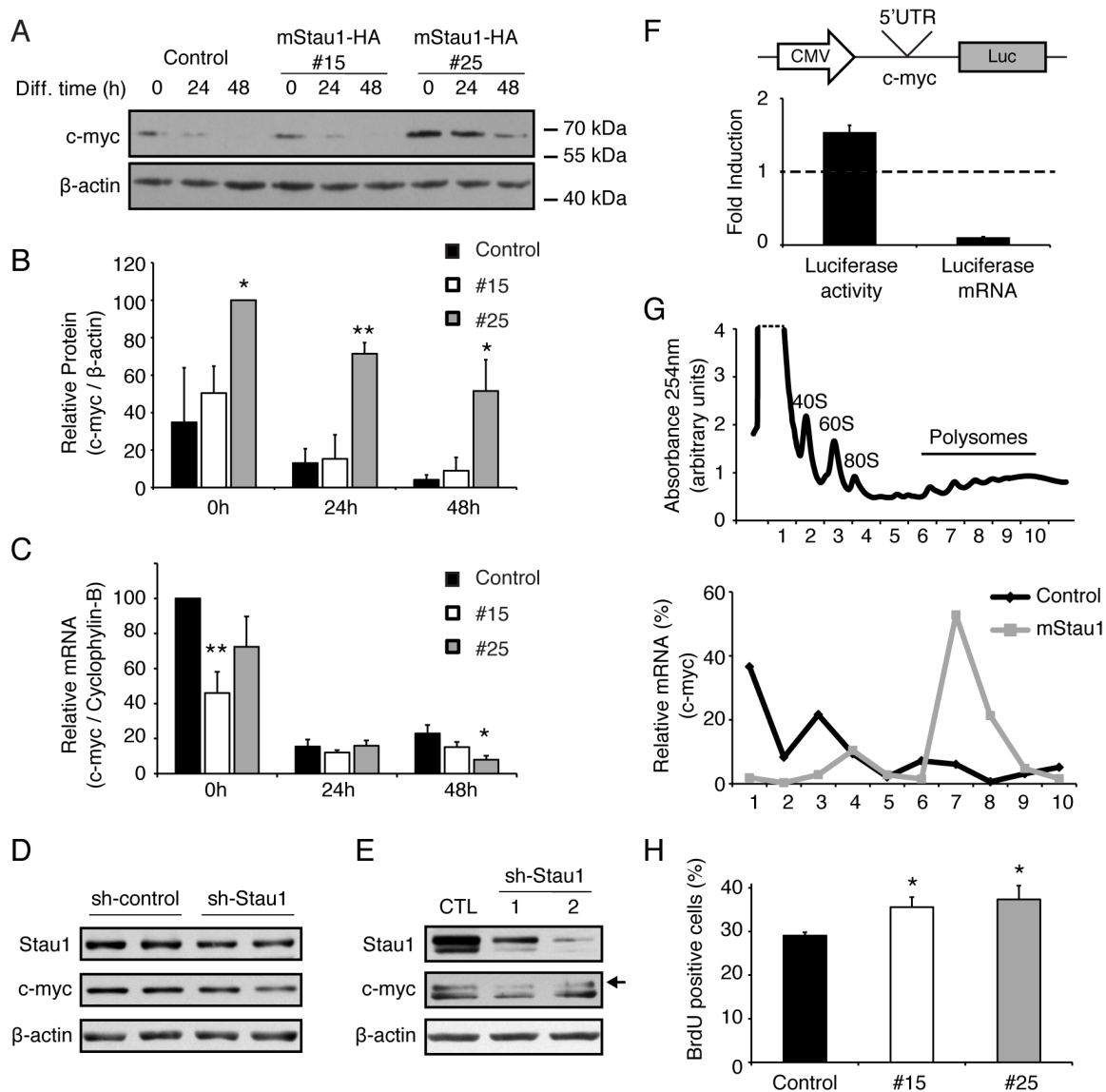


Figure 13. Staufen1 overexpression increases c-myc protein expression levels and cell proliferation. (A) Representative Western blot showing c-myc protein levels during myogenic differentiation of stable C2C12 cells. β -actin was used to show equal loading. (B) Relative quantification of c-myc protein levels normalized to β -actin (n = 3). (C) Relative quantification of c-myc mRNA level in stable cell lines as determined by qRT-PCR. Levels were normalized to Cyclophilin-B (n = 3). (D) C2C12 cells were transfected with sh-Staufen1 or control vectors. Western blots showing decreased

Staufen1 and c-myc protein levels. β -actin was used to show equal loading. **(E)** HSMM primary cells were transduced with sh-Staufen1 or control lentiviruses. Western blots were performed as in (D). The arrow shows the specific c-myc band, as determined in separate experiments by knocking down c-myc expression (data not shown). **(F)** 293T cells were co-transfected with luciferase vectors containing the c-myc 5'UTR and with Staufen1 or control plasmids. Following transfection, the relative activity of luciferase was determined along with luciferase mRNA levels by qRT-PCR. Data are also normalized to luciferase levels in absence of Staufen1 and to an empty vector ($n = 3$). **(G)** Polysome profiling of proliferating stable C2C12 cells. Top: Polysome profile obtained by continuous reading of absorbance at 254 nm. Bottom: Levels of c-myc mRNAs were measured by qRT-PCR from ten 1 ml sucrose gradient fractions. **(H)** Cell proliferation assays performed with stable C2C12 cell lines ($n = 3$). Asterisks indicate significance (* $P \leq 0.05$, and ** $P \leq 0.01$).

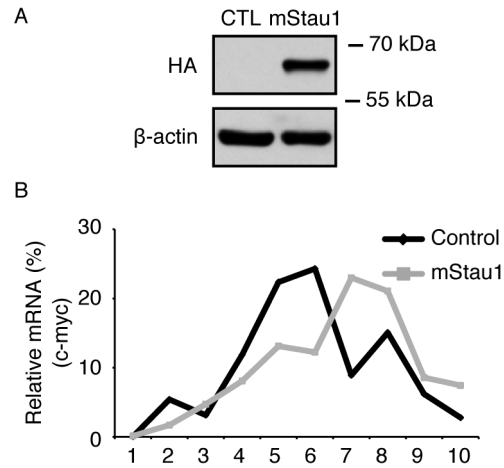


Figure 14. Stau1 overexpression shifts the distribution of c-myc mRNAs to the polysome fractions. C2C12 cells were transiently transfected with pcDNA3 control or Stau1-HA vectors. **(A)** Western blots showing expression of Stau1-HA in transfected cells. β -actin was used as a loading control. **(B)** Polysome profiling as in Figure 13G of control and Stau1-HA transfected proliferating C2C12 cells. Levels of c-myc mRNAs were measured by qRT-PCR from ten 1 ml sucrose gradient fractions.

Discussion

Regulation of Staufen1 and Control of Skeletal Muscle Cell Development

Staufen1 has recently emerged as a multifunctional RNA-binding protein. Although a few studies have shown the importance of Staufen1 in skeletal muscle, little is known about its pattern of expression during embryonic and postnatal development.

Furthermore, its precise role(s) in myogenic differentiation remain(s) fragmentary and conflicting (Bélanger et al. 2003; Kim et al. 2007; Yamaguchi et al. 2008; Gong et al. 2009; Yamaguchi et al. 2012).

Here, we found that expression of Staufen1 is high in E14.5 mouse muscle mass and that it decreases progressively thereafter, becoming expressed at low levels in mature muscles. This pattern follows expression profiles observed during human primary skeletal muscle cells (SkMC) and myoblasts (HSMM), mouse C2C12 differentiation, and muscle degeneration/regeneration. These complementary findings are basically all in agreement, demonstrating that levels of Staufen1 are tightly regulated and decreased during muscle development and differentiation.

Given our convergent findings, we sought to prevent the decrease in Staufen1 expression during myogenesis. Our results show that sustained expression of Staufen1 clearly inhibits muscle differentiation by causing decreases in the expression of multiple key myogenic markers. In addition, our analyses revealed, that Staufen1 overexpression increases c-myc protein levels without a parallel increase in mRNA levels, suggesting in this case, that Staufen1 regulates the translational expression of c-myc. Moreover, downregulation of Staufen1 by shRNAs decreases c-myc protein levels. These results are in agreement with previous findings showing that c-myc protein levels are significantly

reduced upon Staufen1 knockdown in U2OS osteosarcoma cells, while c-myc mRNA remain unaffected (Weidensdorfer et al. 2009). In this context, it has been reported that Staufen1 has the ability to increase translation of specific mRNAs with structured 5'UTRs (Dugré-Brisson et al. 2005). Accordingly, here we show that Staufen1 overexpression increases translation of a luciferase reporter containing the 5'UTR of c-myc, and relocates c-myc mRNAs to the polysomal fraction. Therefore, it appears reasonable to argue that the increase in c-myc expression can be attributed to Staufen1 interacting with the c-myc 5'UTR to promote mRNA translation. Given the known role of c-myc in promoting cellular proliferation while inhibiting differentiation, our results, therefore, uncover a new mechanism by which Staufen1 contributes to the balance between proliferation versus differentiation, and the regulation of muscle cell development.

We also demonstrated that the Staufen1-mediated inhibition of myogenesis cannot be rescued by expression of MyoD. This is consistent with the facts that: i) c-myc protein upregulation is sufficient to inhibit differentiation of myogenic cells; and ii) the differentiation defect caused by c-myc cannot be bypassed by ectopic MyoD expression (Miner & Wold 1991). As increased rates of proliferation are incompatible with the induction of the differentiation program, we propose that Staufen1 overexpression affects the efficiency of myoblasts to differentiate into mature muscle fibers by at least partially promoting cell proliferation via this c-myc dependent pathway.

Is Staufen1 Promoting Staufen1-Mediated Decay During Myogenic Differentiation?

It is thought that Staufen1 controls mRNA stability by a mechanism called SMD. Binding of Staufen1 to a Staufen1-binding site (SBS) located in a 3'UTR triggers mRNA decay

(Y. K. Kim et al. 2005). This mechanism differs from NMD as only Upf1, and not Upf2 or Upf3, is recruited to the target mRNA by Staufen1. SBS can be formed by intramolecular perfect base pairing forming a double stranded stem-loop structure (Kim et al. 2007), or by intermolecular imperfect base pairing between Alu Short Interspersed Elements (SINEs) located in 3'UTRs of mRNAs and in long non-coding RNAs (Gong & Maquat 2011; Wang et al. 2013).

It has been proposed that SMD and NMD are competitive pathways during myogenic differentiation (Gong et al. 2009). Although the levels of Staufen1 and Upf1 are decreased during myogenesis (Wang et al. 2013), the current model suggests that SMD efficiency increases while the impact of NMD decreases during myogenesis due to a reduced affinity of Upf2 for Upf1 (Gong et al. 2009). This pattern of Staufen1 expression and that of its SMD partner Upf1, are thus opposite to the proposed impact of SMD during myogenic differentiation and appear physiologically counterintuitive. Moreover, Pax3 mRNA is a proposed SMD target decreased by Staufen1 in C2C12 myoblasts thereby promoting myogenic differentiation (Gong et al. 2009). Pax3 is highly expressed in skeletal muscle progenitor cells and its downregulation is necessary for these cells to commit to the myoblast lineage (Lagha et al. 2008). However, and in agreement with other groups (Kuang et al. 2006; Collins et al. 2009; C. Liu et al. 2010; Kumar et al. 2009), we were unable to detect Pax3 mRNA expression by qRT-PCR in proliferating C2C12 myoblasts (data not shown). Therefore, while Pax3 downregulation by SMD may constitute an event for muscle progenitor cell determination, it appears unlikely that it controls the myoblast to myotube transition.

In our study, we performed a candidate approach to identify muscle mRNA targets of Stau1 involved in myoblast differentiation. Since expression of endogenous MyoD, MEF2A and MEF2C mRNAs are decreased in stable C2C12 cells overexpressing Stau1, we hypothesized that they may be SMD targets, despite the argument presented above. We used a similar strategy as the one used by the Maquat lab in the discovery of SMD by fusing candidate 3'UTRs downstream of a Firefly luciferase reporter gene (Kim et al. 2007). None of the assessed targets were decayed upon Stau1 overexpression. We thus additionally tested the Arf1 3'UTR as a positive control for SMD. In our experiments, we used both human and mouse Arf1 3'UTRs which contain two 19 bp stem structures necessary to form the SBS (Kim et al. 2007). In contrast to previous work describing Arf1 as a canonical SMD-target (Y. K. Kim et al. 2005; Kim et al. 2007), our results failed to provide any evidence that Arf1 mRNA is destabilized by Stau1 overexpression. While we were finalizing this manuscript, two independent studies by the Moore and DesGroseillers labs using transcriptome-wide approaches also failed to obtain evidence of SMD upon Stau1 overexpression on global mRNAs, and on previously identified SMD targets including Arf1 (Boulay et al. 2014; Ricci et al. 2013). These latter findings are in excellent agreement with ours and together, they raise questions as to the validity and functional significance of the current SMD model.

Impact for Myotonic Dystrophy

Myotonic Dystrophy type 1 (DM1) is caused by the misregulation of RNA-binding proteins. In particular, CUGBP1 and Stau1 are increased while MBNL1 aggregates in the nucleus (Wheeler et al. 2007; Lee & Cooper 2009; O'Rourke & Swanson 2009; Mahadevan 2012; Ravel-Chapuis et al. 2012). Interestingly, MBNL and CUGBP1 are

linked to the post-transcriptional control of myogenic genes during skeletal muscle differentiation, which may alter the differentiation potential of DM1 muscle cells (Amack & Mahadevan 2001; Timchenko, Lakova, et al. 2001; Amack et al. 2002; Squillace et al. 2002; Apponi et al. 2011). Given our current findings, it seems likely that expression of Staufen1 may also be altered in differentiating DM1 muscle thereby further compromising differentiation of these cells. It appears timely to examine the pattern of expression of Staufen1 in developing DM1 muscle and to determine whether it contributes to pathogenic events as these muscle fibers differentiate and regenerate. Such information will provide new mechanistic insights into the complex muscle phenotype seen in DM1 patients.

Materials and Methods

Constructs and Antibodies

The constructs used were pcDNA3 (Invitrogen Life Technologies, Burlington, Canada), mStaufen1-HA3 and hStau1⁵⁵-HA3 (Wickham *et al.*, 1999), and MyoD-Flag-myc expression vector (Y. Liu *et al.* 2010). The 3'UTR of human Arf1, mouse Arf1, MEF2A, MEF2C and c-myc was amplified by RT-PCR from C2C12 cells or mouse muscle extracts using primers containing a restriction site (underlined): hArf1 SBS (fwd-5'-ATTCTCGAGGTGAACGCGACCCCCCTCCCTCTCACTC-3', rev-5'-CAGTCTAGACCAGGTGCCCATGGGCCTACATCCCC-3'); mArf1 (fwd-5'-ATTCTCGAGACCAGACCCCCTCCCTCCC-3', rev-5'-CAGTCTAGAAATAGTTAAGAGACTTTATTCTAA-3') MyoD (fwd-5'-ATATTCTAGATCAGGTGCTTTGAGAGATCG-3', rev-5'-ATATTCTAGATATAAATTAGCGTCTTTATTTCCAACA-3'); MEF2A (fwd-5'-ATATTCTAGAGGCTTCCTGGTTCATGTTTG-3', rev-5'-ATATTCTAGATTCCAAGGTCTGCTTGC-3'); MEF2C (fwd-5'-ATATTCTAGATCTGAAGGATGGGCAACAT-3', rev-5'-ATATTCTAGAAAGAGATGCAGACCCAGATTT-3'); c-myc (fwd-5'-TAAGCAGCTAGCTAAACTGACCTAACTCGAGGAGGAG-3', rev-5'-TGCTTAGTCGACAGTTGGCCCAATTGTATTTTTTCCAATT-3'). PCR products were digested, subcloned into pmirGLO vector (Promega, Madison, Wisconsin, USA) downstream of luciferase. The 5'UTR of c-myc was amplified by RT-PCR using the following primers: (fwd-5'-ATATCTCGAGGATTGGGGTACGCGCTGC-3', rev-5'-ATATCTCGAGCGTCGTGGCTGTCTGCTG-3'). The PCR product was cloned

upstream of luciferase into the modified pGL4.14 vector (Promega, Madison, Wisconsin, USA) containing a CMV promoter. The orientation of inserts was determined by restriction digestion and the integrity of sequences confirmed by sequencing.

A Staufen1 shRNA construct was obtained by cloning the following annealed complementary oligonucleotides: 5'-

GATCCCGGCAACGGTAACTGCCATGTTCAAGAGACATGGCAGTTACCGTTGC
CTTTTTTCCAAA-3' and 5'-

AGCTTTTGGAAAAAAGGCAACGGTAACTGCCATGTCTCTTGAACATGGCAGT
TACCGTTGCCGG-3', into the BamH I - Hind III restriction sites of pRNAT-H1.1/Neo (Genscript) according to manufacturer recommendations.

The antibodies used were anti-HA.11 (16B12, Covance, Montreal, Quebec, Canada), anti-Staufen1, anti-c-myc and anti-GAPDH (AbCam, Toronto, Ontario, Canada), anti-CUGBP1 and anti- β -actin (3B1 and C4, respectively, Santa Cruz Biotechnology, Santa Cruz, California, USA), anti-MyoD (MoAb 5.8A, BD Biosciences, Mississauga, Ontario, Canada), anti-myogenin and anti-MyHC (F5D and MF20, respectively, Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA), and anti-Laminin (Sigma-Aldrich, Oakville, Ontario, Canada). Note that the band observed by Western blots for Staufen1 protein in mouse skeletal muscle cells is slightly above the 55 kDa mark. The band is specific for Staufen1 as its intensity is significantly decreased in protein extracts from cells expressing a shRNA targeting Staufen1 (data not shown).

Mouse Muscle Development and Cardiotoxin Injection

All surgical procedures were performed using aseptic conditions and were in complete agreement with the University of Ottawa Animal Care and Users Committee in

compliance with the Guidelines of the Canadian Council on Animal Care and the Animals for Research Act. For developmental time course experiments, FVB/N mice were used. Skeletal muscles from mouse embryos were recovered at embryonic day E14.5 and E18.5, and in newborns PN1. Given the small size of limbs at these ages, the whole muscle mass of the leg was used. Tibialis anterior (TA) muscles from 14-week old mice were used for adult samples. For degeneration/regeneration experiments, 25 µl of 10^{-5} M cardiotoxin (Latoxan, Rosans, France) were injected into TA muscles of 5 to 6 week-old FVB/N mice to induce muscle degeneration and regeneration as described previously (Clow & Jasmin 2010; Condrea 1974). At different time-points after injection, TA muscles were harvested, frozen in liquid nitrogen and stored at -80°C until further analysis. Contralateral, saline-injected TA muscles were used as controls.

Cell Culture, Transfections, Lentivirus Infections and Stable Cell Lines

HeLa and HEK293T cells (American Type Culture Collection, Manassas, USA) were grown in growth medium (DMEM 10% fetal bovine serum [HyClone, Thermo Fisher Scientific, Ottawa, Ontario, Canada], 100 U/ml penicillin, 100 µg/ml streptomycin). Primary human skeletal muscle cells (SkMC) and myoblasts (HSMM) (Lonza, Allendale, New Jersey, USA) were grown and differentiated according to manufacturer recommendations. Mouse C2C12 cells (American Type Culture Collection, Manassas, Virginia, USA) were maintained as myoblasts in growth medium as previously described (Ravel-Chapuis *et al.*, 2007). To induce myogenic differentiation, cells were allowed to become confluent on matrigel- (BD Biosciences, Mississauga, Ontario, Canada) coated plates, and the medium was switched to differentiation medium (DMEM 2% horse serum [PAA Laboratories, Piscataway, New Jersey, USA], 100 U/ml penicillin, 100 µg/ml

streptomycin). Cell transfections were performed with 1 ug of DNA, Lipofectamine and Plus Reagent (Invitrogen Life Technologies, Burlington, Ontario, Canada) according to the manufacturer's instructions. Stable cell lines were obtained by selection of transfected cells with G418 (1 mg/ml, Gibco Life Technologies, Burlington, Ontario, Canada) until appearance of stably transfected clones. Clones expressing Staufen1-HA were screened by Western blotting, and they were maintained with G418 selection (0.5 mg/ml). Lentivirus production and cell transductions were performed as recently described (Ravel-Chapuis *et al.*, 2012) using pLKO.1-shStau1 vectors (Open Biosystems GE Dharmacon, Lafayette, Colorado, USA).

Immunofluorescence

Cells were fixed for 5 min in 1X PBS containing 1% formaldehyde. Cells were permeabilized for 5 min with 1X PBS, 0.5% Triton and blocked with 1X PBS with 1% BSA. Cells were incubated with the primary antibody diluted in 1X PBS containing 1% BSA and 0.1% Triton for 1 hour at 37°C or overnight at 4°C. Then, the cells were thoroughly washed with 1X PBS, and incubated for 1 hour with Alexa secondary antibodies (Invitrogen Life Technologies, Burlington, Ontario, Canada). Slides were mounted with Vectashield mounting medium (Vector Labs, Burlington, Ontario, Canada) containing DAPI for staining of nuclei. Fluorescent images were visualized by microscopy on a Z1 Axiolmager upright microscope (Carl Zeiss, Toronto, Ontario, Canada). Phase contrast and low magnification fluorescent images were obtained on an Axiovert S100 inverted microscope (Carl Zeiss, Toronto, Ontario, Canada). Images were processed with Adobe Photoshop CS5.

Differentiation Index and Fusion Index

Myoblasts were seeded and allowed to differentiate for 24, 48, 72 or 96 hours before fixation. Cells were immunostained for MyHC and DAPI as described and images were acquired (10 random fields per condition). The fusion index (percentage of nuclei within myotubes having ≥ 3 nuclei) and differentiation index (percentage of nuclei within myotubes plus MyHC-positive mononucleated cells) were then determined.

Western Blotting

Dissected muscle extracts were crushed in liquid nitrogen, and muscle powder resuspended in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors [Complete; Roche, Laval, Quebec, Canada]) or Urea / Thiourea buffer (7 M Urea, 2 M Thiourea, 65 mM Chaps, 100 mM DTT, 10 U DNase I, protease inhibitors [Complete; Roche, Laval, Quebec, Canada]). Protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific, Ottawa, Ontario, Canada) or CB-X Protein Assay kit (G-Bioscience, St. Louis, Missouri, USA). Cells were washed and resuspended in RIPA buffer. 30 μ g of total proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Non-specific binding was first blocked with 1X PBS containing 5% skim milk, and membranes were then incubated with primary antibodies. After thorough washing with 1X PBS with 0.05% Tween, membranes were incubated with HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA). After several washes, signals were revealed using ECL reagents (PerkinElmer, Woodbridge, Ontario, Canada) and autoradiographed with X-Ray films (Thermo Fisher Scientific, Ottawa, Ontario, Canada). Quantifications were performed with the Image Lab Software (Bio-Rad, Mississauga, Ontario, Canada).

RNA Extraction, Reverse Transcription and Real-Time Quantitative PCR

Total RNA was extracted from samples using TRIzol (Invitrogen Life Technologies, Burlington, Ontario, Canada) or TriPure (Roche, Laval, Quebec, Canada). 1 µg of RNA was DNase-treated (Ambion Life Technologies, Burlington, Ontario, Canada), and cDNAs were synthesized using the MuLV Reverse Transcriptase (Applied Biosystem Life Technologies, Burlington, Ontario, Canada). mRNA expression was evaluated by real-time quantitative PCR (MX3005P, Stratagene, La Jolla, California, USA) using the QuantiTect SYBR Green PCR Kit (QIAGEN, Toronto, Ontario, Canada) according to the manufacturer's instructions. The sequences of the primers were as follows: MyoD (fwd-5'-ACTTTCTGGAGCCCTCCTGGC-3', rev-5'-TTTGTTGCACTACACAGCATG-3'); MEF2A (fwd-5'-GAATGCCCAAAGGATAAGCA-3', rev-5'-CAGCATTCCAGGGGAAGTAA-3'); MEF2C (fwd-5'-ATCTGCCCTCAGTCAGTTGG-3', rev-5'-CAGCTGCTCAAGCTGTCAAC-3'); myogenin (fwd-5'-CTACAGGCCTTGCTCAGCTC-3', rev-5'-AGATTGTGGGCGTCTGTACG-3'); c-myc (fwd-5'-GCCCAGTGAGGATATCTGGA-3', rev-5'-ATCGCAGATGAAGCTCTGGT-3'). The measures were normalized to Cyclophilin-B levels (fwd-5'-GATGGCACAGGAGGAAAGAG-3', rev-5'-AACTTTGCCGAAAACCACAT-3'). For Luciferase experiments, qRT-PCRs were performed with Luciferase (fwd-5'-TGCAAAAGATCCTCAACGTG-3', rev-5'-AATGGGAAGTCACGAAGGTG-3') and normalized to human 18S (fwd-5'-GTAACCCGTTGAACCCCAT-3', rev-5'-CCATCCAATCGGTAGTAGCG-3').

Luciferase Reporter Assay

Following transfection, assays for Luciferase enzymatic activity were performed on cell lysates using the Dual Luciferase Reporter Assay System according to the manufacturer's instructions (Promega, Madison, USA). Measures were performed using a luminometer (Lumat LB 9507, Berthold Technologies, Oak Ridge, Tennessee, USA).

Proliferation Assay

Cells were seeded on glass coverslips and left to proliferate overnight. Proliferation was assessed using the 5-Bromo-2'-deoxy-uridine (BrdU) Labeling and Detection Kit I (Roche, Laval, Quebec, Canada) according to manufacturer's instructions, using a 2 hours BrdU incubation time. Coverslips were mounted with Vectashield mounting medium (Vector Labs, Burlington, Ontario, Canada) containing DAPI for staining of nuclei, and visualized as described above.

Polysome Fractionation Experiments

50% confluent proliferating stable cell lines were grown on 150 mm plates. Cells were treated with 0.1 mg/ml cycloheximide (CHX, Sigma-Aldrich, Oakville, Ontario, Canada) for 5 minutes in fresh DMEM. Cells were washed twice with PBS containing CHX and subsequently lysed in a RNA lysis buffer (0.3 M NaCl, 15 mM MgCl₂, 15 mM Tris pH 7.4, 1% Triton X-100, 0.1 mg/mL CHX, 100 U/mL RNAsin). Nuclei and cellular debris were removed by centrifugation steps (3,000 rpm for 5 min at 4°C and 14,000 rpm for 5 min at 4°C). 500 ul of the lysates were layered on continuous sucrose gradients (10–50% sucrose in 15 mM MgCl₂, 15 mM Tris pH 7.4, 0.3 M NaCl). Centrifugations were carried out at 39,000 rpm in a SW41-Ti rotor at 4°C for 90 min. 1 ml fractions were collected at a flow rate of 1 ml/min from top to bottom of the gradient while absorbance

was measured continuously at 254 nm. Samples were digested with proteinase K and total RNA was extracted.

Statistical analysis

Student's *t*-tests were used to determine whether differences between groups were significant. The level of significance was set at $P \leq 0.05$. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$. Means plus/minus SEM are presented throughout, unless otherwise specified.

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Chapter 3: Implications for the RNA-binding protein Staufen1 in Rhabdomyosarcoma

**Novel Roles for Staufe1 in Embryonal and Alveolar Rhabdomyosarcoma via c-
myc-dependent and –independent Events**

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Author Contributions

T.E.C.P., K.A.M., and J.L. performed the experiments. J.M. performed scoring for Stau1 immunohistochemistry on Tissue Microarray. K.C. provided expertise and ERMS tumour biopsy samples. T.E.C.P., A.R.C., J.C. and B.J.J. conceived the project, designed the experiments and T.E.C.P., J.C., and B.J.J. wrote the manuscript.

Abstract

Rhabdomyosarcoma is the most common soft tissue sarcoma in children and young adults. Rhabdomyosarcomas are skeletal muscle-like tumours that typically arise in muscle beds, and express key myogenic regulatory factors. However, their developmental program remains blocked in the proliferative phase with cells unable to exit the cell cycle to fuse into myotubes. Recently, we uncovered a key role for the RNA-binding protein Staufen1 during myogenic differentiation through the regulation of c-myc translation. Given the known implication of c-myc in rhabdomyosarcoma, we hypothesized in the current work that Staufen1 controls rhabdomyosarcoma tumorigenesis. Here, we report for the first time the novel role of Staufen1 in cancer, specifically in rhabdomyosarcoma. We demonstrate that Staufen1 is markedly upregulated in human rhabdomyosarcoma tumours and cell lines as compared to normal skeletal muscle. Moreover, we show that Staufen1 promotes the tumorigenesis of embryonal and alveolar rhabdomyosarcoma subtypes both in cell culture and in animal models. Finally, our data demonstrate that Staufen1 has differential roles in embryonal versus alveolar rhabdomyosarcoma through the control of proliferative and apoptotic pathways, respectively. Together, these results provide the first evidence for Staufen1's direct implication in cancer biology. Accordingly, Staufen1 thus represents a novel target for the development of future therapeutic strategies for rhabdomyosarcoma.

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and young adults (Anderson et al. 1999). RMS cases account for approximately 50% of all paediatric soft tissue sarcomas, and 8% of all pediatric neoplasms (Perez et al. 2011). The World Health Organization's classification for tumours of soft tissue and bone subdivides RMS into four subtypes: embryonal (ERMS), alveolar (ARMS), pleomorphic, and spindle cell/sclerosing RMS, each with distinct genetic, histological and clinical features (Fletcher et al. 2013). The two major forms of RMS are ERMS and ARMS with 2/3 of all RMS cases diagnosed as ERMS. ERMS is most prevalent in children less than 10 years of age. This subtype is genetically heterogeneous with the activation of several oncogenic signaling pathways in combination with the loss of tumour surveillance mechanisms. Although a single mutation for all ERMS cases is not described, many are a result of the loss in heterozygosity at chromosome 11p15.5 (Keller & Guttridge 2013). In contrast, ARMS tumours are commonly found in children as well as young adults. This subtype is often a result of chromosomal translocations $t(2;13)(q35;q14)$ or $t(1;13)(q36;q14)$, which account for approximately 60% or 20% of ARMS cases, respectively. These translocations cause the fusion between the paired box (*PAX*) genes, *PAX3* or *PAX7* and the 3' end of the Forkhead box O1 (*FOXO1*) locus located on chromosome 13 (Davis et al. 1994; Galili et al. 1993; Shapiro et al. 1993). The production of the *PAX3-FOXO1* or *PAX7-FOXO1* fusion protein is oncogenic and drives the formation of ARMS tumours. However, approximately 20% of ARMS tumours are fusion negative despite exhibiting the classic alveolar phenotype (Sorensen et al. 2002; Davicioni et al. 2006; Soleimani &

Rudnicki 2011). This makes it difficult to determine whether ARMS represents multiple clinical and biological entities sharing a common phenotype.

Modern therapeutic regimens combining the use of surgery, radiation therapy and chemotherapy have improved the overall 5-year survival rate. Nonetheless, the 5-year survival rate for ERMS and ARMS remains at approximately 67% and 49%, respectively (Punyko et al. 2005). Moreover, amongst RMS cases in which the tumour is metastatic upon diagnosis, 3-year event-free survival rates are just over 30%, being significantly and adversely influenced by alveolar histology (Oberlin et al. 2008). The treatment plan for metastatic RMS has not improved for several decades and, consequently, there is a clear need to better define the molecular mechanisms driving RMS pathogenesis in an attempt to identify novel and relevant therapeutic targets.

RMS are generally thought of as skeletal muscle-like tumours since they typically arise in muscle beds and express key myogenic regulatory factors (MRFs) (Hettmer & Wagers 2010). Although RMS cells express MRFs, their developmental program remains blocked in the proliferative phase and the cells are unable to exit the cell cycle to fuse into multi-nucleated myotubes and mature myofibers (Tapscott et al. 1993). The Ras/MEK/ERK signaling pathway is known to be highly activated in ERMS leading to an accumulation of c-myc (Martinelli et al. 2009). The increased expression of c-myc is proto-oncogenic since c-myc dimerizes with its binding partner Max to drive expression of oncogenic target genes causing cancer cells to proliferate indefinitely (Blackwood & Eisenman 1991). The inhibition of c-myc via the MEK/ERK inhibitor U0126 in ERMS cells promotes myogenic differentiation resulting in the formation of terminally differentiated muscle-like cells (Marampon et al. 2006). In addition, the down-regulation

of c-myc in ERMS xenografts significantly reduced tumour growth(Marampon et al. 2009) and enhanced radiosensitivity of ERMS tumours (Marampon et al. 2011). More recently, it was described that c-myc drives ERMS transformation and radioresistance by promoting DNA repair (Gravina et al. 2016). Although c-myc expression has not been a primary focus in ARMS, it has also been reported to be amplified in some ARMS cell lines and tumours. In fact, early studies suggested that c-myc expression may be higher in ARMS compared to ERMS (Kouraklis et al. 1999). Such pathways involved in maintaining the proliferative capacity of cells, may thus represent ideal targets for therapeutic intervention focused on rescuing the impaired differentiation program thereby alleviating the malignant characteristics of these cells.

As part of our efforts focused on the role of RNA-binding proteins (RBPs) in neuromuscular biology and on their therapeutic potential for various neuromuscular disorders, we became interested several years ago in examining the impact of Stau1 in skeletal muscle development, at the mature neuromuscular junction, as well as in Myotonic Dystrophy Type I (DM1) (Ravel-Chapuis et al. 2014; Ravel-Chapuis et al. 2012; Bélanger et al. 2003; Bondy-Chorney et al. 2016; Ravel-Chapuis et al. 2016). Stau1 is a multi-functional RBP with several key roles in mRNA localization (Tang et al. 2001), stability (Kim et al. 2007; Gong et al. 2009; Y. K. Kim et al. 2005), translation (Dugré-Brisson et al. 2005; Ravel-Chapuis et al. 2014; Ricci et al. 2013; Sugimoto et al. 2015), the cell cycle (Boulay et al. 2014) and alternative splicing (Ravel-Chapuis et al. 2012; Bondy-Chorney et al. 2016; Sugimoto et al. 2015). There are two protein isoforms of Stau1 generated through alternative splicing of its pre-mRNA, Stau1⁵⁵ and Stau1⁶³, which are ubiquitously expressed (Marión et al. 1999; Wickham et al. 1999). In this

context, we recently described a key functional role of Stau1⁵⁵ in myogenic differentiation of both mouse and human skeletal muscle (Ravel-Chapuis et al. 2014). Our findings showed that Stau1⁵⁵ levels decreased progressively during differentiation of myogenic cells and during embryonic muscle development. Such a reduction in Stau1⁵⁵ expression is necessary for proliferating myoblasts to exit the cell cycle and differentiate (Ravel-Chapuis et al. 2014). Mechanistically, we uncovered that Stau1⁵⁵ overexpression impairs myogenic differentiation by increasing the translation of c-myc, a factor known to delay cell-cycle withdrawal and terminal differentiation of skeletal muscle cells (Ravel-Chapuis et al. 2014; Miner & Wold 1991).

Given the impact of c-myc in the RMS pathology (Martinelli et al. 2009; Schaaf et al. 2010; Blackwood & Eisenman 1991; Marampon et al. 2006; Marampon et al. 2009; Marampon et al. 2011; Gravina et al. 2016), and our recent findings showing that Stau1 regulates c-myc during skeletal muscle differentiation (Ravel-Chapuis et al. 2014), we hypothesize in the current study that Stau1 controls RMS tumorigenicity. To this end, we performed a series of complementary experiments to examine the impact of Stau1 in RMS tumorigenesis. Our findings demonstrate that Stau1 is markedly elevated in ERMS and ARMS. Moreover, we show that overexpression of Stau1 in RMS promotes tumorigenesis of both subtypes in cell culture and xenograft models. However, Stau1 appears to assume differential roles in ERMS versus ARMS through the regulation of proliferative and apoptotic mechanisms, respectively. Collectively, these results show for the first time the direct involvement of Stau1 in cancer biology thereby highlighting its therapeutic potential as a novel target.

Results

Staufen1 is increased in Human Embryonal and Alveolar Rhabdomyosarcoma

To investigate Staufen1 expression in RMS, immunohistochemistry was first performed on a commercially available, high-density tissue microarray. We assessed a total of 26 ERMS, and 24 ARMS tumours as well as 8 normal skeletal muscle samples for Staufen1 expression. Since RMS tumours are thought to originate from a muscle-like cell lineage, skeletal muscle samples are routinely used as controls for the comparison of gene expression in RMS (Hettmer & Wagers 2010). In these experiments, we performed scoring for Staufen1 staining using a 4-point scale where 0-1 is low, 2-3 is moderate, and 4 represents high Staufen1 expression (see additional details in Methods). Representative images are shown in **Figure 15A** and the mean intensity score was calculated for each tissue (**Figure 15B**). Analyses revealed an increase in Staufen1 staining intensity in ERMS ($P < 0.05$) and a trend towards increased expression in ARMS tumours ($P = 0.12$) as compared to normal muscle tissue (**Figure 15B**). It is important to note that in the ARMS samples examined, patient age, treatment history, tumour location and the PAX3/7-FOXO1 status likely contributed to the lack of significance observed in Staufen1 expression. Detailed examination of intensity score distributions demonstrated that a higher percentage of RMS tumours expressed Staufen1 at moderate and high levels (score > 2) as compared to normal muscle (**Table 2**). In fact, 87.5% of normal skeletal muscle tissue had low and 12.5% had moderate expression of Staufen1, consistent with our previous analysis of Staufen1 levels in mouse adult skeletal muscle (Ravel-Chapuis et al. 2014). In contrast, 46.2%, 34.6% and 19.2% of ERMS tumours expressed Staufen1 at low, moderate, or high levels, respectively (**Table 2**). In addition, 66.7% and 33.3% of

ARMS tumours expressed Staufen1 at low and moderate levels, respectively (**Table 2**). In all of the samples, the localization of Staufen1 was predominantly cytoplasmic with a lower level of expression seen in nuclei. While no low-grade tumours (grade I or II) were present in the TMA, the majority of the tumour samples were grade III (n=47) and very few were classified as grade IV (n=3). As a result, correlation of Staufen1 expression across tumour grade was not possible with this sample set.

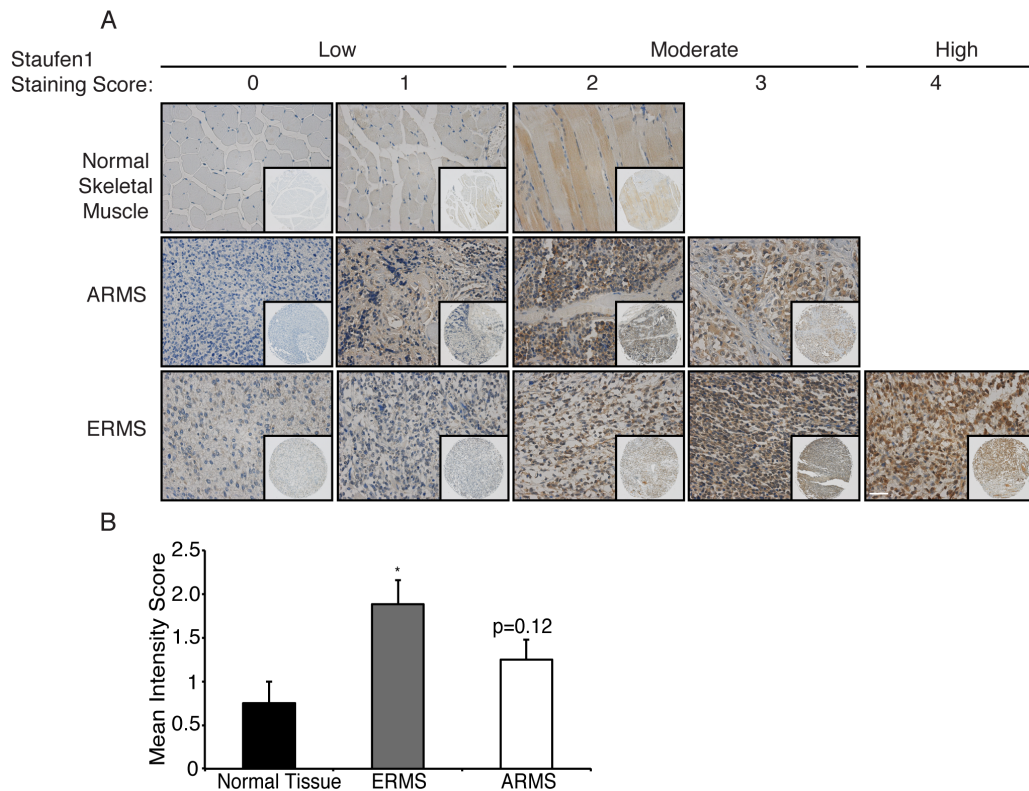


Figure 15. Staufen1 is overexpressed in human rhabdomyosarcoma. Tissue microarrays were used to evaluate Staufen1 expression by immunohistochemistry in human normal skeletal muscle and primary rhabdomyosarcoma core biopsies. **(A)** Intensity scoring of tissues was performed and analyzed using a 4-point scoring system with 0-1 = low expression, 2-3 = moderate expression and 4 = high expression. Representative images of scored Staufen1 staining in normal skeletal muscle tissue (n=8), Embryonal Rhabdomyosarcoma (ERMS) (n=26), and Alveolar Rhabdomyosarcoma (ARMS) (n=24) using anti-Staufen1 antibodies (brown) and hematoxylin stain for nuclei (blue); 20X objective, scale bar = 50 μ m. Insert represents lower magnification images at 5X magnification to visualize total core biopsy sample. **(B)** Quantification of the mean intensity score, data are Mean $\pm$ SEM, *P<0.05; and **(C)** percent score distribution for each tissue.

Table 2. Summary of Staufen1 score intensity distribution.

Score	Normal Tissue (n=8)	Embryonal RMS (n=26)	Alveolar RMS (n=24)
0-1 (Low)	7 (87.5%)	12 (46.2%)	16 (66.7%)
2-3 (Moderate)	1 (12.5%)	9 (34.6%)	8 (33.3%)
4+ (High)	0 (0%)	5 (19.2%)	0 (0%)
P-values		0.11	0.26

Note: P-values calculated by Chi-Squared Test.

Next, using an *in vitro* cell culture system, we examined Staufen1 expression in human primary Skeletal Muscle Cells (SkMC), ERMS (RD) and ARMS (RH30) cells. RD cells are one of the most commonly used ERMS cell line. These cells were developed from a biopsy of pelvic ERMS previously treated with cyclophosphamide and radiation, and they were found to be resistant to treatment (Mcallister et al. 1969). RD cells have 51-hyperdiploid chromosomes and contain several amplifications and mutations in cancer-related genes such as *MYC* amplification (Missiaglia et al. 2009), *NRAS* mutation (Q61H) (Schaaf et al. 2010), and a homozygous mutation of *TP53* (Felix et al. 1992; Miyachi et al. 2009). The RH30 cell line was derived from a bone marrow sample of a 16-year-old male with untreated metastatic ARMS (Douglass et al. 1987). These cells express the t(1;13) translocation and display near-triploid chromosomes (Rodriguez-Perales et al. 2004), amplification of the chromosomal region involving *CDK4* (Berner et al. 1996; Weber-Hall et al. 1996; Khatib et al. 1993), and mutated *TP53* (Felix et al. 1992; Miyachi et al. 2009; Rodriguez-Perales et al. 2004).

Western blot and quantitative RT-PCR (qRT-PCR) were performed to determine Staufen1 protein and mRNA expression, respectively, in ERMS and ARMS cells as compared to SkMCs (**Figure 16A,B**). Results showed that both Stau1⁵⁵ and Stau1⁶³ isoforms were increased at the protein level in ERMS and ARMS with Stau1⁵⁵ being the predominant isoform. Quantification of Stau1⁵⁵ revealed an ~150% and ~95% increase, respectively ($P < 0.01$, **Figure 16A**). Staufen1 mRNA levels were also increased by ~180% and ~132% in ERMS and ARMS cells, respectively ($P < 0.01$, **Figure 16B**). In these same cells, we observed an increase in c-myc, a key mediator of RMS (Martinelli et al. 2009; Schaaf et al. 2010; Marampon et al. 2009; Marampon et al. 2011). C-myc

protein expression was augmented by ~152% and ~46% in ERMS and ARMS cells, respectively ($P < 0.01$, **Figure 16C**). In contrast to the increase in c-myc protein levels, no change in c-myc mRNA expression was detected in ERMS ($P > 0.05$) whereas a decrease was seen in ARMS (~ 60% decrease; $P < 0.05$) (**Figure 16D**). These data are in complete agreement with our recent demonstration that Staufen1 regulates the translation of c-myc (Ravel-Chapuis et al. 2014), thereby maintaining RMS cells in a proliferative state and promoting their tumorigenesis.

These results were confirmed by western blot for Staufen1 expression in human ERMS and ARMS tumours with an ~325% and ~650% increase in Staufen1 levels, respectively, as compared to adult skeletal muscle (**Figure 17A**). In addition, c-myc protein expression was also increased in human primary tumour samples with an ~727% and ~321% increase in ERMS and ARMS, respectively, as compared to adult skeletal muscle (**Figure 17B**). Interestingly, Staufen1 and c-myc levels in RMS tumours appeared similar to those observed in fetal skeletal muscle (**Figure 17A,B**). To demonstrate that the increase of Staufen1 and c-myc in the RD and RH30 cell lines are representative of ERMS and ARMS, we further examined their expression across a panel of RMS cell lines. Our data showed that Staufen1 and c-myc are elevated in the ERMS cell lines RH36 and RH18 as compared to control Human Skeletal Muscle Myoblasts (HSMM) (**Figure 17C,D**). Similarly, Staufen1 and c-myc are increased in the ARMS cell line RH41, consistent with the observed expression in RH30 cells as compared to control cells (**Figure 17C,D**). Most striking is the strong correlation between Staufen1 and c-myc across the cell lines examined denoted by a Pearson correlation coefficient of 0.96 ($P <$

0.01). Together, these data strengthen the conclusion that Staufen1 is increased in ERMS and ARMS.

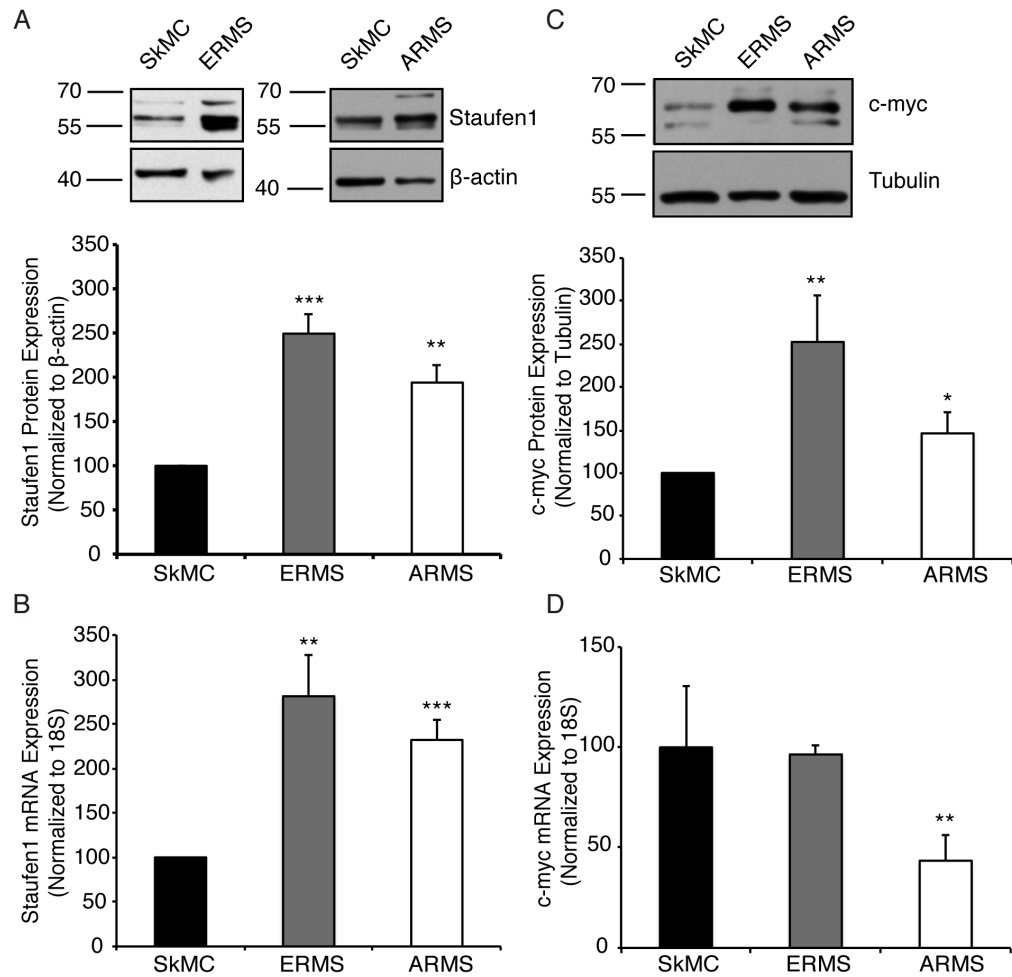


Figure 16. Staufen1 and c-myc are overexpressed in ERMS and ARMS cell lines.

Expression of Staufen1 and c-myc were determined in human skeletal muscle cells (SkMC), ERMS (RD) and ARMS (RH30) cells. (A) Western blot using anti-Staufen1 antibodies and β -actin as a loading control. The predominant Stau1⁵⁵ isoform was quantified (n=4). (B) qRT-PCR using primers specific for Staufen1 mRNAs and normalized to total levels of 18S rRNA (n=6). (C) Western blot using anti-c-myc antibodies and tubulin as a loading control (n=5). (D) qRT-PCR using primers specific for c-myc mRNAs and normalized to total levels of 18S rRNA (n=5). All quantifications are represented as a percentage relative to SkMC (n=5). Data are mean $\pm$ SEM, *P<0.05, **P<0.01, ***P<0.001.

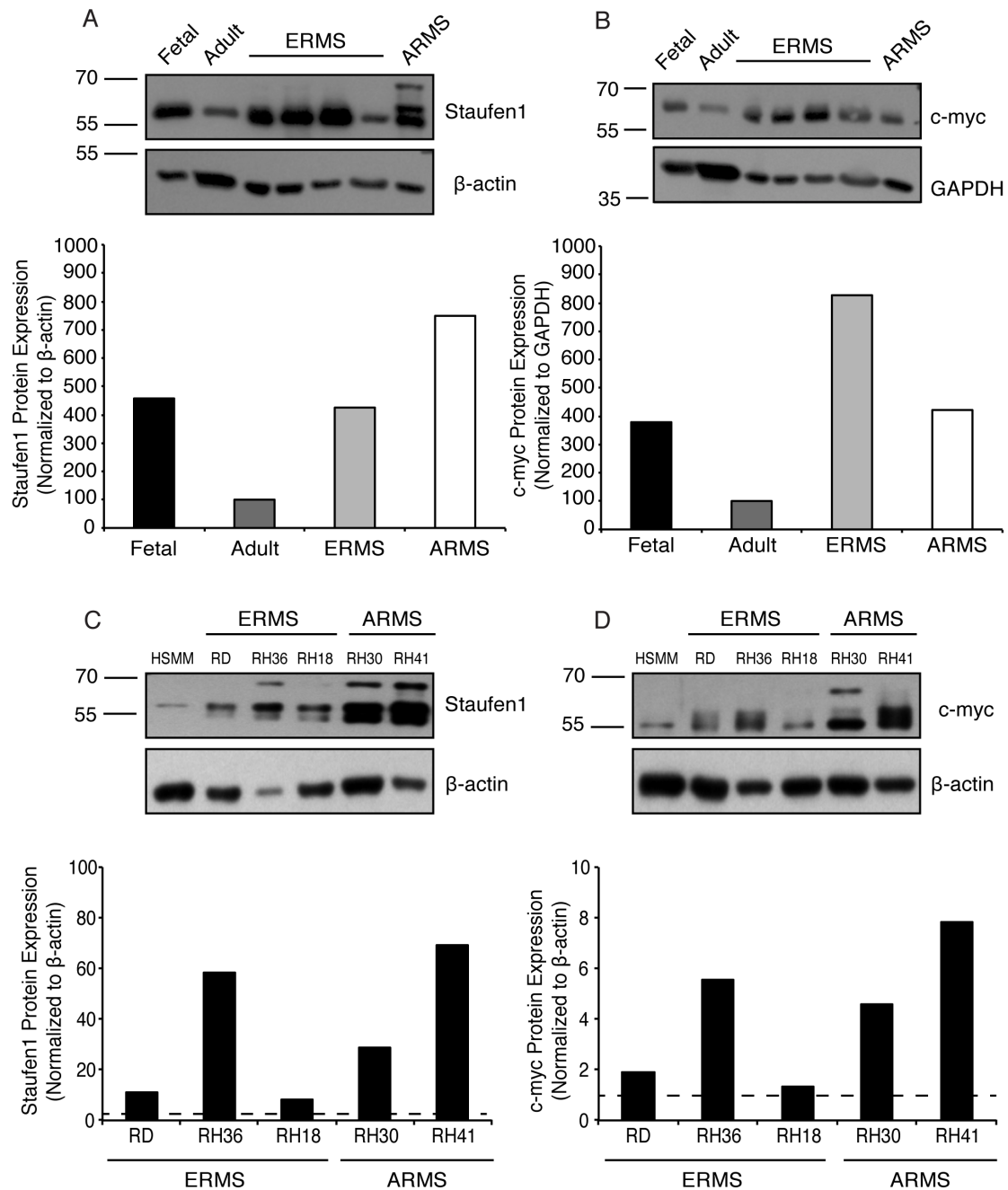


Figure 17. Staufen1 and c-myc are increased in human primary ERMS and ARMS tumours and multiple RMS cell lines. (A) Analysis of Staufen1 expression and (B) c-myc expression by western blot in ERMS (n=4) and ARMS (n=1) human primary tumour samples, normal fetal (n=1) and adult skeletal muscle (n=1) tissues with GAPDH as a

loading control. Quantifications are represented as a percentage relative to adult skeletal muscle. (C) Analysis of Staufen1 and (D) c-myc expression by western blot in ERMS cells (RD, RH36, and RH18) and ARMS cells (RH30, RH41) as compared to control Human Skeletal Muscle Myoblasts (HSMM), (n=2 for all conditions). The Pearson correlation coefficient for Staufen1 and c-myc is $r=0.96$, $P<0.01$, indicating a very strong correlation. Dotted lines represent expression in HSMM cells. Quantifications are represented as a Fold change relative to HSMM cells.

Staufen1 Enhances the Tumorigenic Potential of RMS cells

To assess the impact of Staufen1 in RMS cells, we used a mixture of three independent Staufen1 targeting shRNAs to knockdown Staufen1 in ERMS and ARMS cell lines. Several attempts were made to develop cell lines stably expressing Staufen1-shRNA via antibiotic selection, however these cells were not viable. Therefore, control (CTL) non-targeting shRNA or Staufen1-targeting shRNA lentiviruses were used to transduce RMS cell lines to achieve a Staufen1 knockdown. Western blot analysis confirmed Staufen1 knockdown in both cell lines at 72 hours post-infection (**Figure 18A**). First, we evaluated cell proliferation using flow cytometry to measure bromodeoxyuridine (BrdU) and propidium iodide (PI) staining. Following Staufen1 knockdown, ERMS cells showed an ~30% decrease in BrdU incorporation, which revealed their lower proliferative capacity ($P < 0.01$) as compared to CTL (**Figure 18B,C**). By contrast, ARMS cell proliferation was unaffected (**Figure 18B,C**). Cell cycle analysis was performed on these cells and in agreement with the BrdU incorporation experiments, there was a decrease in the percentage of cells in S-phase ($P < 0.05$, **Figure 18D**).

Next, dual staining of cells with Annexin V and PI was performed and analyzed by flow cytometry to assess apoptosis in RMS cells expressing Staufen1-shRNA. Western blot analysis demonstrated reduced Staufen1 levels in both cell lines (**Figure 19A**). Our data showed that apoptosis in ERMS cells was unaffected. However, ARMS cells showed a significant increase in Annexin V staining (~126%, $P < 0.05$) indicating that Staufen1 protects ARMS cells from apoptosis (**Figure 19B,C**). These data indicate that Staufen1 has different roles in regulating ERMS and ARMS tumorigenesis.

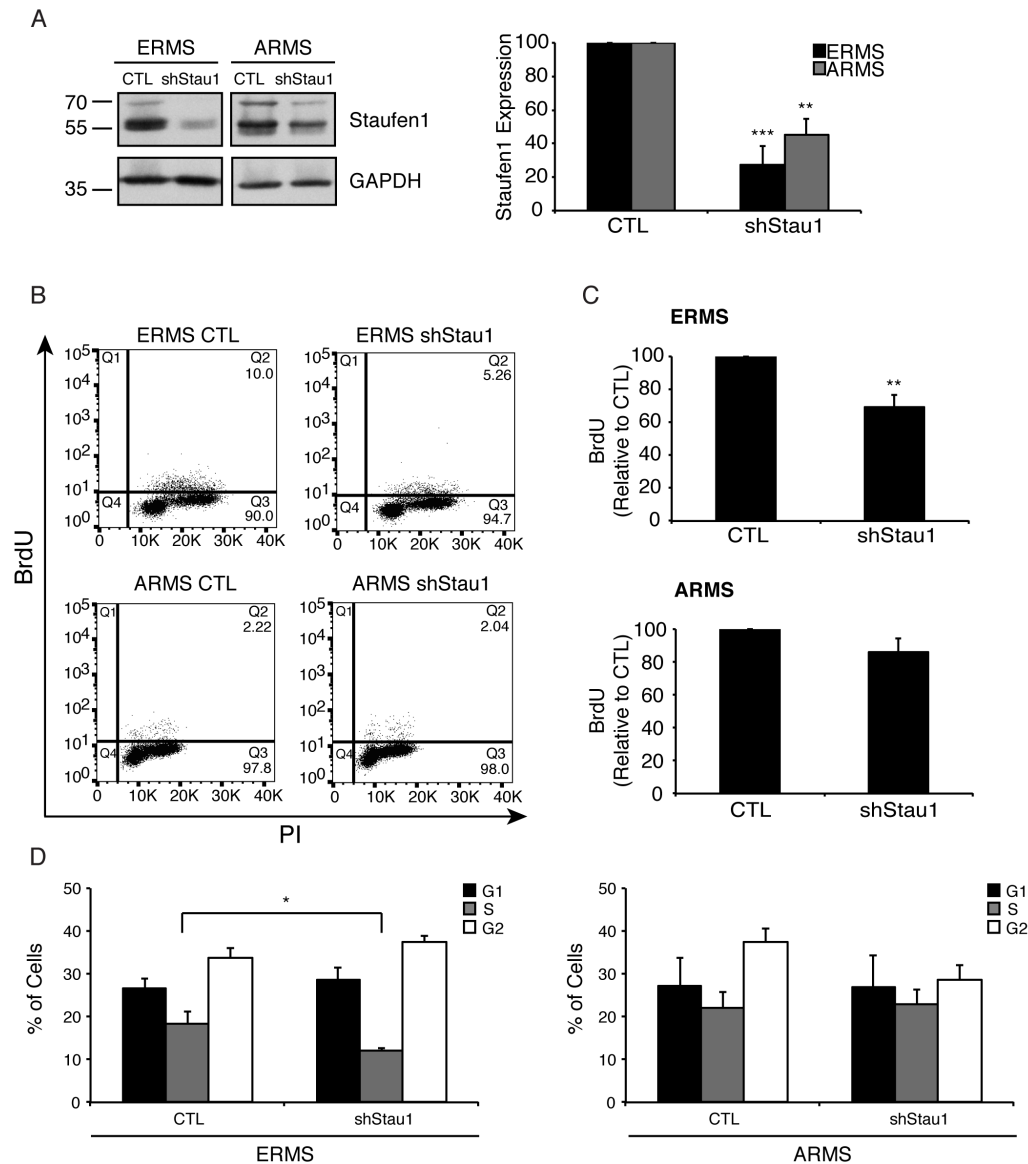


Figure 18. Staufen1 regulates the proliferation and cell cycle of ERMS cells. Analysis of cell proliferation was performed after 72 hours of Control (CTL) or Staufen1-shRNA expression (shStau1) in ERMS and ARMS cells. **(A)** Representative western blot of Staufen1 expression with GAPDH as a loading control in ERMS (RD) and ARMS (RH30) cells. Quantification of n=4 and n=3, respectively, is represented as a percentage of the CTL. **(B)** Proliferation was assessed by two-parameter flow cytometry and representative dot plots following 2 h of BrdU incorporation and Propidium Iodide (PI)

staining in ERMS (RD) and ARMS (RH30) cells expressing CTL or Staufen1-shRNAs. **(C)** Quantification of BrdU incorporation is represented as a percentage relative to CTL in ERMS (RD) and ARMS (RH30) cells, n=4 and n=3, respectively. **(D)** Cell cycle analysis was examined by flow cytometry and the percentage of the cells in G1, S and G2 phases are indicated. Data are Mean $\pm$ SEM, *P<0.05, **P<0.01, ***P<0.001.

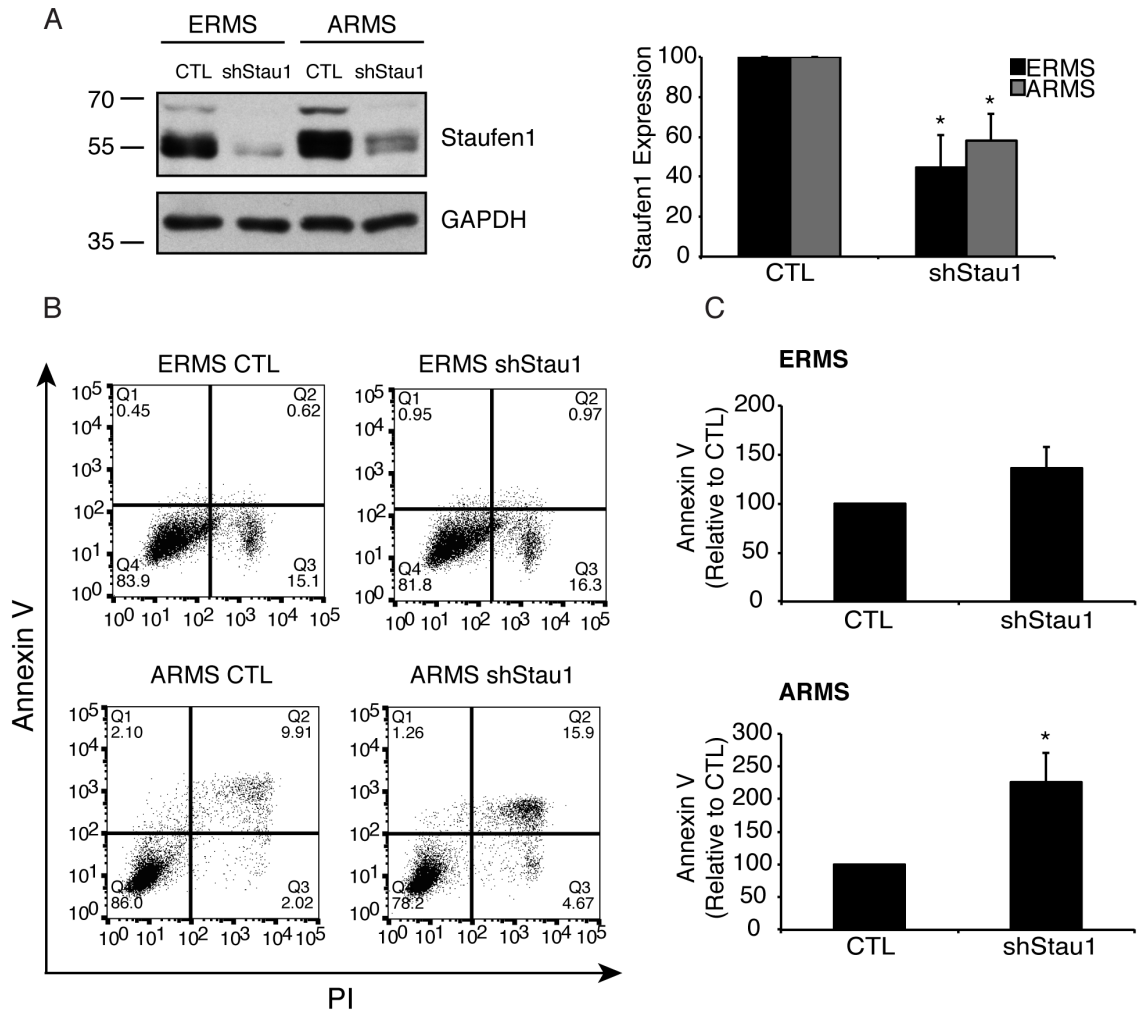


Figure 19. Stau1 regulates apoptosis in ARMS cells. Analysis of apoptosis was performed after 72 hours of Control (CTL) or Stau1-shRNA expression (shStau1) in ERMS and ARMS cells. (A) Representative western blot of Stau1 expression with GAPDH as a loading control in ERMS (RD) and ARMS (RH30) cells. Quantification of n=3 is represented as a percentage of the CTL. (B) Apoptosis was assessed by two-parameter flow cytometry and representative dot plots of CTL and Stau1-shRNA expressing ERMS (RD) and ARMS (RH30) cells stained with Annexin V and Propidium Iodide (PI). (C) Quantification of Annexin V staining is represented as a percentage relative to CTL (n=3). Data are Mean $\pm$ SEM, *P<0.05.

To determine the role of Staufen1 on the metastatic potential of RMS, we investigated the impact of Staufen1 knockdown on the invasion and migration properties of these cells. Western blotting confirmed an ~85% and ~65% reduction of Staufen1 in ERMS and ARMS cells, respectively (**Figure 20A**). ERMS and ARMS cells were plated into transwell chambers containing a porous membrane coated with or without matrigel. In the absence of matrigel, the knockdown of Staufen1 decreased both ERMS and ARMS cell motility by ~70% and ~30%, respectively ($P < 0.01$, **Figure 20B**). Furthermore, we assessed the invasion of cells in the presence of matrigel and observed that both ERMS and ARMS cell invasion was decreased by ~85% and ~70%, respectively ($P < 0.001$, **Figure 20B**). The facts that both cell motility and invasion were decreased in ERMS and ARMS cells with Staufen1 knockdown support the idea that Staufen1 has a dual role in these RMS subtypes.

Next, we performed migration assays to further evaluate the impact of Staufen1 on RMS cell motility and migration. Western blotting confirmed Staufen1 knockdown of ~100% efficiency in both cell lines (**Figure 20C**). Cells were seeded into culture inserts for 24 hours and then a 500 μm cell free gap was created by removing the insert. Removal of the insert represented time 0 hour. Migration of the cells was subsequently evaluated at 6 hour-intervals for a total of 30 hours. Results of these experiments revealed that upon Staufen1 knockdown, ERMS and ARMS cells showed significant reductions in migration at almost all time points as compared to CTL cells (**Figure 20D**). These data support the notion that the lack of effect on ARMS cell motility observed in the motility/invasion assays was due to reduced Staufen1 knockdown efficiency.

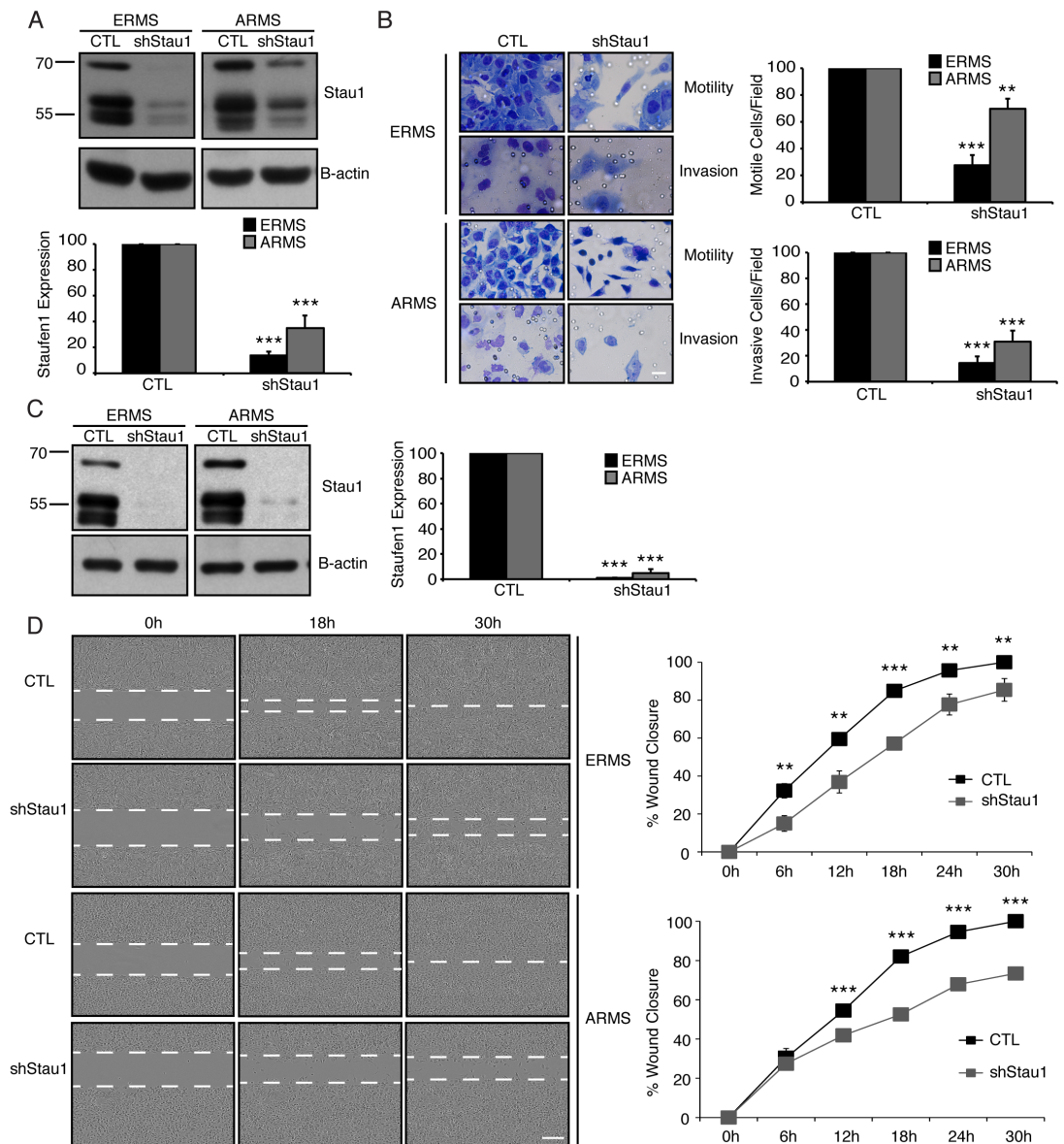


Figure 20. Staufen1 knockdown inhibits RMS cell invasion and migration. Motility and Invasion assays were performed following 48 hours (h) of Control (CTL) or Staufen1-shRNA (shStau1) expression. **(A)** Western blot analysis of Staufen1 expression showing a representative blot of Staufen1 with β -actin as a loading control in ERMS (RD) and ARMS (RH30) cells. **(B)** Cells were seeded into transwell chambers containing membranes coated with or without Matrigel and incubated for 72 h and 48 h for ERMS

(RD) and ARMS (RH30) cells, respectively. Representative images of cells that passed through the transwell chamber at 40X magnification, scale bar = 20 μ m, are displayed. Average number of cells/field of view is quantified for cell motility (no matrigel) and invasion (matrigel) as a percentage relative to CTL. Migration assays were performed after 48 h of Staufen1 knockdown and (C) is a representative western blot and quantification of Staufen1 expression with β -actin as a loading control in ERMS (RD) and ARMS (RH30) cells. (D) Cells were seeded into culture insert wells for 24 h, following removal of insert, a 500 μ m cell free gap represents 0 h, and consecutive photos were taken at 6 h intervals for a total of 30 h. Representative images at 10X magnification, scale bar = 300 μ m. Quantification of the gap is represented as % wound closure normalized to cell confluency. Quantifications are for all data are n=3. Data are Mean $\pm$ SEM, **P<0.01, ***P<0.001.

Decreasing Staufen1 Expression Inhibits RMS Tumour Growth *in vivo*

Based on the role of Staufen1 in RMS cells in culture and the increase in Staufen1 expression observed in RMS cells and tumours, we next determined whether Staufen1 knockdown impacted RMS tumour growth *in vivo*. To this end, we performed flank injections of ERMS and ARMS cells expressing CTL- or Staufen1-shRNAs. Western blotting demonstrated an ~75% and ~50% knockdown in ERMS and ARMS cells, respectively, prior to injection (**Figure 21A**). Tumour growth was monitored until an ethical endpoint of 72 and 31 days for ERMS and ARMS, respectively. Strikingly, ERMS cells expressing Staufen1-shRNAs did not form tumours (**Figure 21B**). The right flank injected with CTL ERMS cells formed large tumours as compared to the left flank injected with Staufen1-shRNA expressing ERMS cells (**Figure 21B,C; top panel**). Although Staufen1-shRNA expressing ARMS cells formed tumours, these were significantly smaller as compared to CTL tumours (**Figure 21B; bottom panel**). Total tumour weight was determined and ARMS tumours expressing Staufen1-shRNA weighed ~25% less ($P < 0.05$) than contralateral CTL tumours (**Figure 21C; bottom panel**). Tumour volume was monitored throughout the growth period and consistent with our endpoint results, these data showed that Staufen1-shRNA expressing ERMS and ARMS tumours had decreased tumour volumes (**Figure 21D**). It is important to note that in ARMS tumours, there was large variability between animals towards the end of the time-course and thus only a trend was observed from days 24-31. Analyses of Staufen1 expression at endpoint ARMS tumours demonstrated a ~30% decrease, which is less than the ~50% knockdown observed pre-injection (**Figure 22A**). In addition, hematoxylin and eosin staining of CTL and shStau1 tumours demonstrated that the tumours appear similar

(Figure 22B). Based on these data, we hypothesize that the injected cells with significant Stau1 knockdown were removed via apoptosis and the surviving cells formed smaller tumours consistent with those in CTL conditions. Together, these findings demonstrate that the increased expression of Stau1 in RMS promotes tumour formation *in vivo*.

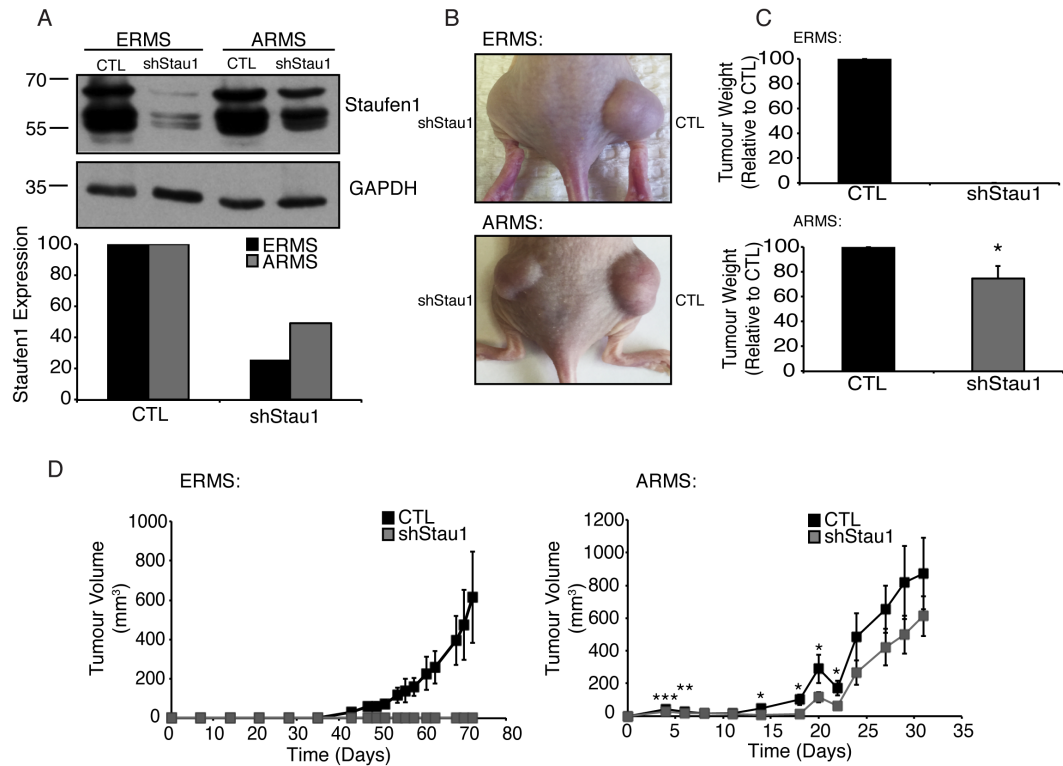


Figure 21. Staufen1 knockdown inhibits RMS tumour growth in a xenograft model.

Flank injections of 6-week-old female SCID-488 mice with ERMS (RD) or ARMS (RH30) cells following 72 hours of Control (CTL) or Staufen1-shRNA (shStau1) expression. To determine Staufen1 knockdown pre-injection, cells were collected for western blot analysis with (A) anti-Staufen1 antibodies and GAPDH as a loading control in ERMS (RD) and ARMS (RH30) cells. Quantification is represented as a percentage relative to CTL (n=1, batch culture). (B) Representative endpoint images of ERMS (RD) and ARMS (RH30) at 72 days and 31 days post-injection, respectively. (C) Tumour weight from CTL or shStau1 at endpoint for ERMS (RD) (n=7) and ARMS (RH30) cells (n=6). (D) Tumour volume measurements of ERMS (RD) and ARMS (RH30) tumours over time (n=7 and n=6, respectively). Data are Mean $\pm$ SEM, *P<0.05, **P<0.01, ***P<0.001.

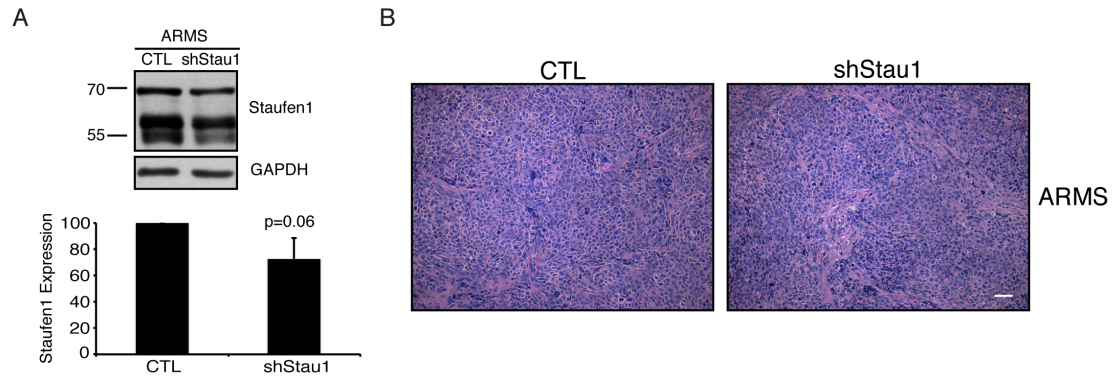


Figure 22. Staufen1 expression in end-point ARMS xenograft tumours. Control (CTL) and Staufen1-shRNA (shStau1) expressing ARMS tumours were dissected and paraffin-embedded at day 31 post-injection. (A) Staufen1 expression in end-point ARMS tumours was examined by western blot using anti-Staufen1 antibodies and anti-GAPDH antibodies as a loading control. Quantification is represented as a percentage relative to CTL (n=6) and data are Mean $\pm$ SEM. (B) Representative sections of CTL and shStau1 ARMS tumours stained with Hematoxylin and Eosin, scale bar = 50 μ m.

Differential Role for Staufen1 in ERMS and ARMS

Our data demonstrate that Staufen1 has differential roles in ERMS and ARMS. Based on our previous findings that Staufen1 regulated c-myc translation (Ravel-Chapuis et al. 2014) and given the well-known implication of c-myc in RMS (Martinelli et al. 2009; Schaaf et al. 2010; Marampon et al. 2009; Marampon et al. 2011), we investigated the impact of Staufen1 on c-myc expression in ERMS and ARMS. First, we evaluated c-myc expression in ERMS and ARMS cells expressing Staufen1-shRNA. Western blot analysis demonstrated an ~99% and ~95% decrease, in Staufen1 expression, respectively (**Figure 23A**). We observed an ~28% reduction in c-myc protein expression in ERMS cells upon Staufen1 knockdown ($P < 0.01$, **Figure 23B**). These data are consistent with our previous study showing a decrease in c-myc expression in C2C12 and HSMM cells expressing Staufen1-shRNA (Ravel-Chapuis et al. 2014). Conversely, c-myc expression was unaffected following Staufen1 knockdown in ARMS cells (**Figure 23B**). Next, we analyzed expression of p14^{ARF}, a downstream target of c-myc to determine if an ~28% decrease in c-myc is sufficient to impair c-myc function. Our data showed that p14^{ARF} was decreased, but only in ERMS cells with reduced Staufen1 expression (~29% decrease, $P < 0.01$, **Figure 23C**). Interestingly, the relative expression of both c-myc and p14^{ARF} are decreased similarly in ERMS cells (**Figure 23B,C**). Such a reduction was not observed in ARMS, which actually showed increased p14^{ARF} expression (~65% increase, $P = 0.1$, **Figure 23C**).

To further evaluate the differential roles of Staufen1 in ERMS and ARMS, we performed polysome-profiling experiments with CTL and Staufen1-shRNA expressing cells. The optical density profiles for each sucrose gradient were measured at 254 nm and

profiles for ERMS and ARMS cells are depicted (**Figure 23E,D**). The top of the sucrose gradient contains free mRNAs and the 40S, 60S and 80S ribosomal subunits, while mRNAs associated with polysomes are concentrated at the bottom of the gradient. Next, we examined the expression profile of c-myc mRNAs across each gradient by qRT-PCR. Our data demonstrate that c-myc mRNAs are poorly translated in ERMS cells expressing Staufen1-shRNA (**Figure 23D**). These findings are entirely consistent with our previous work in C2C12 cells(Ravel-Chapuis et al. 2014) and support the notion that Staufen1 regulates c-myc translation in ERMS cells. In contrast, Staufen1 knockdown in ARMS cells only modestly affects c-myc mRNA localization since it remained concentrated in the polysome fractions (**Figure 23E**). Together, these data highlight the impact of Staufen1 in RMS, and further demonstrate that Staufen1 regulates ERMS and ARMS tumorigenesis via c-myc-dependent and -independent events.

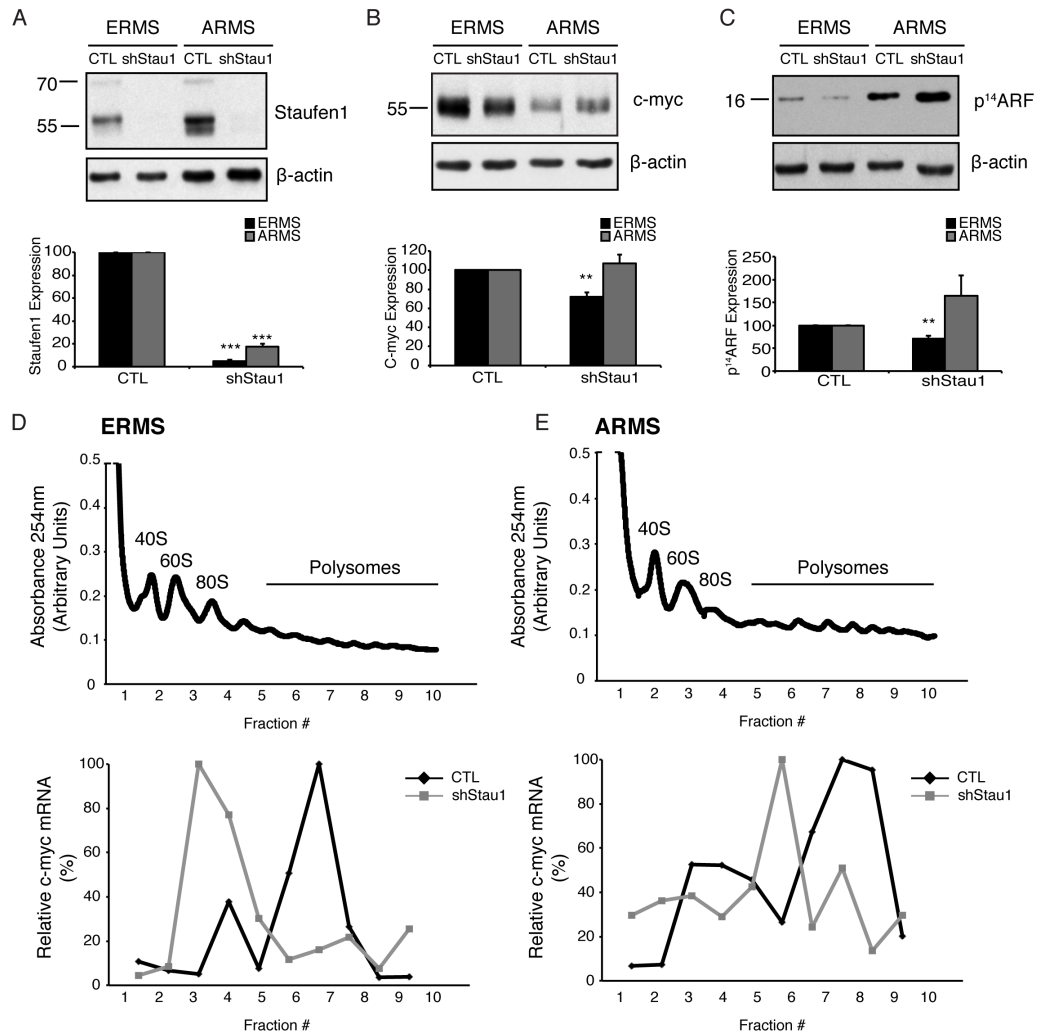


Figure 23. Staufen1 has differential roles in ERMS and ARMS via c-myc-dependent and -independent events, respectively. Western blot analysis of ERMS (RD) and ARMS (RH30) cells following 48 hours of Control (CTL) or Staufen1-shRNA (shStau1) expression for (A) Staufen1 expression (B) c-myc expression and (C) p¹⁴ARF. All quantifications are normalized to β -actin and represented as a percentage of the Control (CTL) with n=3. Polysome profiling of (D) ERMS (RD) and (E) ARMS cells expressing CTL or shStau1. Polysome profile obtained by continuous absorbance readings at 254 nm (Top) and c-myc mRNA expression measured by qRT-PCR from 10 x 1 mL sucrose gradient fractions (Bottom). Data are Mean $\pm$ SEM, *P<0.05, **P<0.01, ***P<0.001.

Discussion

Given the well documented impact of c-myc in RMS tumorigenicity (Martinelli et al. 2009; Schaaf et al. 2010; Blackwood & Eisenman 1991; Marampon et al. 2006; Marampon et al. 2009; Marampon et al. 2011; Gravina et al. 2016), and our recent findings showing that Staufen1 regulates c-myc during skeletal muscle differentiation (Ravel-Chapuis et al. 2014), we hypothesized in the current study that Staufen1 controls RMS tumorigenicity. We thus performed a series of complementary experiments to examine whether Staufen1 controls c-myc expression in two RMS subtypes, and determined the impact of this regulatory pathway on tumorigenesis. Results showed that Staufen1 is overexpressed in ERMS and ARMS tumours and cell lines. Similarly, c-myc protein but not mRNA was also increased in both subtypes. Moreover, Staufen1 specifically regulates the proliferation and apoptosis of ERMS and ARMS cells, respectively, thereby highlighting the differential roles of Staufen1 in these RMS subtypes. Finally, knockdown of Staufen1 decreased invasion and migration in ERMS and ARMS cultures, while also inhibiting tumour growth *in vivo*. Together, our data provide the first direct evidence for the impact of Staufen1 in cancer biology.

Converging lines of evidence indicate that Staufen1 plays an important regulatory role during skeletal muscle differentiation as well as in neuromuscular disorders (Bélanger et al. 2003; Kim et al. 2007; Yamaguchi et al. 2008; Gong et al. 2009; Yamaguchi et al. 2012; Ravel-Chapuis et al. 2012; Ravel-Chapuis et al. 2014; Bondy-Chorney et al. 2016; Ravel-Chapuis et al. 2016). In particular, we showed that Staufen1 is down-regulated during myogenesis and embryonic muscle development, but that its expression remains preferentially high within the post-synaptic sarcoplasm of neuromuscular junctions

(Bélanger et al. 2003; Ravel-Chapuis et al. 2014). Moreover, sustained expression of Staufen1 causes myoblasts to differentiate poorly as evidenced by inhibition of fusion and a decrease in several differentiation markers including MyoD, myogenin, MEF2A and MEF2C (Ravel-Chapuis et al. 2014). Similarly, Yamaguchi et al. reported that Staufen1 negatively regulates muscle differentiation (Yamaguchi et al. 2008) via regulation of Dvl2 mRNAs (Yamaguchi et al. 2012). In addition, it has also been suggested that Staufen1 controls myogenesis through a process referred to as Staufen1-mediated decay (SMD) (Kim et al. 2007; Gong et al. 2009)²². Taken together, results from these studies clearly highlight the role of Staufen1 during myogenesis.

In our recent work, we uncovered that Staufen1 increases expression of c-myc through a translational mechanism that involves its 5'UTR. An increase of c-myc, in turn, delays cell cycle exit thereby causing impaired differentiation (Ravel-Chapuis et al. 2014). Such regulation of c-myc translation by Staufen1 is consistent with the ability of Staufen1 to promote translation of target mRNAs via secondary structures formed within 5'UTRs (Dugré-Brisson et al. 2005). Along those lines, more recent work further showed that Staufen1 can regulate translation via secondary structures located throughout mature transcripts (Ricci et al. 2013; Sugimoto et al. 2015). Of relevance, a similar role for Staufen1 in regulating c-myc expression was reported in a study focused on IGF2BP1 in osteosarcoma U2OS cells (Weidensdorfer et al. 2009). In the latter study, Staufen-containing mRNPs co-purified with IGF2BP1-containing mRNPs, and Staufen1 knockdown decreased c-myc protein expression without parallel alterations in mRNA levels (Weidensdorfer et al. 2009). Our current results obtained with ERMS cells are thus entirely consistent with these earlier studies showing that Staufen1 exerts a profound

translational regulatory influence on c-myc mRNAs. The importance of translational regulation has been described for other RBPs such as Y-box binding protein 1 (YB-1), which increases translation of specific target mRNAs causing, for example, enhanced metastatic potential of breast cancer cells (Evdokimova et al. 2009) and drives sarcoma invasion and metastasis (Somasekharan et al. 2015). In the context of RMS, it was recently shown that IGF2BP1 regulates translation of mRNAs encoding cellular inhibitor of apoptosis 1 (cIAP1) thus impacting ERMS cell survival and drug resistance (Faye et al. 2015).

The role of c-myc in driving oncogenesis has been extensively studied for several years (Oster et al. 2002). More specifically, the reduction of c-myc through the Ras/MEK/ERK signaling greatly reduced ERMS malignancy and radioresistance (Martinelli et al. 2009; Schaaf et al. 2010; Blackwood & Eisenman 1991; Marampon et al. 2006; Marampon et al. 2009; Marampon et al. 2011; Gravina et al. 2016). Although c-myc protein expression is increased in both ERMS and ARMS cell lines and primary tumour samples, it appears that Staufen1 has different roles in regulating its expression. In ERMS cells, Staufen1-mediated regulation of c-myc is consistent with the expected translational regulation as discussed above and the decrease in proliferation we observed. By contrast, knockdown of Staufen1 in ARMS cells is unable to modulate c-myc expression. However, reduction of Staufen1 in ARMS increased apoptosis of ARMS cells in culture, without affecting proliferation, and also inhibited tumour growth in a xenograft model. A similar effect of Staufen1 knockdown was observed in neuronal cells since disruption of any component of the TDP-43/FMRP/Staufen1 complex, including with a siRNA against Staufen1, sensitized these cells to apoptosis (Yu et al. 2012).

Combined with our findings, these data indicate that in addition to Staufen1's role in controlling exit/entry of the cell cycle (Boulay et al. 2014; Ravel-Chapuis et al. 2014), Staufen1 can also regulate apoptosis in specific cellular contexts. Altogether, these data not only highlight the differential roles of Staufen1 in RMS, but also strengthen the importance of Staufen1-regulated c-myc events.

Through activation of p14^{ARF}, c-myc modulates the activity of the tumour suppressor protein p53, inducing apoptosis (Zindy et al. 1998). Although the ERMS RD cell line used contains a homozygous mutation in the *TP53* gene, rendering it non-functional, the ARMS RH30 cell line contains a heterozygous mutation leaving one functional *TP53* allele (Felix et al. 1992; Hinson et al. 2013). In the current study, the knockdown of Staufen1 failed to regulate c-myc expression in ARMS cells. Given the increased p14^{ARF} expression, it seems that this may be sufficient to activate p53 and increase apoptosis in ARMS cells. Therefore, sustained c-myc expression and increased p14^{ARF} in ARMS, despite the Staufen1 knockdown, likely contributes to the increased apoptosis observed in these cells.

In recent years, Staufen1 has emerged as a multi-functional RBP involved in several key aspects of RNA metabolism including mRNA localization (Tang et al. 2001), stability (Kim et al. 2007; Gong et al. 2009; Y. K. Kim et al. 2005), translation (Dugré-Brisson et al. 2005; Ravel-Chapuis et al. 2014; Ricci et al. 2013; Sugimoto et al. 2015), and alternative splicing (Ravel-Chapuis et al. 2012; Bondy-Chorney et al. 2016; Sugimoto et al. 2015). Therefore, it seems most likely that Staufen1 regulates other target mRNAs in ARMS, which act in combination with c-myc regulated p53-dependent apoptosis, to amplify the apoptotic response. In this context, several groups have

performed large scale screens to identify Staufen1-interacting proteins and mRNA binding sites across various cell types, adding to the complexity of Staufen1-regulated events (Furic et al. 2008; Milev et al. 2012; Ricci et al. 2013; Sugimoto et al. 2015). In addition, small and large-scale screens have also been performed on ARMS cells and tumours to better understand the impact of the PAX3- or PAX7-FOXO1 fusion proteins (Pandita et al. 1999; Shern et al. 2014; Khan et al. 2001; Khan et al. 1998; Schaaf et al. 2005; Romualdi et al. 2006; Seki et al. 2015). Comparative analysis of Staufen1-regulated mRNAs with the disrupted genes and molecular pathways caused by the oncogenic fusion proteins may identify potential Staufen1 targets relevant for ARMS. For example, *FGFR1*, *KRAS*, *NRAS*, *MDM2*, and *CDK4* are commonly misregulated in fusion-positive ARMS (Rodriguez-Perales et al. 2004; Khan et al. 2001) and, interestingly, each contains at least one Staufen1-binding site in their coding sequence (CDS) or 3'UTR (Sugimoto et al. 2015). The identification of all Staufen1 targets will prove invaluable for comprehensively deciphering its regulatory function in fusion-positive ARMS.

Recent work from DesGroseillers and colleagues demonstrated the cell cycle-dependent regulation of Staufen1 expression in various cancerous cell lines (HCT116, U2OS, and HEK293T). Based on these observations, the authors hypothesized that Staufen1 may participate in the cell cycle progression of cancer cells (Boulay et al. 2014). Our recent study describing the impact of Staufen1-dependent regulation of c-myc expression in skeletal muscle also indicated that Staufen1 is involved in controlling the proliferative capacity of cells (Ravel-Chapuis et al. 2014). Here, using multiple approaches, we thus provide direct evidence that Staufen1 regulates key tumorigenic

features of cancer cells both *in vitro* and *in vivo* thereby revealing for the first time its crucial involvement in cancer biology. Such a novel function of Stau1 in RMS clearly highlights its potential as a new and relevant target for the development of therapeutic strategies based on Stau1 modulation that are much needed for this devastating pediatric cancer.

Methods

Constructs and Antibodies

The constructs used were pLKO.1-TRC cloning vector, a gift from David Root (Addgene #10878), pLKO.1-TRC-shStau1 (Clone ID: TRCN0000102306, Clone ID: TRCN0000102308 and Clone ID: TRCN0000102309) (GE Healthcare Life Sciences, Ontario, Canada), pMD2.G (Addgene # 12259) and psPAX2 (Addgene # 12260) were gifts from Didier Trono.

The antibodies used were anti-Staufen1 (ab73478, Abcam, Ontario, Canada), anti-c-myc (ab11917, Abcam, Ontario, Canada), anti-p¹⁴ARF/CDKN2A (NB200-111, Novus Biologicals, Ontario, Canada), anti- β -actin (#47778, Santa Cruz Biotechnology, CA, USA), anti-Tubulin (T5168, Sigma-Aldrich, Ontario, Canada) and anti-GAPDH (ab8245, Abcam, Ontario, Canada).

Cell Culture, Transfection, and Lentivirus Production and Infection

Clonetics Skeletal Muscle Cells (SkMC) and Human Skeletal Muscle Myoblasts (HSMM) were cultured in according to manufacturer instructions (Lonza, NJ, USA). Embryonal rhabdomyosarcoma RD cells (CCL-136) and HEK 293T cells (CRL-3216) were cultured according to manufacturer instructions (American Type Culture Collection, VA, USA). Alveolar rhabdomyosarcoma RH30 cells (CRL-2061; American Type Culture Collection, VA, USA) and the RH36, RH18 and RH41 cells, which were a gift from Dr. P. Houghton (Department of University of Hematology-Oncology, St Jude Children's Research Hospital), were cultured in Multicell RPMI 1640 1X with L-Glutamine (Wisent Bioproducts, Quebec, Canada) supplemented with 15% HyClone FBS

and 1% HyClone Penicillin-Streptomycin (Thermo Fisher Scientific, Ontario, Canada). All cell cultures were incubated at 37°C, 5% CO₂.

Lentiviral particles were produced in HEK-293T cells by transfection of CTL or shRNA vectors and packaging vectors psPAX2 and pMD2.G using Lipofectamine 2000 (Invitrogen, Ontario, Canada) according to the manufacturers protocol. Medium containing viral particles was collected by centrifugation (1000rpm, 5 min) and filtered (0.45 µm). Cells were infected twice with equal volumes of virus and media containing 8 µg/ml Hexadimethrine Bromide (Sigma-Aldrich, Ontario, Canada). Cells were collected 48-72 h post-secondary infection.

Western Blotting

Cells were lysed in RIPA buffer as previously described (Ravel-Chapuis et al. 2014), sonicated and centrifuged (13000 rpm, 10 min). Protein concentration was determined with the Bicinchoninic Acid protein assay kit (Thermo Fisher Scientific, Ontario, Canada) and 10-30 µg of protein was separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Ontario, Canada). Membranes were then blocked and incubated with antibodies as previously described (Ravel-Chapuis et al. 2014). For endogenous Stau1 expression in RMS cells, only the predominant Stau1⁵⁵ isoform was quantified whereas total Stau1 expression was quantified following knockdown.

RNA Extraction, Reverse Transcription, and Real-Time quantitative PCR

RNA extraction, reverse transcription and qRT-PCR were performed as previously described (Ravel-Chapuis et al. 2014). Primer sequences were as follows: Stau1 (fwd 5'-AACGGAAGTTGCCTGTGAAT-3', rev 5'-AGGGGCGGTAAGTTCTTCAG-3'), c-myc (fwd 5'-CCTACCCTCTCAACGACAGC-3', rev 5'-

CTCTGACCTTTTGCCAGGAG-3') and 18S (fwd 5'-GTAACCCGTTGAACCCCAT-3', rev 5'-CCATCCAATCGGTAGTAGCG -3').

Human Samples

Frozen and formalin-fixed, paraffin-embedded human pediatric RMS tumours and normal skeletal muscle samples were obtained from either the Children's Hospital of Eastern Ontario or from the Ontario Tumour Bank after institutional ethics board approval. Biological materials were provided by the Ontario Tumour Bank, which is funded by the Ontario Institute for Cancer Research. All primary tumour samples were collected following informed consent and all experiments were performed in accordance with relevant guidelines and regulations. Control human muscle lysates were purchased (Novus Biologicals, Ontario, Canada). TMA slides were purchased from US Biomax, Inc. (Rockville, MD, USA).

Immunohistochemistry

Formalin-fixed, paraffin-embedded TMA slides were deparaffinized in xylene and rehydrated through a 100-70% ethanol gradient. Heat induced epitope retrieval was done at 110°C, 12 min with citrate buffer pH 6.0 and 3% H₂O₂ was used to block endogenous peroxidases. Sections were blocked with Background Sniper blocking reagent (BioCare Medical, CA, USA) and then incubated with anti-Staufen1 antibodies for 1 h at room temperature (1:500 antibody dilution). The MACH4TM + DAB detection system was used according to manufacturers instruction (BioCare Medical, CA, USA). Nuclei were counter-stained using Hematoxylin. A pathologist analyzed and scored the sections for staining intensity and a 4-point scale was developed to score Staufen1 staining in the tissues, where 0-1 is low, 2-3 is moderate, and 4 represents high Staufen1 expression. In

most cases, the percentage of cells positive for Staufen1 staining was ~90-100%. Only in four cases was the percentage of positive cells below ~20% and these tumours were scored as 0-1 staining intensity since these tumour cells had a high nuclear/cytoplasmic ratio, making it difficult to analyze with light microscopy. Therefore, the assessment of percentage of positive cells was deemed to be non-contributory.

Proliferation Assay

Cell proliferation was quantified by flow cytometry using 5-bromo-2'-deoxyuridine (BrdU) incorporation. Cells were seeded on 60 mm culture plates (7.5×10^5), infected with CTL or Staufen1-shRNA lentivirus and maintained at 70% confluency. BrdU (30 μ M) (Invitrogen, Ontario, Canada) was added to cultures 72 h post-secondary infection and incubated for 2 h. Labeled cells were washed with sterile PBS and collected using HyClone Trypsin 0.25% (1X) solution. Cells were washed and fixed with 5 mL of cold 70% ethanol for 30 min at -20°C . Cells were centrifuged (1200 rpm, 7 min), washed and collected by centrifugation (1200 rpm, 7 min). Cells were resuspended in 1 mL of sterile PBS containing 0.5 mg/mL RNase A (QIAGEN, Ontario, Canada) and incubated for 30 min at 37°C , then washed with 1 mL of sterile PBS and centrifuged (1200 rpm, 7 min). Samples were resuspended in 1 mL of cold 0.1 N HCl (Sigma-Aldrich, Ontario, Canada) containing 0.7% Triton X-100 (Sigma-Aldrich, Ontario, Canada) and incubated at 4°C for 15 min. Subsequent washing with 1 mL of sterile PBS, centrifugation (1200rpm, 7min) and resuspension in 1 mL of sterile H_2O , incubated at 97°C for 15 min and then 4°C for 15 min. Next, cells were resuspended in 1 mL of 0.5% Tween 20 (Sigma-Aldrich, Ontario, Canada) and were collected by centrifugation (1200 rpm, 7 min). Pellets were suspended in 100 μ l of HBT (0.5% Tween 20, 5%w/v BSA in sterile PBS) containing

Alexa Fluor[®] 488 anti-BrdU antibody (1:20) (Invitrogen, Ontario, Canada) and incubated at room temperature for 30 min. 1 mL of HBT was added and cells were centrifuged (1200 rpm, 7 min). Finally, cells were suspended in 0.5 mL of 20 µg/mL PI (Sigma-Aldrich, Ontario, Canada) and 100 µg/mL RNase A (QIAGEN, Ontario, Canada), transferred to 5 mL Falcon[®] polystyrene round bottom tubes (Thermo Fisher Scientific, Ontario, Canada) and incubated at 4°C for 30 min. Stained cells were analyzed using the Beckman MoFlo[®] Astrios[™] or the BD LSRFortessa[™] flow cytometers. Data analysis was performed using FlowJo software.

Apoptosis Assay

Cells were seeded in 60 mm culture plates (7.5×10^5), infected with CTL or Staufen1-shRNA lentivirus and maintained at 70% confluency. Cells were co-stained 72 h post-secondary infection using the Alexa Fluor[®] 488 Annexin V/Dead Cell Apoptosis Kit with Alexa Fluor[®] 488 annexin V and PI for Flow Cytometry (Thermo Fisher Scientific, Ontario, Canada) according to manufacturer instructions. Stained cells were analyzed using the Beckman MoFlo[®] Astrios[™] or the BD LSRFortessa[™] flow cytometers. Data analysis was performed using FlowJo software.

Motility and Invasion Assay

Infected cells (2.5×10^4) were seeded in serum free medium in Corning[®] BioCoat[™] Control Inserts (#354578) or Corning[®] GFR Matrigel[®] Basement Membrane Matrix Invasion Chambers (#354480) (VWR International, Ontario, Canada) containing growth medium in the bottom chamber. RD and RH30 cells were incubated for 72 and 48 h, respectively. Cells were fixed and stained with the Shandon[™] Kwik-Diff[™] Stain (Thermo Fisher Scientific, Ontario, Canada). Cell motility and invasion were assessed

according to manufacturer instructions. Six random fields of view were imaged and analyzed using Northern Eclipse Software (NES, Expix Imaging, Ontario, Canada).

Migration Assay

RD and RH30 cells were infected with CTL or Staufen1-shRNA lentivirus for 48 h post-secondary infection and were seeded into culture inserts (RD CTL 7.5×10^5 cells/ml; RD shStau1 1×10^6 cells/ml; RH30 CTL; shStau1 5×10^5 cells/ml) to form a confluent monolayer (Ibidi, Munich, Germany). Inserts were removed 24 h post-seeding and washed with PBS and imaged in real time for 30 h using the Incucyte[®] ZOOM system (Essen Bioscience, MI, USA). The gap was measured using Northern Eclipse Software (NES, Expix Imaging, Ontario, Canada). The average distance at 3 points was calculated. These values were normalized to the % confluence of cells.

Xenograft Mouse Model

Animal experimental protocols were approved by the University of Ottawa Animal Care Committee and were in accordance with the Canadian Council of Animal Care Guidelines. RD and RH30 cells were infected with lentivirus encoding CTL or Staufen1-shRNA and collected 72 h post-secondary infection. Subcutaneous tumours were established by performing flank injections with 2.5×10^6 and 5×10^6 RD and RH30 cells, respectively, on randomly selected 6-week-old female SCID hairless mice SHC strain 488 (Charles River, Saint-Constant, Canada). The right flank injection contained pLKO.1-TRC-Control expressing cells whereas the left flank injection contained pLKO.1-Staufen1-shRNA expressing cells. Tumour width and length were measured throughout experiment (unblinded) and tumour volume was calculated using $[(L \times W^2)/2]$ where L is the tumour length and W is the tumour width. One animal was excluded as no

tumours formed during the experimental timeline. Each mouse represented a biological replicate, and both littermates and non-littermates were used.

Polysome Profiling

RD and RH30 cells were infected with two rounds of CTL or Staufen1-shRNA and were grown at 50-60% confluency on 150 mm culture plates. Two plates per condition were treated at 48 h post-secondary infection with 0.1 mg/ml Cycloheximide (CHX) (Sigma-Aldrich, Ontario, Canada) for 5 min in DMEM. Cells were washed twice with ice cold 1X PBS pH 7.4 containing 0.1 mg/ml CHX, collected by centrifugation (1000 rpm x 5 min 4°C) and resuspended in 500 uL RNA lysis buffer (0.3 M NaCl, 15 mM MgCl₂, 15 mM Tris pH 7.4, 1% Triton X-100, 1 mM Dithiothreitol, 0.1 mg/mL CHX, 100 U/mL RNasin). Cells were incubated on ice for 10 min and lysed using a 20 gauge needle. Nuclear debris was removed by centrifugation at 5000 rpm x 10 min at 4°C followed by a subsequent centrifugation at 13 000 rpm for 10 min at 4°C to remove cellular debris. Next, 500 uL of cell lysate was layered on the top of continuous sucrose gradients (15-45% sucrose in 0.3 M NaCl, 15 mM MgCl₂, 15 mM Tris pH 7.4). Ultracentrifugation of samples was performed at 39 000 rpm in a SW41-Ti rotor for 90 min at 4°C with no brake. We collected 1 mL fractions using the BRANDEL Density Fractionation System. Samples were digested with proteinase K and total RNA was extracted and analyzed by qRT-PCR.

Statistical Analysis

All experiments were performed with a minimum of $n \geq 3$ biological replicates unless otherwise stated. The data were analyzed using the student's t-test. Significance was set

at $P \leq 0.05$ with $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$. Error bars represent standard error of the mean (SEM).

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Chapter 4: Impact of Sustained Staufe1 Expression in Postnatal Skeletal Muscle

**Muscle-specific Expression of the RNA-binding Protein Staufen1 Induces
Progressive Skeletal Muscle Atrophy via Regulation of Phosphatase Tensin
Homolog**

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Author Contributions

T.E.C.P., E.B.C., and J.M.R. performed the experiments and analyzed the data. T.E.C.P., A.R.C., J.C. and B.J.J. conceived the project, designed the experiments and T.E.C.P. and B.J.J. wrote the manuscript.

Abstract

Converging lines of evidence have now highlighted the key role for post-transcriptional regulation in the neuromuscular system. In particular, several RNA-binding proteins are known to be misregulated in neuromuscular disorders including myotonic dystrophy type 1, spinal muscular atrophy and amyotrophic lateral sclerosis. In this study, we focused on the RNA-binding protein Staufen1, which assumes multiple functions in both skeletal muscle and neurons. Given our previous work that showed a marked increase in Staufen1 expression in denervated muscle, in embryonic and undifferentiated skeletal muscle, as well as in myotonic dystrophy type 1 muscle samples from both mouse models and humans, we investigated the impact of sustained Staufen1 expression in postnatal skeletal muscle. To this end, we generated a skeletal muscle-specific transgenic mouse model using the muscle creatine kinase promoter to drive tissue-specific expression of Staufen1. We report that sustained Staufen1 expression in postnatal skeletal muscle causes a myopathy characterized by significant morphological and functional deficits. These deficits are accompanied by a marked increase in the expression of several atrophy-associated genes and by the negative regulation of PI3K/AKT signaling. We also uncovered that Staufen1 mediates PTEN expression through indirect transcriptional and direct post-transcriptional events thereby providing the first evidence for Staufen1-regulated PTEN expression. Collectively, our data demonstrate that Staufen1 is a novel atrophy-associated gene, and highlight its potential as a biomarker and therapeutic target for neuromuscular disorders and conditions.

Introduction

Over the past 15 years, there has been increasing interest in determining the role of post-transcriptional regulation within the neuromuscular system. In this context, several studies have highlighted the importance of RNA-binding Proteins (RBPs) in regulating post-transcriptional events, particularly as many of these proteins are misregulated in neuromuscular disorders thereby negatively impacting on the pathophysiology and phenotypic outcomes associated with these diseases (Lukong & Fatimy 2012). In myotonic dystrophy Type I (DM1) for example, a core group of RBPs are misregulated including Muscleblind like splicing regulator 1 (MBNL1) and CUGBP Elav-like family member 1 (CUGBP1 also called CELF1), which in turn causes disruption of several post-transcriptional events contributing to the etiology of the disease (Timchenko et al. 2004; Ho et al. 2005; Ward et al. 2010; Kanadia et al. 2003; Kanadia et al. 2006; Chamberlain & Ranum 2012; Suenaga et al. 2012). Similarly, the ELAV family of RBPs that includes HuD and HuR, are important in spinal muscular atrophy (SMA) (Hubers et al. 2011; Farooq et al. 2009; Farooq et al. 2013) as well as in amyotrophic lateral sclerosis (ALS) (Milani et al. 2013; Fallini et al. 2012) where they control post-transcriptional events driving key hallmarks of these diseases.

The double-stranded RBP Staufen, is a multi-functional protein involved in several post-transcriptional events that affect target mRNAs. Staufen was first identified for its role in oogenesis and central nervous system development in *Drosophila*. In invertebrates, Staufen is necessary for correct localization of maternal mRNAs to the anterior and posterior poles of oocytes (St Johnston et al. 1991) as well as in the asymmetric localization of mRNAs in neuroblasts (Li et al. 1997; Schuldt et al. 1998). In

fact, the expression of Staufen is required for the transport and translation of *oskar* mRNAs during pole plasm formation, a critical step in germ-line formation and abdomen development in *Drosophila* (Breitwieser et al. 1996).

In mammals, there are two homologs of Staufen, namely, Staufen1 and Staufen2, with Stau1⁵⁵ and Stau2⁵⁹ (corresponding to their molecular mass in kDa) representing the primary Staufen splice variants (Bélanger et al. 2003). While Staufen1 is ubiquitously expressed, Staufen2 is predominantly expressed in the brain (Duchaine et al. 2002; Marión et al. 1999; Wickham et al. 1999). In neurons, both Staufen1 and Staufen2 are somatodendritically localized with Staufen-associated mRNPs moving bidirectionally along dendritic microtubules regulating mRNA localization and activity-dependent translation (Kiebler et al. 1999; Köhrmann et al. 1999; Tang et al. 2001; Krichevsky & Kosik 2001). In addition, Staufen1 mRNPs are important for the processing and transport of transcripts, including those encoding BC1 RNA and Calcium (Ca^{2+}) / calmodulin-dependent kinase II (CaMKII) α -subunit in dendrites of hippocampal neurons (Mallardo et al. 2003). These events are critical for the regulation of synaptic strength and maintenance of altered connectivity, which underlie hippocampus-dependent learning and memory (Marión et al. 1999). These studies, clearly highlight the multi-functional nature of Staufen1 and its importance in the development, maintenance and plasticity of neurons.

Several years ago, we reported that Staufen1 and Staufen2 are regulated during myogenesis and that their expression is elevated in denervated skeletal muscle (Bélanger et al. 2003). Others have later confirmed our original observations that Staufen1 is developmentally regulated in muscle (Gong et al. 2009; Chang et al. 2012; Yamaguchi et

al. 2008; Yamaguchi et al. 2012). More recently, we demonstrated that Staufen1 is abundant in undifferentiated myoblasts and embryonic muscles, and that its progressive decrease during myogenesis and in early postnatal muscle is essential for normal muscle development (Ravel-Chapuis et al. 2014). Along those lines, we also recently demonstrated that Staufen1 levels are markedly increased in muscle samples from both DM1 mouse models and human patients, correlating with disease severity (Ravel-Chapuis et al. 2012).

Based on these findings showing increased expression of Staufen1 in several physiologically-relevant conditions, and the necessity for its down-regulation in mature muscle, we hypothesize in the present work that sustained expression of Staufen1 in postnatal muscle triggers the activation of pathways that negatively impact adult muscle fibers. To this end, we generated and characterized a muscle-specific Staufen1 transgenic mouse model using the Muscle Creatine Kinase (MCK) promoter/enhancer regulatory cassette. We report that sustained Staufen1 expression causes a myopathy characterized by morphological and functional deficits. These changes are accompanied by increased expression of the atrogenes, Muscle Atrophy F-box Protein (MAFbx, also called Atrogin-1) and the Muscle-Specific RING Finger 1 protein (MuRF1). Our data further revealed that Staufen1 is a negative regulator of the PI3K/AKT signaling pathway through the regulation of Phosphatase Tensin Homolog (PTEN) expression. Accordingly, Staufen1 may represent an attractive biomarker and therapeutic target for neuromuscular diseases and conditions.

Results

Generation of a Skeletal Muscle-Specific Staufen1 Transgenic Mouse Model

To determine the role of sustained Staufen1 expression in skeletal muscle *in vivo*, we developed a skeletal muscle-specific Staufen1 transgenic mouse model. A vector was engineered containing the MCK enhancer/promoter regulatory cassette to direct tissue-specific expression of the coding sequence of mouse Stau1⁵⁵ with three C-terminal HA-tags and a polyadenylation signal (**Figure 24A**). The MCK enhancer/promoter was selected as it is widely used to generate muscle-specific expression of transgenes in skeletal muscle (Sternberg et al. 1988; Shield et al. 1996; Lyons et al. 1991; Donoviel et al. 1996; Amacher et al. 1993). The construct was linearized via restriction digest, purified and inserted into more than one hundred fertilized FVB/N embryos by pronuclear injection. Positive founder mice were identified by genotyping via PCR and two independent hemizygous transgenic lines, Tg-551 and Tg-6898, were generated through subsequent crossing with Wild-Type (WT) FVB/N mice. Various tissues from offspring were analyzed by western blot using anti-HA antibodies to confirm skeletal muscle-specific transgene expression in both Tg-551 and Tg-6898 lines (**Figure 24B**). Specifically, our data showed a marked expression of Staufen1-HA in the Tibialis Anterior (TA) and Extensor Digitorum Longus (EDL) muscles, with lower to no expression in Diaphragm (Dia), and Soleus (SOL) muscles (**Figure 24B**). As expected, no expression of the transgene was observed in non-skeletal muscle tissues such as the heart and brain (**Figure 24B**). These data are in agreement with the expected expression pattern caused by the MCK promoter/enhancer as suggested in the literature (Sternberg et

al. 1988; Tai et al. 2011). Here, this novel transgenic mouse model is referred to as MCK-Staufen1-HA.

Histological and Functional Analyses of MCK-Staufen1-HA Skeletal Muscle

We first performed histological analyses on Tg-551 and Tg-6898 mice to investigate the effect of sustained Staufen1 expression in postnatal skeletal muscle. Hematoxylin and Eosin (H&E) staining was performed on TA muscle cross-sections from 4-, 8- and 16-week-old Tg-551 and Tg-6898 transgenic mice (**Figure 24C**). Cross-sectional area (CSA) of TA muscle fibers was measured and the frequency distribution of CSA demonstrated that across all ages studied, both Tg-551 and Tg-6898 mouse lines have an increased frequency of small fibers concomitant with a decreased frequency of larger fibers as compared to WT littermates (**Figure 24D**). The Variance Coefficient (VC) of CSA was also calculated to assess fiber size variability. By 16 weeks of age, we noted an ~1.3-Fold increase ($P < 0.01$) in both transgenic lines (**Figure 24E**). In addition, there was an age-dependent decrease in the mean CSA in muscles from Tg-551 and Tg-6898 mice (**Figure 24F**). Finally, our analysis further revealed a significant increase in the percent of muscle fibers containing central nuclei, which progressively increased ($P < 0.001$) in an age-dependent manner (**Figure 24G**).

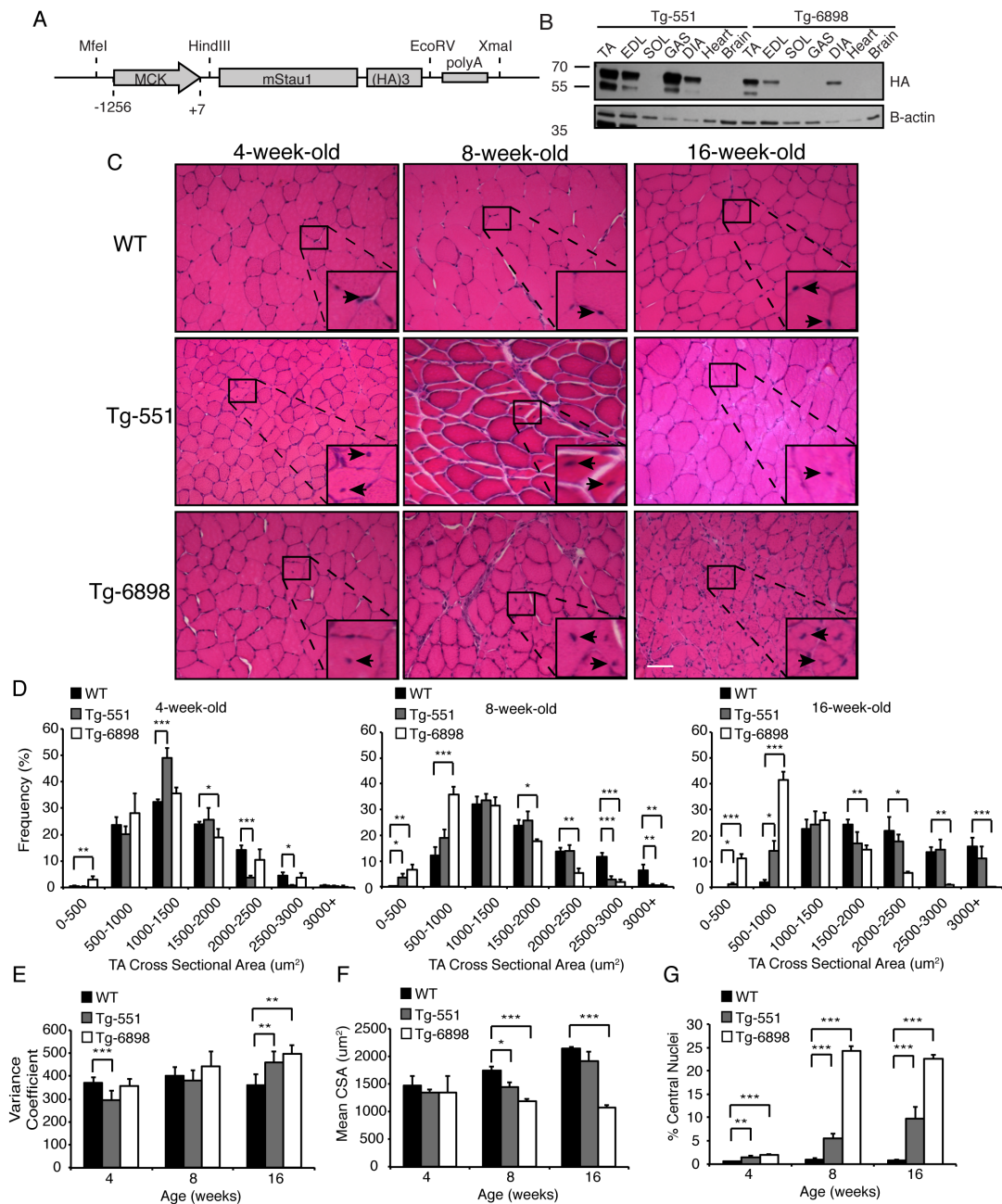


Figure 24. MCK-Staufen1-HA mice develop central nucleation, fiber variability and decreased myofiber size. (A) Schematic diagram of the MCK-Staufen1-HA linearized transgene. (B) Western blot using anti-HA antibodies to demonstrate the tissue specific pattern of 4-week-old Tg-551 and Tg-6898 Tibialis Anterior (TA) muscles with β -actin

as a loading control. **(C)** Representative hematoxylin and eosin staining of TA muscle cross sections (10 μm) from WT (FVB/N), Tg-551 and Tg-6898 4-week-old, 8-week-old and 16-week-old mice. Black arrows show central nuclei, scale bar = 50 μm . **(D)** Fiber cross sectional area (CSA) in μm^2 displayed as a frequency distribution (% of Total) of TA muscle fibers. **(E)** Variance Coefficient, **(F)** Mean CSA in μm^2 and **(G)** % Central Nuclei from 4-week-old WT (n=11), Tg-551 (n=5), Tg-6898 (n=6); 8-week-old WT (n=5), Tg-551 (n=5), Tg-6898 (n=4); and 16-week-old WT (n=5), Tg-551 (n=5), Tg-6898 (n=3). Data are means $\pm$ SEM. *P < 0.05, **P < 0.001, ***P < 0.0001, Student T-Test.

To complement these data, histological analyses of EDL muscles from 16-week-old WT, Tg-551, and Tg-6898 mice were conducted. H&E staining was performed on muscle cross-sections (**Figure 25A**) and CSA of muscle fibers was measured. The frequency distribution of EDL CSA showed an increased frequency of small fibers together with a decreased frequency of larger fibers (**Figure 25B**). The VC was also calculated and, as observed in TA muscles, both Tg-551 and Tg-6898 mouse lines showed an increased VC ($P < 0.001$ and $P < 0.01$, respectively; **Figure 25C**). In addition, the mean CSA of EDL fibers was decreased by ~32% and ~50% ($P < 0.001$ and $P < 0.01$) in Tg-551 and Tg-6898 mice, respectively (**Figure 25D**). EDL muscles from both transgenic mouse lines also displayed a significant increase in the percent of muscle fibers containing central nuclei (**Figure 25E**). These data obtained using EDL muscles, are in complete agreement with those from TA muscles, strengthening the notion that the increased fiber size variability, decreased CSA, and increased central nucleation are a direct consequence of sustained muscle-specific Stauf1 expression.

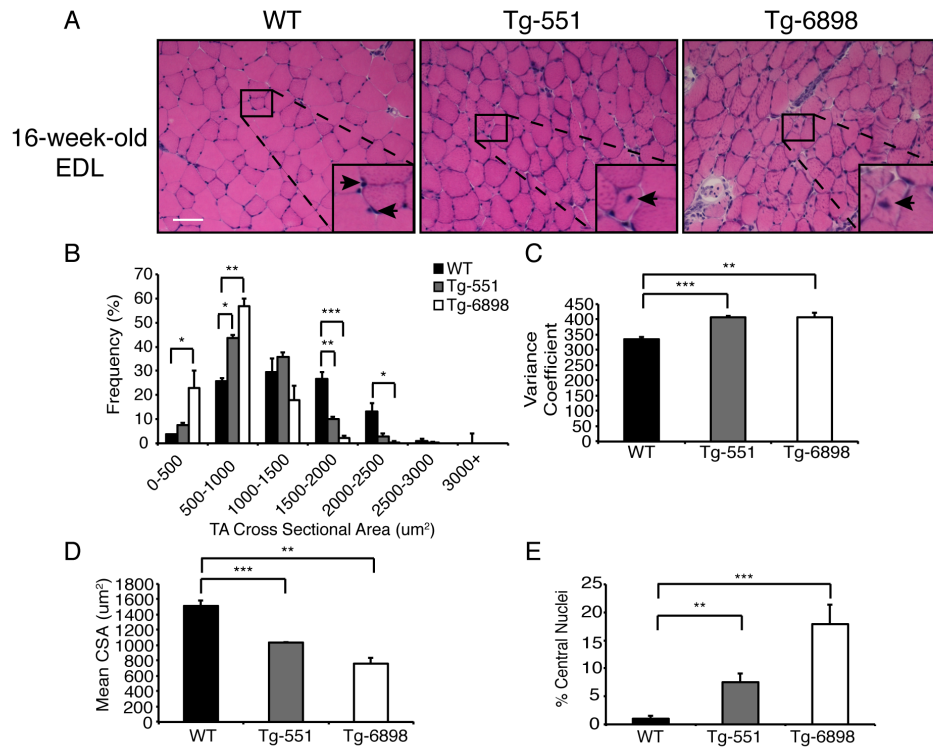


Figure 25. Analyses of adult MCK-Staufen1-HA EDL muscles. (A) Representative hematoxylin and eosin staining of Extensor Digitorum Longus (EDL) 10µm cross-sections, black arrows indicate central nuclei, scale bar = 50 µm. (B) Fiber cross sectional area (CSA) in µm², displayed as a frequency distribution (% of Total), (C) Variance Coefficient, (D) Mean cross sectional area in µm² and (E) % Central Nuclei of 16-week-old WT (n=3), Tg-551 (n=3), Tg-6898 (n=3) EDL muscle fibers. Data are means ± SEM. *P < 0.05, **P < 0.001, ***P < 0.0001, Student T-Test.

Since we observed an increase in central nucleation, we next investigated the expression of embryonic myosin heavy chain (MHC_{emb}), a known marker for muscle regeneration (Sartore et al. 1982; Schiaffino et al. 2015). Immunofluorescent staining performed on 8-week-old TA cross-sections showed an increase ($P < 0.001$) in the number of fibers expressing MHC_{emb} in both Tg-551 and Tg-6898 mice compared to WT (**Figure 26A**). In addition, we observed an ~ 2 -Fold increase ($P < 0.05$) in myosin heavy chain (MHC) Type I-positive fibers in TA muscles of Tg-6898 mice (**Figure 26B**), with no significant ($P > 0.05$) change in the number of fibers expressing MHC Type II A and B in either transgenic line (data not shown). Altogether, the increased expression of MHC_{emb} and central nucleation indicate ongoing regeneration as a result of sustained expression of *Staufen1* in mature muscles.

To determine whether muscle function was altered as a result of increased *Staufen1* expression, a cohort of WT, Tg-551 and Tg-6898 mice was studied at 4, 8, and 16 weeks of age. First, total body weight was measured and revealed that Tg-6898 mice had decreased body weight at all ages examined culminating with an $\sim 21\%$ decrease ($P = 0.06$) by 16 weeks (**Figure 27A**). Analysis of forelimb grip strength showed a progressive decline in force reaching an $\sim 34\%$ decrease ($P < 0.01$) in 16-week-old Tg-6898 mice (**Figure 27B**). Although Tg-551 grip strength was unchanged ($P > 0.05$) at 4 and 8 weeks of age, there was a significant decrease of $\sim 15\%$ in 16-week-old mice ($P < 0.05$, **Figure 27B**). Given the decreased total body weight in Tg-6898 mice, we also compared forelimb grip strength to body weight. As expected, our data demonstrate that there was no significant change in grip strength following normalization to total body weight in Tg-551 or Tg-6898 mice at all ages examined ($P > 0.05$, **Figure 27C**). These

data suggest that the sustained skeletal muscle-specific expression of Stauf1 leads to progressive muscle weakness due to decreased muscle size.

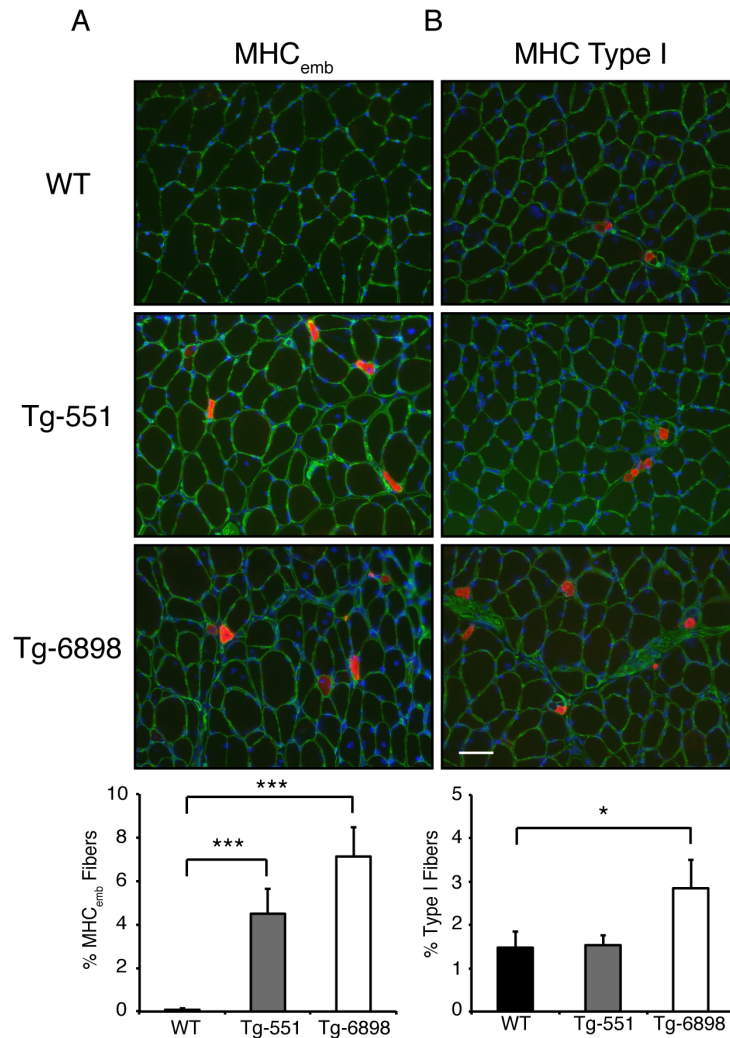


Figure 26. Fiber type composition of MCK-Staufen1-HA mice. Myosin Heavy Chain (MHC) isoforms **(A)** embryonic MHC (MHC_{emb}) and **(B)** MHC Type I fibers were detected in Tibialis Anterior (TA) 10μm muscle cross sections from 8-week-old WT (n=6), Tg-551 (n=4), and Tg-6898 (n=3) mice by immunofluorescent staining. Representative images are shown, scale bar = 50 μm and percentage of total fibers was calculated. Data are Means ± SEM. **P < 0.001, ***P < 0.0001, Student T-Test.

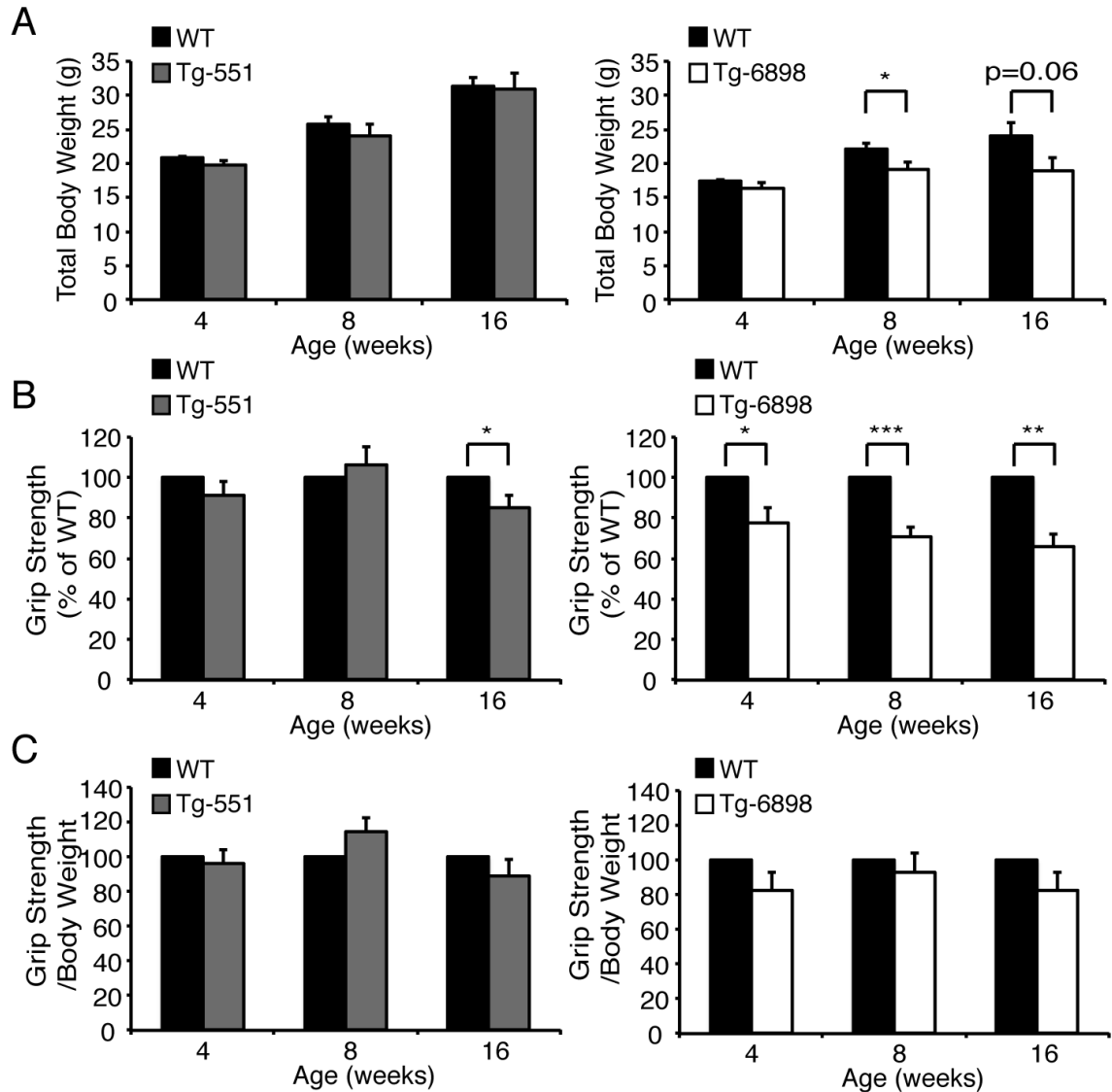


Figure 27. MCK-Staufen1-HA mice display impaired grip strength. A cohort of mice were tested at 4, 8, and 16 weeks of age to measure (A) Total body weight in grams (g). (B) Forelimb Grip Strength represented as a percentage relative to WT and (C) forelimb grip strength normalized to total body weight and represented as a percentage relative to WT. Tg-551 (n=5), Tg-6898 (n=4) and respective WT littermates (n=5 and n=4, respectively) were used. Data are means $\pm$ SEM. *P < 0.05, **P < 0.001, Student T-Test.

To further characterize the impact of sustained *Staufen1* expression on muscle function, we performed a series of *ex-vivo* experiments using EDL muscles of 16-week-old transgenic mice as we perform routinely (Miura et al. 2009; Ljubicic et al. 2011; Al-Rewashdy et al. 2014; Péladeau et al. 2015). First, wet muscle weight was measured to determine if the observed decrease in total body weight is a result of lower muscle mass. Indeed, EDL muscle weights of Tg-6898 mice were decreased by ~23% ($P < 0.01$, **Figure 28A**). Next, we examined the contractile properties of EDL muscles. Decreased tetanic force, which represents the maximum force a muscle can generate, was observed in Tg-6898 as compared to Tg-551 and WT mice ($P = 0.1$) (**Figure 28B**). The maximum tetanic force values were then normalized to CSA. After normalization, no significant change ($P > 0.05$) in Tg-551 and Tg-6898 EDL muscles as compared to WT mice were observed (**Figure 28C**). These data are consistent with grip strength measurements that showed decreased force in Tg-551 and Tg-6898 mice, an effect that was lost following normalization to total body weight. Similarly, twitch force, the force generated by a single action potential, was significantly decreased ($P < 0.05$) in Tg-6898 mice but was unchanged in EDL muscles following normalization to CSA (**Figure 28D,E**). In addition to these functional deficits, there was an ~8% increase ($P=0.05$) in the time to peak twitch force (tPEAK) and an ~10-15% increase ($P=0.01$ and $P=0.09$) in the time to half relaxation ($T_{1/2}$ Relax) of EDL muscles from Tg-551 and Tg-6898 mice, respectively (**Figure 28F,G**). Force frequency relationships generated following nerve stimulation showed a shift of ~15Hz towards lower stimulation frequencies for EDL muscles from Tg-551 and Tg-6898 mice as compared to WT (**Figure 28H**). The increased twitch contraction times (i.e. both tPEAK and $T_{1/2}$ Relax) are in agreement with this ~15Hz shift

and indicate that muscle fibers with elevated Staufen1 expression fuse at lower frequencies. Finally, a series of eccentric contractions was imposed on EDL muscles via stimulation at 200Hz. Interestingly, there was a trend for the loss of maximal tetanic force over the 12 contractions to be smaller for Tg-551 and Tg-6898 mice as compared to WT, but the difference was not significant ($P > 0.05$, **Figure 28I**). These latter data indicate that sarcolemma integrity is not compromised in EDL muscles with sustained Staufen1 expression.

Muscle atrophy is a phenomenon where the rapid loss of muscle proteins occurs in response to decreased protein synthesis and increased protein degradation. Since we observed an increased frequency of small fibers in Tg-551 and Tg-6898 mice compared to WT, we analyzed the expression of key atrophy-associated genes by quantitative RT-PCR (qRT-PCR) using primers specific for FOXO3a, MAFbx, and MuRF1 mRNAs. In 4-week-old Tg-551 TA muscles, we observed an ~1.4-Fold, ~3.7-Fold, and ~3.1-Fold increase ($P < 0.05$, $P < 0.001$, and $P < 0.01$) of FOXO3a, MAFbx, and MuRF1 expression, respectively (**Figure 29A, B, C**). Atrogene expression is known to only be required in the earlier stages of the atrophic program (Bodine et al. 2001; Gomes et al. 2001). In agreement with this, we observed in our transgenic mice increased ($P < 0.05$) atrogene expression at 4 and 8 weeks of age in comparison to WT, with no change or even reduced expression at 16 weeks of age (**Figure 29A, B, C**). A similar pattern of expression was seen in TA muscles from Tg-6898 mice (data not shown). Together, these data show that sustained expression of Staufen1 in skeletal muscle activates the atrophy program.

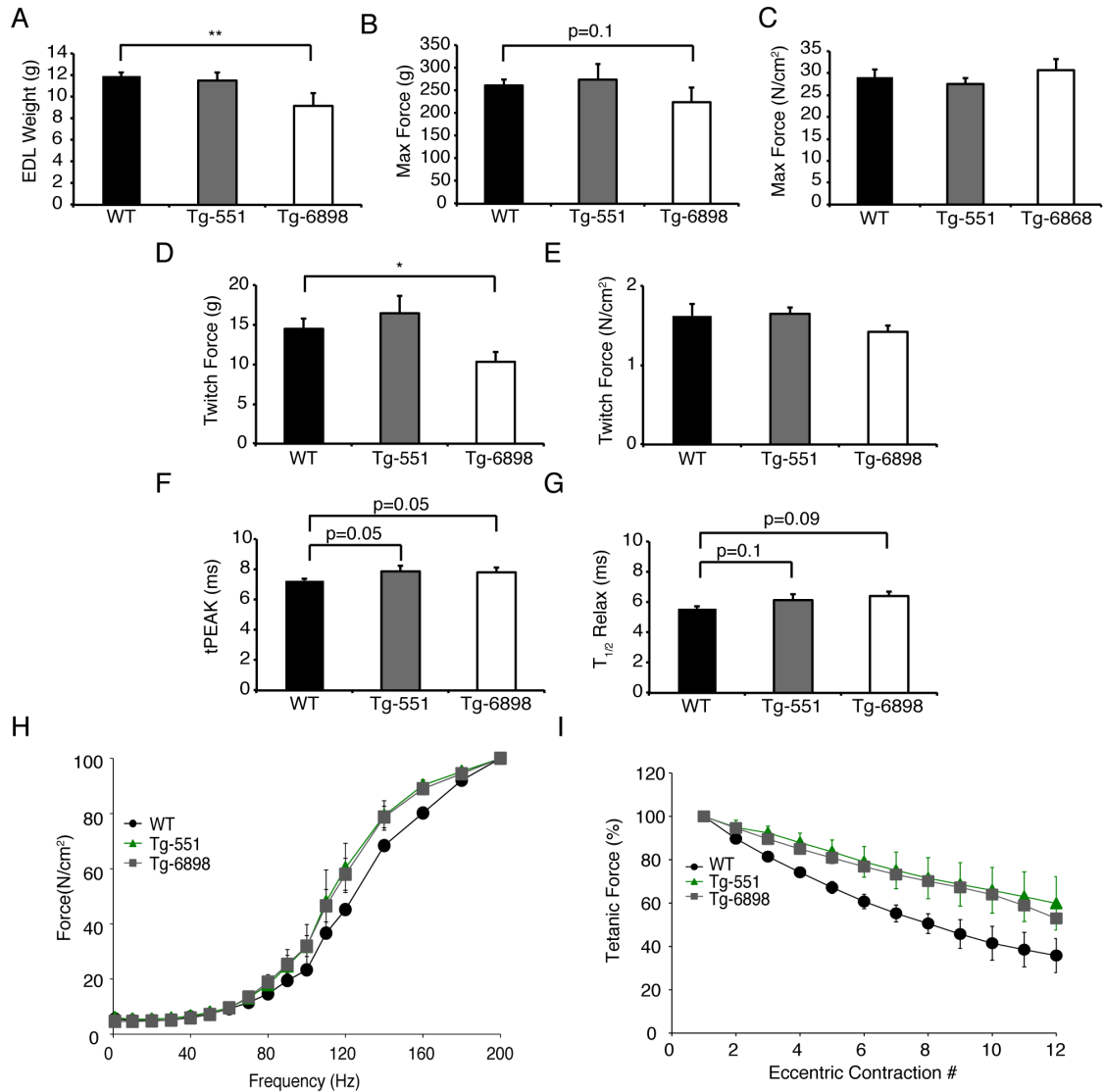


Figure 28. Impact of sustained Staufen1 expression on skeletal muscle contractile performance. A series of ex-vivo experiments were performed on WT, Tg-551, and Tg-6898 16-week-old Extensor Digitorum Longus (EDL) muscles. **(A)** Wet muscle weights were measured in grams (g). **(B)** Maximum tetanic force output of EDL muscles following nerve stimulation at 200Hz and **(C)** Maximum tetanic force output of EDL muscles following nerve stimulation at 200Hz normalized to muscle cross sectional area (CSA). **(D)** Maximum twitch force of EDL muscles and **(E)** Maximum twitch force of EDL muscles normalized to CSA. **(F)** Twitch contraction time to Peak tetanic force

(tPeak) measured in milliseconds (ms). (G) Twitch contraction time to half relaxation ($T_{1/2}$ Relax) measured in ms. (E) Force frequency curves following nerve stimulation from 0 to 200Hz and (F) peak tetanic force measured during 12 repeated eccentric contractions in EDL muscles at 200Hz for 16-week-old WT (n=5), Tg-551 (n=3) and Tg-6898 (n=3) mice. Data are means $\pm$ SEM. *P < 0.05, **P < 0.01, Student T-Test.

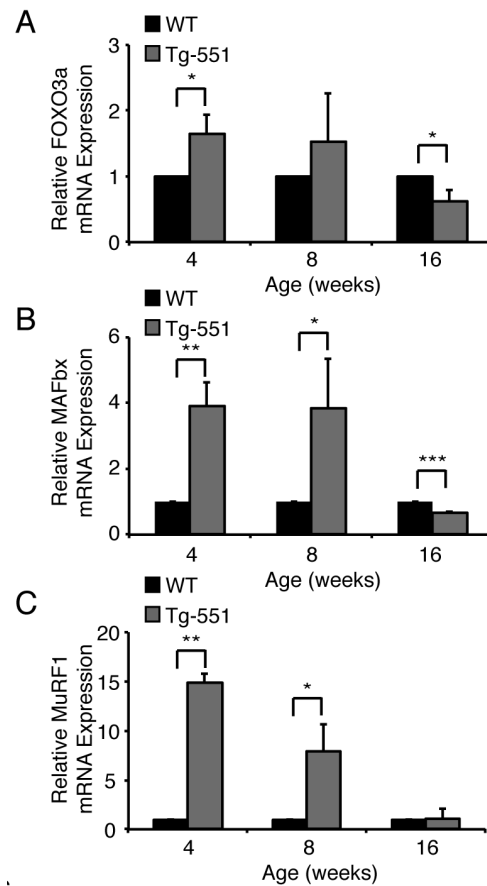


Figure 29. Increased expression of atrogenes in MCK-Staufen1-HA skeletal muscle.

Tibialis Anterior (TA) muscles were analyzed for (A) FOXO3a (B) MAFbx and (C) MuRF1 relative mRNA expression by qRT-PCR using primers specific for target mRNAs in 4-week-old WT (n=5), Tg-551 (n=6); 8-week-old WT (n=5), Tg-551 (n=5); and 16-week-old WT (n=5), Tg-551 (n=5) mice. Data are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$, Student T-Test.

Morphological and Functional Examination of the Neuromuscular Junction

Due to the induction of atrogene expression and the presence of atrophic fibers, it was important to examine the integrity of neuromuscular junctions (NMJ) of MCK-Staufen1-HA mice to determine if the atrophic phenotype was caused, at least partially, by abnormal NMJ structure and function. First, fluorescent staining of isolated single TA muscle fibers was performed using α -Bungarotoxin (α -BTX). Examination of NMJ staining revealed no overt abnormalities in 8-week-old Tg-551 and Tg-6898 mice as compared to WT (**Figure 30A**). Quantification of NMJ size showed that Tg-6898 mice had a non-significant ($P > 0.05$) decrease in endplate area, which could be expected since a population of transgenic muscle fibers has decreased CSA (**Figure 30B**). To functionally test NMJ, maximal tetanic force following nerve stimulation (also shown in **Figure 28C**) was compared to direct muscle stimulation (**Figure 30C**). Our data showed that maximum tetanic force of EDL muscles stimulated at 200Hz via the nerve or directly was similar in WT, Tg-551 or Tg-6898 mice (**Figure 30C**). These data demonstrate that the NMJ of MCK-Staufen1-HA mice are unaffected by the sustained expression of Staufen1. Together with the fact that we did not observe evidence of extra synaptic BTX staining along muscle fibers (**Figure 30A**), these findings suggest that the morphological, biochemical and functional changes seen in muscles from MCK-Staufen1-HA mice, are not caused by denervation.

To further complement these findings, we denervated hind limb muscles of WT and Tg-551 mice by severing the sciatic nerve to determine if MCK-Staufen1-HA mice respond to denervation-induced muscle atrophy in a manner analogous to that observed in WT mice. Total RNA was collected from innervated and short-term denervated TA

muscles and qRT-PCR was performed to analyze expression of atrogenes. Our data demonstrate that both MAFbx and MuRF-1 mRNA expression was markedly induced in TA muscles from WT as well as Tg-551 mice as compared to muscles from the contralateral, innervated leg (**Figure 30D,E**). Specifically, we observed an overall ~3- to 5-Fold increase in MAFbx and MuRF1 mRNAs ($P < 0.05$) in WT and Tg-551 mice at Day 1 and 2 post-denervation (**Figure 30D,E**). In both WT and Tg-551 mice, the pattern of expression of atrogenes decreased towards levels seen in innervated muscles by Day 7, as expected based on the literature (data not shown) (Bodine et al. 2001; Bodine & Baehr 2014). Therefore, these data demonstrate that hindlimb muscles from both WT and MCK-Staufen1-HA mice responded similarly and as expected to denervation, thereby further supporting the notion that the muscle atrophy observed in our transgenic mice is not a result of denervation.

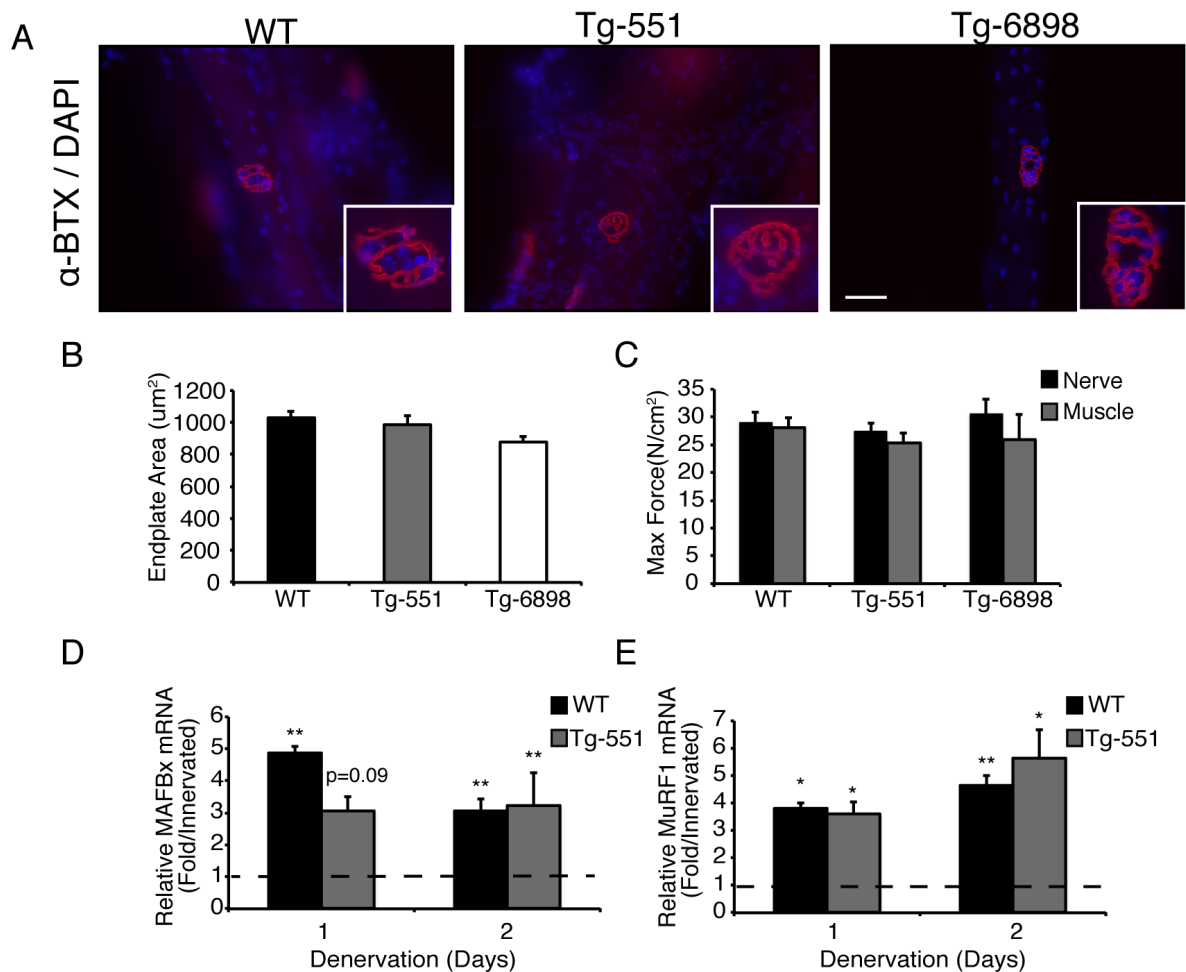


Figure 30. Morphological and functional analyses of the neuromuscular junctions in MCK-Staufen1-HA skeletal muscle. (A) Representative images of the neuromuscular junctions (NMJ) of 8-week-old Tibialis Anterior (TA) muscles of WT (n=3), Tg-551 (n=3), and Tg-6898 (n=3) taken at 20X magnification, scale bar = 50 μ m. Inserts represent 40X magnification. (B) Mean endplate area in μ m². (C) Maximal force output of 16-week-old mouse Extensor Digitorum Longus (EDL) muscles following nerve or direct muscle stimulation of 200Hz. Total RNA was extracted from denervated and contralateral innervated TA muscles from 8-week-old WT and Tg-551 mice and analyzed

for **(D)** MAFbx and **(E)** MuRF1 relative mRNA expression by qRT-PCR using primers specific for target mRNAs. Values are relative to innervated muscle (dashed line). Data are means $\pm$ SEM, *P < 0.05, **P < 0.01, Student T-Test relative to contralateral innervated muscles.

Sustained Staufen1 Expression Modulates the Atrophic Response via c-myc and PTEN Regulation

In a last set of experiments, we sought to determine possible mechanisms by which Staufen1 triggers the atrophic process in mature muscles. Previously, we showed that Staufen1 overexpression impairs skeletal muscle differentiation in cultured cells through enhanced translation of c-myc (Ravel-Chapuis et al. 2014). In this context, it has been reported that *PTEN* is a transcriptional target of c-myc (Kaur & Cole 2013). PTEN is a known negative regulator of the PI3K/AKT signaling cascade and as a result, promotes skeletal muscle atrophy. To determine if sustained Staufen1 expression induces atrophy through this pathway, we first examined c-myc protein levels by western blot. In agreement with our previous findings (Ravel-Chapuis et al. 2014), our data showed an ~2-Fold increase of c-myc ($P < 0.05$ and $P=0.08$, respectively) in EDL muscles from 8-week-old Tg-551 and Tg-6898 mice (**Figure 31A**). Next, we also examined PTEN protein and mRNA expression by western blot and qRT-PCR, respectively. In EDL muscles from 8-week-old Tg-551 and Tg-6898 mouse lines, PTEN levels were upregulated by ~2-Fold ($P < 0.01$) as compared to WT (**Figure 31B**). Similarly, PTEN mRNA levels were also increased by ~3-Fold ($P < 0.01$) in muscles from Tg-551 and Tg-6898 mice (**Figure 31C**). To verify that the increased levels of PTEN were sufficient to regulate AKT signaling, expression of phosphorylated AKT (Ser473) and total AKT was determined by western blot and a ratio of phosphorylated AKT to total AKT (p-AKT/AKT) was calculated. Our analysis showed decreased p-AKT/AKT levels in 8-week-old EDL muscle (~70%, $P=0.01$, **Figure 31D**) from Tg-551 and Tg-6898 mice. Taken together, these data indicate that sustained Staufen1 expression leads to increased

c-myc expression *in vivo* which in turn, activates transcription of *PTEN* ultimately affecting downstream AKT signaling as previously reported (Kaur & Cole 2013).

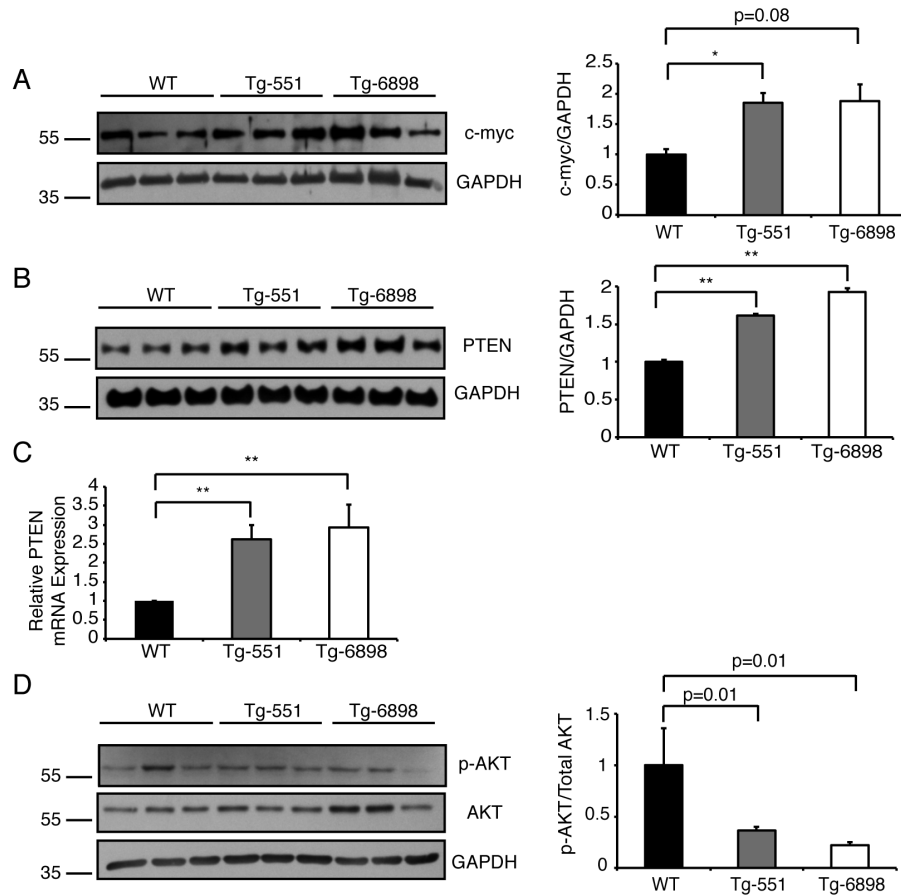


Figure 31. Sustained muscle-specific Staufen1 expression negatively regulates the PI3K/AKT signaling pathway. Total protein or RNA was extracted from Extensor Digitorum Longus (EDL) muscles of 8-week-old WT (n=3), Tg-551 (n=3) and Tg-6898 (n=3) mice. **(A)** Western blot analysis was performed using anti-c-myc antibodies and gapdh as a loading control. **(B)** Western blot analysis was performed using anti-PTEN antibodies and gapdh as a loading control. **(C)** qRT-PCR was performed using primers specific for PTEN mRNAs and normalized to GAPDH mRNAs. **(D)** Western blot analysis for phosphorylated Ser473 AKT, and Total AKT; gapdh was used as a loading control. Data are means $\pm$ SEM. *P < 0.05, **P < 0.01, Student T-Test.

Given the multi-functionality of Staufen1, we also determined whether Staufen1 could regulate PTEN expression via alternate mechanisms. Interestingly, two separate high-throughput screens predict *PTEN* to be a Staufen1-associated mRNA (Furic et al. 2008; Sugimoto et al. 2015). In fact, Sugimoto et al. recently identified a Staufen1-binding secondary structure in the 3'UTR of *PTEN* mRNA (Sugimoto et al. 2015). Since these screens were performed in human cell systems, we first compared the predicted human and mouse Staufen1-binding site (SBS) of the *PTEN* 3'UTR using RNAfold with the minimum free energy determination (**Figure 32A**). The SBS predicted to form within the human and mouse *PTEN* 3'UTR are classified as a long range duplex with a length >500 nt, which Staufen1 has a high affinity for (Sugimoto et al. 2015) (**Figure 32A**). Since these secondary structures represent classic SBSs, we thus hypothesized that Staufen1 also regulates *PTEN* mRNAs post-transcriptionally via the 3'UTR.

To directly test this idea, we used mouse C2C12 myoblasts grown in culture. First, we performed RNA-Immunoprecipitation (RIP) experiments with HA antibodies using C2C12 cells transfected with a vector containing the mouse Staufen1-HA construct (mStaufen1-HA) to determine if Staufen1 interacts with endogenous *PTEN* mRNAs. Results from these experiments showed an ~6-Fold enrichment of endogenous *PTEN* mRNAs in protein extracts obtained from immunoprecipitated mStaufen1-HA cells ($P < 0.05$) as compared to extracts obtained following immunoprecipitation (IP) with control IgG (**Figure 32B**). By contrast, GAPDH mRNAs were not enriched ($P > 0.05$) in the mStaufen1-HA IP (**Figure 32B**). Finally, we determined whether Staufen1 functionally regulates PTEN expression via its 3'UTR since this region of the mature transcript contains the SBS (Sugimoto et al. 2015). For this, C2C12 myoblasts were first transfected

with constructs containing a luciferase reporter upstream of either the human or mouse *PTEN* 3'UTR together with mStaufen1-HA construct or empty control vector. Analysis of luciferase activity demonstrated that Staufen1-HA expression increased the activity of the reporter containing the human or mouse *PTEN* 3'UTRs (**Figure 32C**). Together, these findings demonstrate that Staufen1 also increases PTEN expression via the 3'UTR.

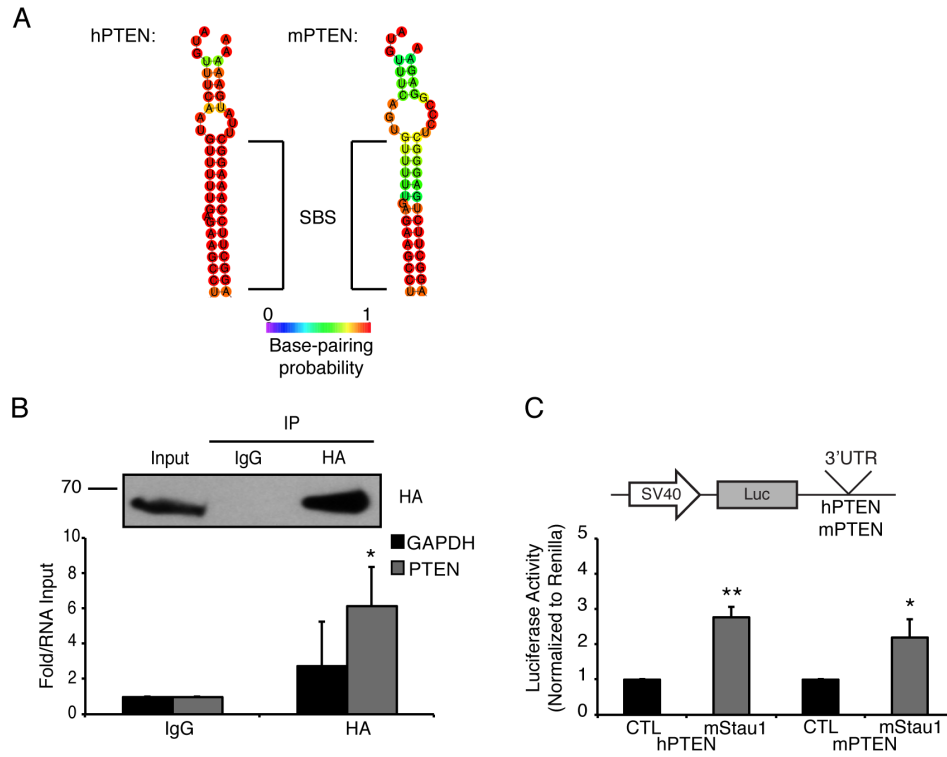


Figure 32. Staufen1 interacts with PTEN mRNAs and enhances PTEN expression via the 3'UTR. (A) Comparison of the human PTEN (hPTEN) and mouse PTEN (mPTEN) Staufen1 binding site (SBS) as predicted by RNAfold Vienna package version 2.2.7 (B) C2C12 cells transfected with mStaufen1-HA and RNA Immunoprecipitation using anti-HA antibodies. Immunoprecipitation was verified by western blot with anti-HA antibodies. Presence of co-immunoprecipitated PTEN mRNAs was determined by qRT-PCR and gapdh mRNAs were detected as a control (n=4). (C) Luciferase activity of hPTEN and mPTEN 3'UTR upon mStaufen1-HA overexpression as compared to an empty vector control (CTL), all values are normalized to Renilla luciferase expression. Data are means $\pm$ SEM. *P < 0.05, **P < 0.001, Student T-Test.

Discussion

Over the past decade, converging lines of evidence have highlighted the critical role of post-transcriptional regulation in skeletal muscle development and plasticity, as well as in several neuromuscular disorders. In this context, many RBPs have received considerable attention because of their direct involvement in the etiology of DM1, SMA and ALS (Lukong & Fatimy 2012). We recently demonstrated that the double-stranded RBP Staufen1 is up-regulated during early stages of muscle development, in denervated muscle and in DM1 skeletal muscle (Bélanger et al. 2003; Ravel-Chapuis et al. 2014; Ravel-Chapuis et al. 2012). Therefore, it became important to examine the impact of sustained Staufen1 expression in mature muscle in an attempt to gain insight into the role of Staufen1 under these varied conditions. Here, we thus generated a transgenic mouse model designed to drive skeletal muscle-specific expression of Staufen1. We report that MCK-Staufen1-HA mice develop a progressive myopathy characterized by morphological and functional deficits. Moreover, these effects appear to be mediated by a marked increase in the expression of the atrogenes, MAFbx and MuRF1, and by the negative regulation of PI3K/AKT signaling via PTEN up-regulation. In addition to revealing the novel function of Staufen1 in muscle plasticity *in vivo*, these findings further indicate that Staufen1 may thus represent an attractive biomarker and therapeutic target for neuromuscular diseases and conditions.

Skeletal muscle atrophy has been heavily studied over the last several decades and is a condition often observed in multiple clinical settings including diabetes (Price et al. 1996), cancer cachexia (Llovera et al. 1994; Baracos et al. 1995; Temparis et al. 1994), neuromuscular disorders (Vignaud et al. 2010; Timchenko 2013; Léger et al. 2006;

Bricceno et al. 2012), chronic disuse (Chopard et al. 2009), and sarcopenia (Evans & Campbell 1993). In most forms of muscle atrophy, the rapid loss of muscle protein is a result of a decrease in protein synthesis paralleled by an increase in protein degradation rates. Early studies demonstrated, for example, that an increase in protein degradation contributes to the loss of muscle mass and myofibrillar proteins following denervation and glucocorticoid treatment (Goldberg 1969). Despite recent advances in our understanding of cellular and molecular events involved in skeletal muscle atrophy, treatments for muscle wasting diseases and conditions are still a challenge today.

The ubiquitin-proteasome pathway is activated during muscle atrophy and contributes to the increase in proteolysis (Jagoe et al. 2002). Fifteen years ago, MAFbx and MuRF1 were identified as novel E3 ubiquitin ligases and as key regulators of muscle atrophy (Bodine et al. 2001; Gomes et al. 2001). Their expression is restricted to striated muscle, and is relatively low in resting and normal states. However, their levels can be rapidly increased in response to atrophic stimuli causing muscle loss (Bodine et al. 2001; Bodine & Baehr 2014). Interestingly, expression of Stauf1 in normal mature skeletal muscle is low and essentially mirrors that of both MAFbx and MuRF1 (Bodine et al. 2001; Perera et al. 2012; Bodine & Baehr 2014). Skeletal muscle-specific MuRF1 transgenic mice do not develop muscle wasting, as expected, suggesting that upstream events are also required to induce muscle atrophy (Hirner et al. 2008). Moreover, mice lacking functional MAFbx or MuRF1 display no overt phenotypes in normal muscle but denervation of these muscles fail to cause a loss of muscle mass as compared to WT mice (Bodine et al. 2001). These findings are important because in our study, steady-state levels of both MAFbx and MuRF1 are markedly increased in MCK-Stauf1-HA skeletal

muscle, which in turn causes a progressive muscle atrophy. Therefore, it seems reasonable to postulate that *Staufen1* represents a novel atrophy-associated gene, and that its expression is required for activation of the full atrophic response in skeletal muscle.

Although the intricacies of several cellular and molecular pathways regulating skeletal muscle atrophy are still being unraveled, a number of regulatory factors and processes appear central to the atrophic response. Genetic activation of AKT is sufficient to inhibit atrophy-associated expression of MuRF1 and MAFbx mRNAs via regulation of FOXO transcription factors (Stitt et al. 2004; Sandri et al. 2004; Lee et al. 2004), highlighting the importance of this signaling pathway in the maintenance of muscle mass. It has previously been reported that c-myc is a negative regulator of the PI3K/AKT pathway through the transcriptional up-regulation of *PTEN* (Kaur & Cole 2013). Since *Staufen1* enhances the expression of c-myc in cultured myogenic cells (Ravel-Chapuis et al. 2014) as well as *in vivo* (this study), we hypothesized that *PTEN* transcript levels are increased through transcriptional regulation by c-myc (see model in **Figure 33**). *Staufen1* is also predicted to bind *PTEN* mRNAs through a putative SBS located within the 3'UTR (Furic et al. 2008; Sugimoto et al. 2015). Here, we also provide the first direct evidence for the interaction between *Staufen1* and endogenous *PTEN* mRNAs, and demonstrate that *Staufen1*-regulated *PTEN* expression occurs via the 3'UTR. We therefore propose a model whereby *Staufen1* regulates *PTEN* expression through an indirect transcriptional mechanism involving c-myc and a direct post-transcriptional event, ultimately increasing *PTEN* expression to levels that are sufficient to inhibit PI3K/AKT signaling thereby inducing muscle atrophy (**Figure 33**).

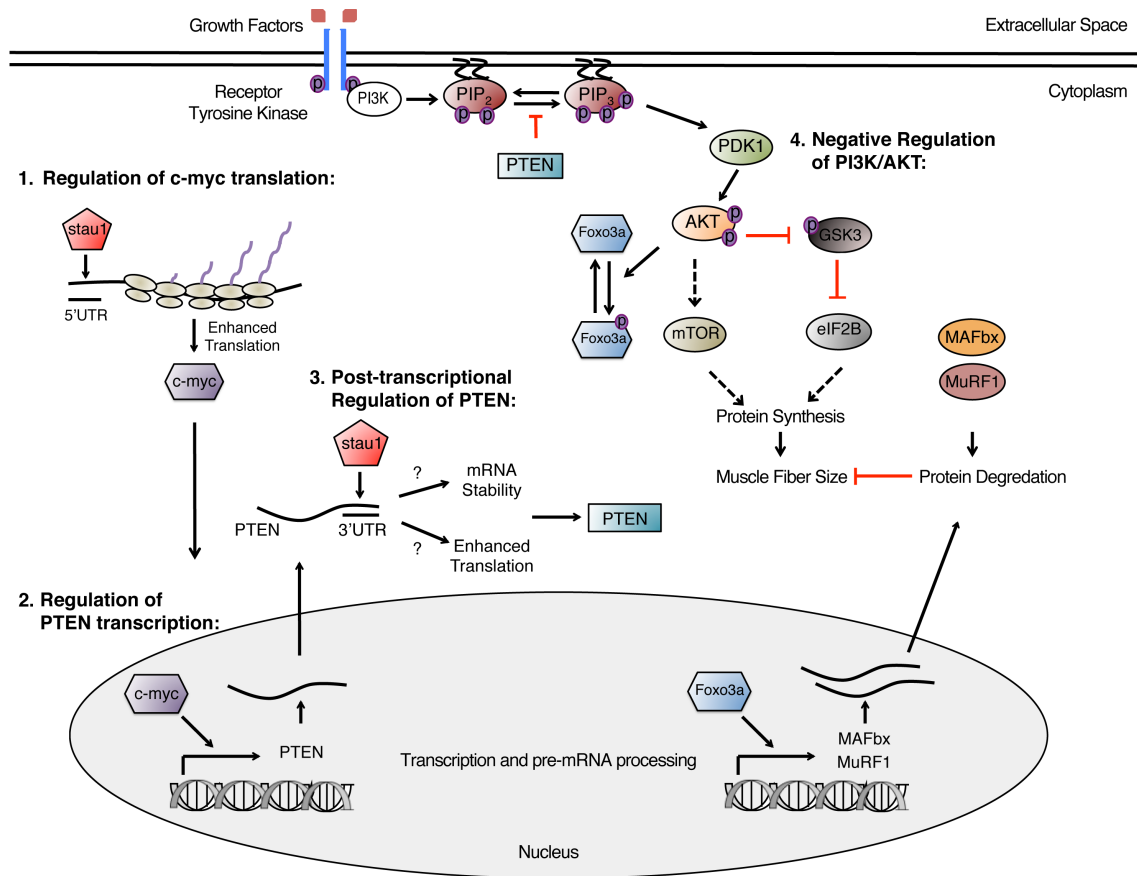


Figure 33. Proposed model for Staufen1-regulated skeletal muscle atrophy. Staufen1 promotes skeletal muscle atrophy through a series of complementary cellular events: (1) Staufen1 enhances c-myc translation via the 5'UTR and (2) an increase in c-myc expression causes the up-regulation of *PTEN* transcription. (3) Staufen1 post-transcriptionally regulates *PTEN* mRNAs through the 3'UTR and (4) as a result, *PTEN* expression is increased and negatively regulates the PI3K/AKT signaling pathway. Collectively, these Staufen1-regulated events result in increased expression of the atrogenes, MAFbx and MuRF1, and induce skeletal muscle atrophy.

It has been proposed that Staufen1 binds to SBSs of similar structure to the one located in the *PTEN* 3'UTR as to elicit an mRNA decay mechanism (Y. K. Kim et al. 2005; Kim et al. 2007; Gong et al. 2009; Gong & Maquat 2011; Wang et al. 2013). Staufen1-mediated mRNA decay (SMD) is an mRNA decay pathway believed to induce mRNA degradation by Staufen1 binding to secondary structures in 3'UTRs of target mRNAs and recruitment of the nonsense-mediated decay factor, Upf1 (Y. K. Kim et al. 2005; Kim et al. 2007; Gong et al. 2009; Gong & Maquat 2011; Wang et al. 2013). The *Arf1* mRNA has been proposed as a canonical SMD target since it contains a SBS in its 3'UTR (Y. K. Kim et al. 2005; Kim et al. 2007). However, there is evidence indicating that SMD does not occur on *Arf1* mRNAs^{30,72}. For example, we recently demonstrated that *Arf1* mRNAs are not a SMD target in our cultured experiments with myogenic or HeLa cells (Ravel-Chapuis et al. 2014). In addition, a separate large-scale screen performed by Ricci et al. identified several SBSs within the 3'UTR of target mRNAs but the authors also demonstrated that modulating Staufen1 levels had little to no effect on mRNA expression including that of *Arf1* (Ricci et al. 2013). By contrast, Staufen1 has been shown to enhance the stability of *XBPI* mRNAs via a SBS in the 3'UTR (Sugimoto et al. 2015). Our current findings are thus in agreement with the latter study as we demonstrate that Staufen1 increases the expression of *PTEN* mRNAs through the 3'UTR. Collectively, these studies question the validity of the current SMD model under varied cellular and physiological contexts, and highlight the complexity and importance for Staufen1-mediated post-transcriptional regulation in skeletal muscle.

As mentioned above, we previously described a key role for Staufen1 in DM1. DM1 is caused by the expansion of CTG trinucleotide repeats in the 3'UTR of the dystrophin

myotonic protein kinase (*DMPK*) gene (Brook et al. 1992; Fu et al. 1992; Mahadevan et al. 1992). The mutant CUG mRNAs with expanded repeats (CUG^{exp}) aggregate in nuclei causing an accumulation of RNA foci and the sequestration and misregulation of several transcription factors and RBPs (Lee & Cooper 2009). As a result, an RNA toxic effect is observed and altered expression, metabolism and alternative pre-mRNA splicing of a large subset of mRNAs collectively lead to many of the symptoms seen in DM1 (O'Rourke & Swanson 2009). In particular, it is thought that a decrease in the functional availability of MBNL1, through its sequestration by nuclear RNA foci, together with an up-regulation of CUGBP1, contribute directly to the complexity of the DM1 phenotype associated with aberrant alternative splicing (Timchenko et al. 2004; Ho et al. 2005; Ward et al. 2010; Kanadia et al. 2003; Kanadia et al. 2006; Chamberlain & Ranum 2012; Suenaga et al. 2012). Multiple mouse models have been generated to define the impact of MBNL1 and CUGBP1 on skeletal muscle and their implications for DM1 (Timchenko et al. 2004; Ho et al. 2005; Ward et al. 2010; Kanadia et al. 2003; Kanadia et al. 2006; Chamberlain & Ranum 2012; Suenaga et al. 2012). Although these models recapitulate several DM1 features, not all of the DM1 symptoms can be attributed to MBNL1 loss or CUGBP1 overexpression.

In attempts to identify additional RBPs involved in DM1, we recently demonstrated that Stauf1 binds to *DMPK* mRNAs (Ravel-Chapuis et al. 2012). We also reported in these studies that Stauf1 is overexpressed in skeletal muscle samples from DM1 mouse models and human patients and that it regulates alternative pre-mRNA splicing of the insulin receptor (*INSR*) and the chloride voltage-gated channel 1 (*CLCN1*) mRNAs (Ravel-Chapuis et al. 2012). More recently, we expanded these findings and

characterized Staufen1 as a disease modifier in DM1 through its regulation of multiple alternative-splicing events and stress granule formation (Ravel-Chapuis et al. 2016; Bondy-Chorney et al. 2016). Our current findings demonstrating the involvement of Staufen1 in muscle atrophy and causing functional deficits, indicate that the increased expression of Staufen1 as we observed in DM1 muscle may, at least partially, be the cause for the mild myopathy encountered in DM1. Together, these findings clearly highlight the importance of Staufen1 as central in the complex DM1 pathology.

An interesting question raised by our findings is whether Staufen1 is also involved in SMA and ALS (Ning et al. 2010; Little et al. 2015; Kirby et al. 2011; Yang et al. 2014). In support of such involvement, there is increasing evidence indicating a role for PTEN in SMA and ALS. In fact, PTEN depletion in healthy and SMN-deficient motor neurons promotes survival and axonal growth (Ning et al. 2010). Moreover, systemic knockdown of PTEN increases the lifespan of SMA mice (Little et al. 2015). Similarly, treatment of ALS motor neurons with a siRNA against PTEN increases cell survival by protecting motor neurons from excitatory transmissions (Kirby et al. 2011; Yang et al. 2014). The control of PTEN expression in muscle by Staufen1 may thus also occur in neurons, thereby contributing to the SMA and ALS pathologies. Accordingly, Staufen1 may in fact have broad impact on several additional neuromuscular diseases, which clearly warrants further studies to gain a better understanding of the mechanisms causing neurodegeneration and the potential of Staufen1 as a therapeutic target and biomarker.

Materials and Methods

Constructs and Antibodies

The constructs used were pcDNA3.1 (Invitrogen Life Technologies, Burlington, Canada), mStaufen1-HA3(Wickham et al. 1999), human pGL3-Control-PTEN-3'UTR-Wt was a gift from Joshua Mendell (addgene plasmid # 21326), mouse pGL3-PTEN-3'UTR was a gift from Jeffrey Rosen (addgene plasmid #28104), and phRGtk-luc (E6291, Promega, Madison, WI, USA). The mouse MCK enhancer/promoter regulatory cassette (-1256 to +7) (Amacher et al. 1993) and mStaufen1-HA3 was amplified by RT-PCR from MCK-nls-LacZ (Duclert et al. 1993) and pcDNA3.1-mStaufen1-HA3 (Ravel-Chapuis et al. 2014; Wickham et al. 1999), respectively, using primers containing a restriction site (underlined): MCK Enh/Prom (fwd 5'-GACCAATTGGCCACTACGGGTCTAGGC-3', rev 5'-CAGAAGCTTGGCAGCCCCTGTGCCCCT-3'). mStaufen1-HA3 (fwd 5'-CAGAAGCTTGGTACGAGCTCGGATCCTCT-3', rev 5'-GACGATATCACTGAGCAGCGTAATCTGGAA-3'). The backbone vector pcDNA3.1 and PCR products were digested and purified using QIAquick PCR purification kit (Qiagen #28104). The MCK enhancer/promoter fragment was first subcloned into pcDNA3.1 to form pcDNA3.1-MCKenh/prom. Next mStaufen1-HA was subcloned into pcDNA3.1-MCKenh/prom to form pcDNA3.1-MCKenh/prom-mStaufen1-HA3 (referred to as MCK-Staufen1-HA). The orientation of inserts were determined by restriction digestion and the integrity of sequences was confirmed by sequencing.

The antibodies used were anti-embryonic myosin (F1.652, DSHB Hybridoma, Iowa, USA), anti-Myosin Heavy Chain Type I (BA-D5, DSHB Hybridoma, Iowa, USA), anti-laminin (L9393, Sigma-Aldrich, Oakville, Ontario, Canada) anti – HA.11 clone 16B12

(Covance, Montreal, Canada), anti – phospho Akt (Ser473) (#4060, Cell Signaling Technology, Massachusetts, USA), anti-Akt (pan) (#4691, Cell Signaling Technology, Massachusetts, USA), anti-PTEN (#9188, Cell Signaling Technology, Massachusetts, USA), anti-Mouse IgG (M-8642, Sigma-Aldrich, Oakville, Ontario, Canada), anti- β -actin (#47778, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GAPDH (ab8245, Abcam, Toronto, Canada).

Generation of Transgenic Animals

All animal experimental protocols were approved by the University of Ottawa Institutional Animal Care Committee and were in accordance with the Canadian Council of Animal Care guidelines. For MCK-Staufen1-HA transgenic mice, linearized MCK-Staufen1-HA via restriction digest was inserted into more than one hundred fertilized FVB/N embryos by pronuclear injection (Transgenic Core Facility, University of Ottawa, Faculty of Medicine). Positive hemizygous founder mice were identified by PCR using transgene-specific primers (fwd 5'-CACTTAGTTTAGGAACCAGTG-3', rev 5'-CGACCAGAGGAGGGAAGAG-3') and then crossed with FVB/N WT mice (The Jackson Laboratory) to generate individual hemizygous transgenic lines. Genotyping of transgenic animals was performed by Genomic DNA isolation from progeny tail or ear clippings (Macherey-Nagel Inc, PA, USA) and were screened by PCR using transgene-specific primers (EZ BioResearch PCR Ready Mix). The primer set mentioned above was used to detect a single band migrating at 345bp and additional primers were designed to amplify both endogenous Staufen1 (870bp) and exogenous MCK-Staufen1-HA (137bp) (fwd 5'-GCCAGAGTACATGCTCCTTAC-3', rev 5'-TGTTCTCAGCAGCATTACGC -3') to confirm genotyping.

Forelimb Grip Strength

Mice used for these experiments were handled regularly. The mice acclimatized to the testing room for 30 minutes (min) prior to testing. The mice were placed near the triangular grid of the Chatillion DFE II (Columbus Instruments, Columbus, USA) for 60 seconds (s) to habituate to the meter. Next, the mouse was moved towards the meter until it had a firm grip. The mouse was gently moved along a horizontal plane relative to the triangular grid at a speed of approximately 2.5 cm/second until it released the bar. The value of the maximal peak force was recorded. This process was repeated six times for each animal, with 10–15 s intervals between each trial.

Eccentric Contractions and Force Frequency

EDL muscles were constantly immersed in physiological saline solution containing (in mM): 118.5 NaCl, 4.7 KCl, 2.4 CaCl₂, 3.1 MgCl₂, 25 NaHCO₃, 2 NaH₂PO₄, and 5.5 D-glucose. All solutions were continuously bubbled with 95% O₂-5% CO₂ to maintain a pH of 7.4. Physiological solution entered the 0.7 ml muscle chamber at a rate of 15 ml/min and at a temperature of 37°C. EDL muscles were attached horizontally to a fix hook at one end and to a force-length transducer at the other end (model 300, Aurora Scientific, Aurora, Canada). Force and muscle length were recorded using a Keithley data acquisition board (model KPCI-3104, Cleveland, USA) at a sample rate of 5 KHz. Tetanic force, defined as the increase in force elicited by a stimulation, was calculated as the difference in force measured just prior to a contraction and the maximum force during a contraction.

Muscle length was first adjusted to give maximum tetanic force and then muscles were allowed to equilibrate for 30 min. During that time, contractions were elicited every

100 s with 200 ms train of 0.3 ms, 10 V square pulses at 200 Hz. Electrical stimulations were generated by a Grass S88X stimulator (Grass Technologies, West Warwick, USA) connected to a SIV-U stimulation isolation unit. EDL muscles were used in pairs: one for force-frequency measurements the other for the effects of eccentric contractions (EC). Force-frequency measurement was carried out using the same stimulation protocol described above except that the stimulation frequencies were varied between 1 and 200 Hz. For EC, the stimulation frequency was kept at 200 Hz but the duration was prolonged to 700 ms long tetanic contraction, which were elicited at an interval of 2 min with a 10% muscle lengthening at a velocity of 0.5 Length/s applied during the last 200 ms.

Hematoxylin and Eosin Staining and Immunofluorescence

For Hematoxylin and Eosin staining, muscles were dissected, embedded in Tissue-Tek OCT compound (VWR, Mississauga, Ontario, Canada) and frozen in melting isopentane pre-cooled with liquid nitrogen. Samples were stored at -80°C until use. Muscle cross sections of 10 µm thick were stained with Hematoxylin and Eosin, dehydrated through a series of ethanol washes (70%, 95%, 100%), cleared with toluene, mounted using Permount (Fisher Scientific, Ottawa, Canada) and visualized via light microscopy under 20X magnification. Analysis of 5 cross-sectional views was performed using Northern Eclipse Software (NES, Empix Imaging, Mississauga, Ontario, Canada). The central nuclei percentage was calculated by $[(\text{number of centrally nucleated fibers} / \text{total fibers}) \times 100\%]$. Cross-Sectional Area (CSA) of each fiber was measured using NES, and the variance coefficient was calculated by $(\text{Variance coefficient } Z = 1000 \times \text{standard deviation of muscle fiber CSA} / \text{mean muscle fiber CSA})$.

Fiber typing was performed on muscle cross sections of 10 μ m thick and were stained using the M.O.M Immunodetection kit (#BM-2202, Vector Laboratories, Ontario, Canada). Sections were incubated with primary antibodies against MHC_{emb}, and MHC Type I for 30 min at room temperature. A Texas Red conjugated streptavidin detection system was applied (1:500) for fluorescent detection. Sections were then incubated with anti-laminin antibodies (1:800) for 30 min at room temperature. Following 3 x 5 min washes with 1X Phosphate Buffered Saline (PBS) pH 7.4, sections were incubated with Alexa Fluor 488 antibodies (1:500) (Invitrogen Life Technologies, Burlington, Canada). The slides were mounted with Vectasheild mounting medium containing DAPI (Vector Laboratories, Ontario, Canada) and visualized using a Zeiss AxioImager.M2 microscope. MHC positive fibers were quantified using Northern Eclipse Software (NES, Empix Imaging, Mississauga, Ontario, Canada) and percentage of MHC positive fibers was determined by [(number of MHC positive fibers/total fibers) x 100%].

For NMJ staining, TA muscles were dissected from WT, Tg-551 and Tg-6898 mice, fixed in 4% formaldehyde at room temperature for 1 hour. Approximately 30 single fibers were microdissected from multiple regions of the muscle and permeabilized in 2% Triton X-100 in 1X PBS pH 7.4 for 30 min at room temperature. Fibers were blocked in 4% BSA in 1% Triton X-100/1X PBS pH 7.4 solution for 30 min at room temperature. Next, fibers were incubated with Alexa 594 conjugated anti-Bungarotoxin antibodies (1:500) (Invitrogen Life Technologies, Burlington, Canada) for 1 h at room temperature with shaking. Fibers were washed 6 x 10 min in 1X PBS pH 7.3 + 0.01% Triton X-100 and mounted on a slide with mounting medium containing DAPI (Vector Laboratories, Ontario, Canada). Slides were visualized using a Zeiss AxioImager.M2 microscope and

at least 20 NMJs were quantified per condition using Northern Eclipse Software (NES, Empix Imaging, Mississauga, Ontario, Canada).

Hindlimb Surgical Denervation

Mice were anesthetized using isoflurane and the left sciatic nerve was severed with a 2-3 mm section removed to avoid reinnervation. Sterile clips were used to close the skin and the contralateral innervated leg served as a control. Muscles were collected 24 and 48 h post-denervation and immediately frozen in liquid nitrogen. All muscle samples were stored at -80°C for subsequent analysis.

Western Blot

Frozen muscle samples were crushed in liquid nitrogen and a portion of the powder was resuspended in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1X protease inhibitor cocktail, 1X PhosphoStop [Roche, Laval, Quebec]) and centrifuged at 13000rpm, 10 min. Or Muscle Lysis Buffer (20mM HEPES, 10mM NaCl, 1.5mM MgCl₂, 1mM DTT, 20% Glycerol, 0.1% Triton X-100) for phosphorylated protein analysis, which were then centrifuged at 1200 x g, 5min. Protein concentration was determined using the Bicinchoninic Acid protein assay kit (Thermo Fisher Scientific, Ottawa, Canada). Cells were washed with sterile 1X PBS pH 7.4 and resuspended in the appropriate lysis buffer. 30 µg of protein was separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Mississauga, Ontario, Canada). Non-specific binding was blocked using 5% skim milk in 1X PBS + 0.05% Tween 20 or 5% BSA for phosphorylated protein analysis. Membranes were incubated with primary antibody dilutions in 1% skim milk or 1% BSA for 1 hour at room temperature or overnight at 4°C. Membranes were washed 3 x 10 min with 1X PBS + 0.05% Tween 20

and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Note: for HA-tag detection in mouse models, anti-mouse light chain-specific secondary antibodies were used (111-035-174, Jackson ImmunoResearch Laboratories Inc, PA, USA). Membranes were washed 3 x 10 min with 1X PBS + 0.05% Tween 20 and signal detection was performed using PierceTM ECL Western Blotting Substrate and auto radiographed with x-ray film (Thermo Fisher Scientific, Ottawa, Canada). Quantification was performed using Image Lab Software (Bio-rad, Mississauga, Ontario, Canada).

RNA Extraction, Reverse Transcription, and Real-Time quantitative PCR

Total RNA was extracted from samples using TRIpure Isolation Reagent (Sigma-Aldrich, Oakville, Ontario, Canada). Extracted RNA was treated for 1 h at 37°C with DNase I (Ambion Life Technologies, Burlington, Ontario, Canada). Reverse Transcription was performed on DNase I treated samples in a reaction mixture (5 mM MgCl₂, 1× PCR buffer, 1 mM dNTP, 1 U/ml RNase inhibitor, 5 U/ml Moloney murine leukemia virus reverse transcriptase and 2.5mM random hexamers) (Applied Biosystems, CA, USA) and incubated at 42°C, 45 min; 95°C, 5 min. mRNA expression was analyzed by real-time quantitative PCR using QuantiTect SYBR Green PCR kit (QIAGEN, Toronto, Canada) and the MX3005p real-time PCR system (Stratagene, La Jolla, CA, USA) according to manufacturers instructions. Primer sequences were as follows: FOXO3a (fwd 5'-TCAGAATGAAGGCACGGGCA-3', rev 5'-TGGAGAGCTGGGAAGGACTG -3'), MuRF1 (fwd 5'-TGTCTGGAGGTCGTTTCCG -3', rev 5'-ATGCCGGTCCATGATCACTT-3'), MAFbx (fwd 5'-AGCGACCTCAGCAGTTACTGC-3', rev 5'-CTTCTGGAATCCAGGATGGC-3')

PTEN (fwd 5'-GAAAGGGACGGACTGGTGTA-3', rev 5'-CGCCACTGAACATTGGAATA -3'), and normalized to GAPDH (fwd 5'-GGGTGTGAACCACGAGAAAT-3', rev 5'-CCTTCCACAATGCCAAAGTT-3').

RNA Immunoprecipitation

C2C12 cells (7.2×10^5) were plated on 100 mm culture plates and incubated for 24 h at 37°C with 5% CO₂ in a humidified chamber. Cells were then transfected with 12.5 µg of DNA and Lipofectamine 2000 (Invitrogen Life Technologies, Burlington, Ontario, Canada) according to the manufacturers instructions. Following transfection (48 h), myoblasts were washed with 1X PBS pH 7.4 and fixed in fresh 1% formaldehyde for 10 min at room temperature, the reaction was quenched using 0.25 M glycine in 1X PBS pH 7.4 for 5 min at room temperature. Cells were washed with 1X PBS pH 7.4 and resuspended in low stringency RIPA Buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 1% NP40, 0.25% Sodium Deoxycholate, 1mM EDTA, 1X protease inhibitor cocktail). Cross-linked complexes were solubilized via 4 x 15 s sonication pulses, insoluble debris was removed by centrifugation (13,000 rpm x 10 min at 4°C). Equal amounts of lysate were pre-cleared with protein A/G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA), 0.4 µg/µl of competitor tRNA, and 0.08 µg/µl of salmon sperm DNA. 3 µg of specified antibodies were bound to A/G plus agarose beads rotating at 4°C for 1 h. Pre-cleared lysates were then immunoprecipitated using antibody-bound beads rotating overnight at 4°C. Beads were washed 5 x 10 min with low stringency RIPA buffer followed by 2 x 10 min washes with TE Buffer (10mM Tris-HCl pH7.4, 1mM EDTA). Beads were then collected and resuspended in 100 µL of Elution Buffer (50mM Tris-HCl pH7.4, 5mM EDTA, 10mM DTT, 1% SDS). Crosslinking was reversed at 70°C for 5 h.

Samples were analyzed by western blot to determine immunoprecipitation efficiency, and the remaining samples were analyzed by qRT-PCR.

Luciferase Reporter Assay

Cells were harvested 48 h post-transfection and the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used according to the manufacturer instructions. Briefly, C2C12 cells were co-transfected with human or mouse PTEN 3'UTR luciferase constructs, mStaufen1-HA3, and Renilla luciferase. First, firefly luciferase expression was detected, and normalized to renilla expression as a transfection efficiency control.

Statistical Analysis

The data were analyzed using student t-tests. The level of significance was set at $p \leq 0.05$. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$. Error bars represent standard error of the mean (SEM).

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Chapter 5: Staufen1 Regulates Multiple Alternative Splicing Events in DM1

Skeletal Muscle

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

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Author Contributions

In the following manuscript, the project was conceived and designed by E.B.C., T.E.C.P., A.R.C., R.K., B.C., B.J.J., and J.C. The data presented in Figure 35 and Figure 36 was performed by E.B.C and T.E.C.P. The lentiviral and retroviral particles were generated by E.B.C., T.E.C.P., and used for the infection of the cell lines for the RT-PCR screen and validation experiments. The RT-PCR screen was performed by R.K. and B.C. The data presented in Figure 32-34, Figure 37-39, and Figure 41 were generated and analyzed by E.B.C. while Figure 40 was generated and analyzed by E.B.C., L.R., and M.P. Finally, the manuscript was written by E.B.C., T.E.C.P., B.C., B.J.J., and J.C.

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 Stau1 (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 *CLCN1*, 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.

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 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.

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 (Pan et al. 2008; Wang et al. 2008; Djebali et al. 2012; Merkin et al. 2012; Barbosa-Morais et al. 2012). Generation of these variants by alternative splicing is a major mechanism responsible for the complexity of the transcriptome and proteome observed in eukaryotes (Nilsen & 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 (Shepard & Hertel 2008; Raker et al. 2009). 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 (Sen et al. 2009; Du et al. 2010; 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 & Zoghbi 2005; Ranum & Cooper 2006), but more recent studies suggest the existence of numerous additional splicing defects in DM1 tissues (Dhaenens et al. 2011; Klinck et al. 2014; Fugier et al. 2011). 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 (Ephrussi et al. 1991; Wickham et al. 1999; Micklem et al. 2000; Bélanger et al. 2003; Martel et al. 2010; Kim et al. 2007; Thomas et al. 2009; Martel et al. 2006; Sugimoto et al. 2015; Ravel-Chapuis et al. 2012; Johnston & Nüsslein-Volhard 1992). In mammals, Stau1 pre-mRNA is alternatively spliced to produce two major forms Stau1⁵⁵, Stau1⁶³ and one variant reported to not bind RNA, Stau1ⁱ (Wickham et al. 1999; Duchaine et al. 2002; Furic et al. 2008). 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 (Sugimoto et al. 2015; Ricci et al. 2013; Laver et al. 2013; De Lucas et al. 2014). 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 (Sugimoto et al. 2015; Ricci et al. 2013; Laver et al. 2013; De Lucas et al. 2014). 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.

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 (Ravel-Chapuis et al. 2012), 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 (**Figure 34A**). 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 (**Figure 34B**). This reduction of Stau1 levels caused a significant ~10% decrease in the relative inclusion of *INSR* exon 11 (**Figure 34**

B). 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 (**Figure 35A**).

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. 2009; Sen et al. 2010). These splicing proteins, similar to Stau1, are misregulated in DM1 (Savkur et al. 2001; Philips et al. 1998; Kanadia et al. 2003; D. H. Kim et al. 2005). 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 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 (**Figure 34 C,D** and **Figure 35 B,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.

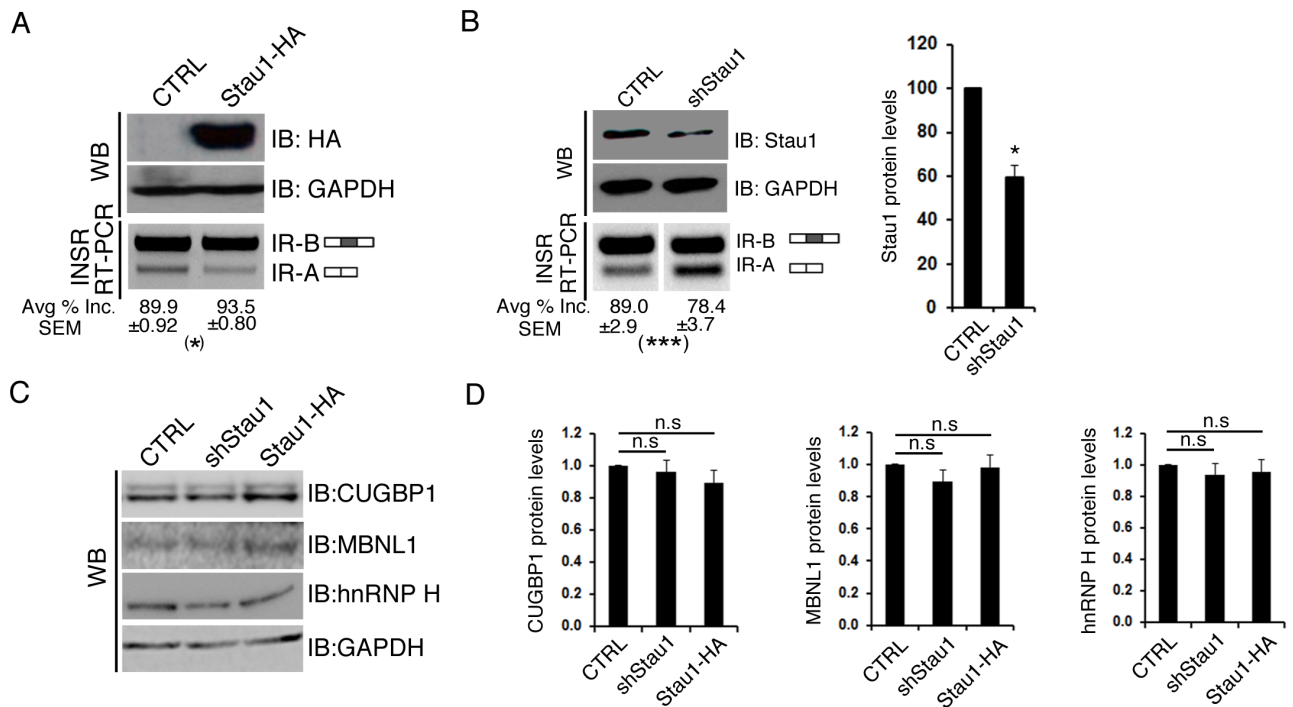


Figure 34. Stau1 levels regulate the pre-mRNA splicing of the human INSR in HeLa cells. (A) pGIPZ (CTRL) or Stau1-HA (Stau1-HA) plasmids were transiently transfected into HeLa cell lines and total RNA and protein lysate was collected after 48 hours. RT-PCR using primers specific to the human endogenous *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 ≥ 3 independent experiments. Error bars represent SEM * = $p < 0.05$, ** = $p < 0.01$.

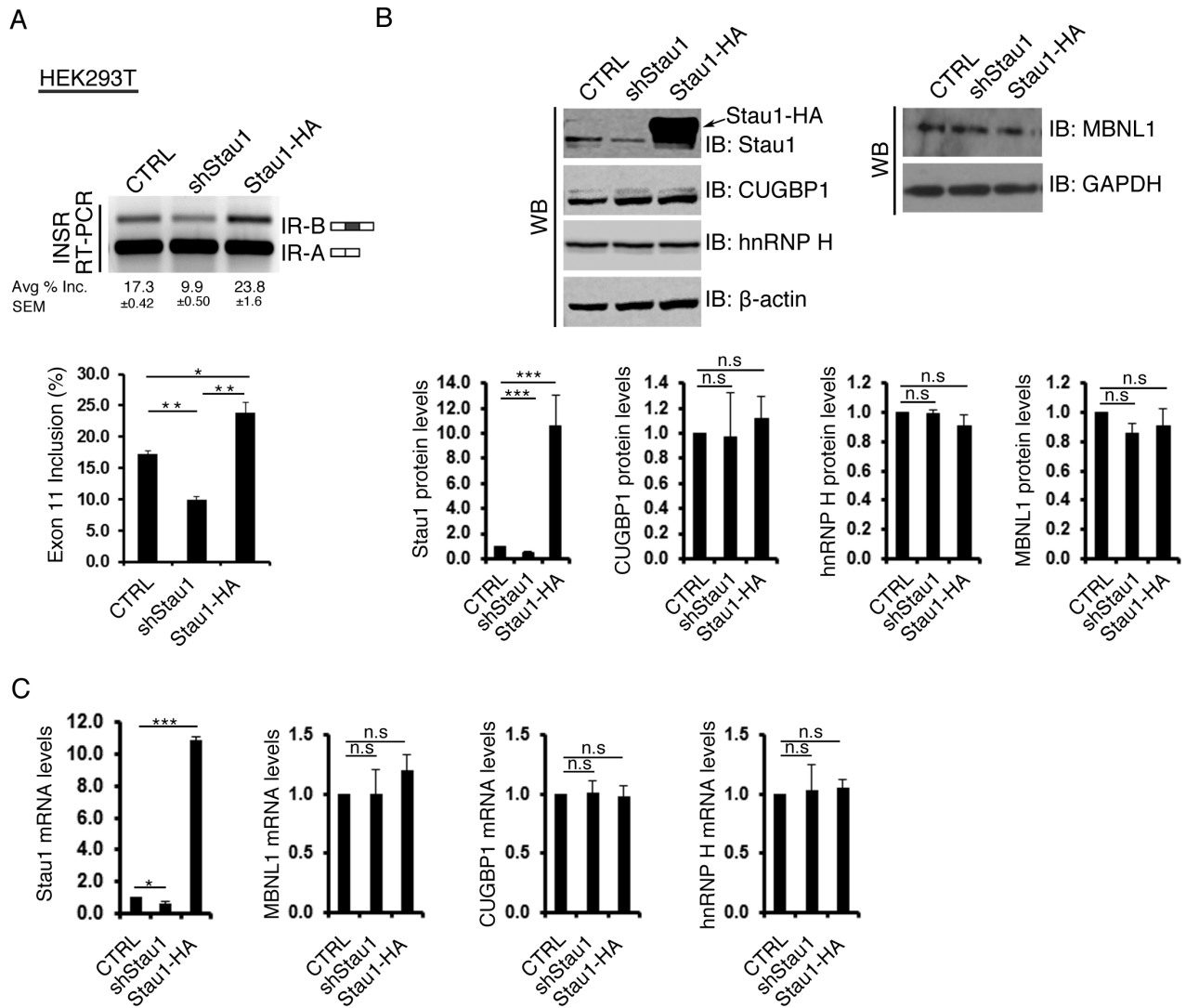


Figure 35. Stau1 levels regulate the pre-mRNA splicing of the human INSR in HEK293T cells. (A) pGIPZ (CTRL), shStau1 or Stau1-HA (Stau1-HA) plasmids were transiently transfected into HEK293T cell lines and total RNA and protein lysate was collected after 48 hours. RT-PCR using primers specific to the 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 * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

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 (Kelley et al. 2014; Sugimoto et al. 2015; Laver et al. 2013; De Lucas et al. 2014; Ricci et al. 2013; Elbarbary et al. 2013). 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 (**Figure 36A**). 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 (Sen et al. 2009; Sen et al. 2010; Kosaki et al. 1998): WT and Δ Alus (in which all three Alu elements are deleted) (**Figure 36A**). HeLa cells were co-transfected with Stau1-HA and either the WT or the Δ Alus 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 (**Figure 36B**). The amount of Stau1-HA bound to WT IR-minigene demonstrated a significant ~2-fold enrichment over the IgG control (**Figure 36B**; black bars). This degree of association was greatly reduced with the Δ Alus IR-

minigene (**Figure 36B**; 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 Δ Alus. Overexpression of Stau1-HA induced an ~5% increase in exon 11 inclusion in the WT IR-minigene (**Figure 36C**), 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 (**Figure 36D**). 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 (**Figure 36C 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.

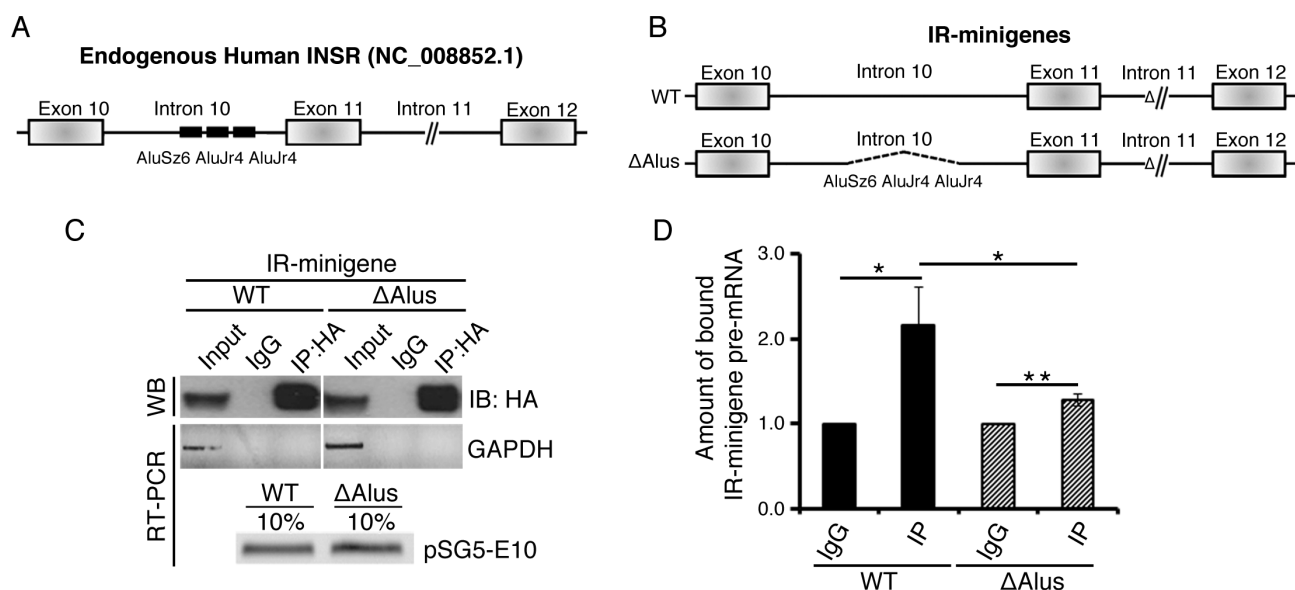


Figure 36. Stau1 regulates splicing of INSR exon 11 through an interaction with Alu elements in intron 10. (A) The genomic DNA sequence of the human INSR (NG_008852.1) was used to assess the Alu elements located in intron 10. Introns are not to scale and this is indicated in intron 11 (//). The IR-minigene 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 Δ Alus 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 Δ Alus IR-minigene. IR-minigene splicing was determined by RT-PCR. The average of ≥ 3 independent experiments was used. Error bars represent SEM * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

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 (**Figure 37A**). Semi-quantitative RT-PCR and Western blot analysis using *MyoD* specific primers and MyoD antibodies confirmed the overexpression of *MyoD* mRNA and protein (**Figure 37 B,C**). Moreover, western blot analysis with anti-HA tag antibodies confirmed Stau1-HA overexpression (**Figure 37D**). 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 (**Figure 38 A,B**). Although several ASEs were not affected by Stau1-HA overexpression (e.g. *ITGA7a*; **Figure 38B**), a number of ASEs showed important changes in both WT and DM1 conditions (e.g. *INSR*; **Figure 38B**). 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 (Δ PSI). A threshold of a Δ PSI $\geq 10\%$ was established to denote relevant changes in splicing regulated by Stau1 (**Figure 38C,D** and **Appendix A Table 3**). 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 common in both conditions (**Figure 38D**). Similar trends were seen in both WT and DM1 conditions where the majority of Stau1-regulated ASEs showed a Δ PSI between 10-30% upon Stau1-HA overexpression. Accordingly, only a few ASEs showed large ($\geq 50\%$) Δ PSI when Stau1-HA was overexpressed, compared to GFP CTRL, such as *INSR* and *NRG1* (**Figure 38 E,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.

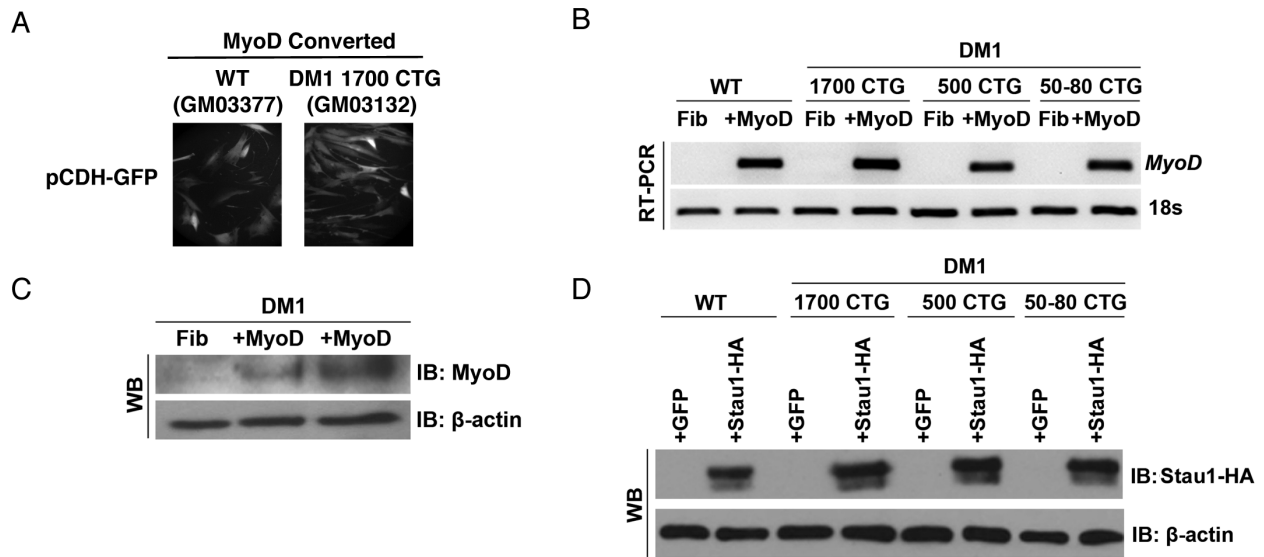


Figure 37. Confirmation of MyoD expression and overexpression of Stau1-HA protein in GM0 cells. (A) Representative image of GFP positive MyoD converted WT and DM1 cell lines. (B) One WT (GM03377) and three DM1 (GM03132, GM03987, GM03991) primary fibroblast cell lines were converted to myoblasts using 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.

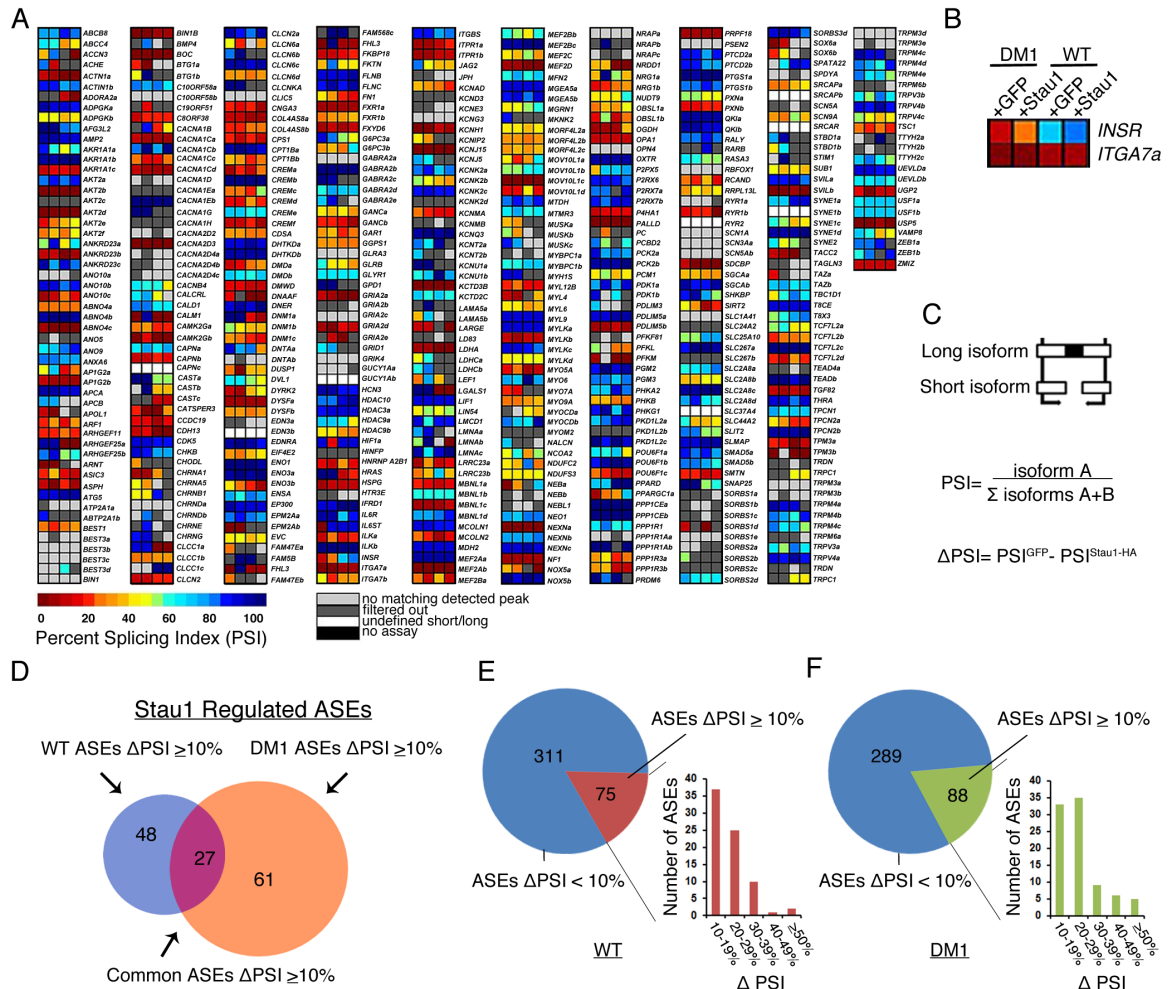


Figure 38. Analysis of high-throughput RT-PCR splicing screen. (A) Raw data output displayed as heatmap from RT-PCR splicing screen. A total of 489 AEs were analysed in the high-throughput RT-PCR Screen. Any AEs that showed a blank reading for Percent Splicing Index (PSI) in any of the four conditions was excluded. **(B)** Enlarged image of two AEs in DM1 and WT conditions. **(C)** PSI represented here as a ratio between the long isoform and the short isoform. The changed in PSI (Δ PSI) was calculated for each ASE in both conditions. **(D)** Total number of AEs that showed a PSI change $\geq 10\%$ with Stau1-HA overexpression in WT and DM1. **(E-F)** Stau1 and non-

Stau1 (<10% Δ PSI) regulated AEs. Bar graphs display the Δ PSI groups (according to percent change) of Stau1-regulated AEs in both WT and DM1.

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 $\geq 10\%$ 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 (**Figure 39A,B**). This suggests that promoting 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 (**Figure 39C** and **Appendix A Table 3**). 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 (**Figure 39D** and **Appendix A Table 3**). 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 (**Figure 39E**). 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.

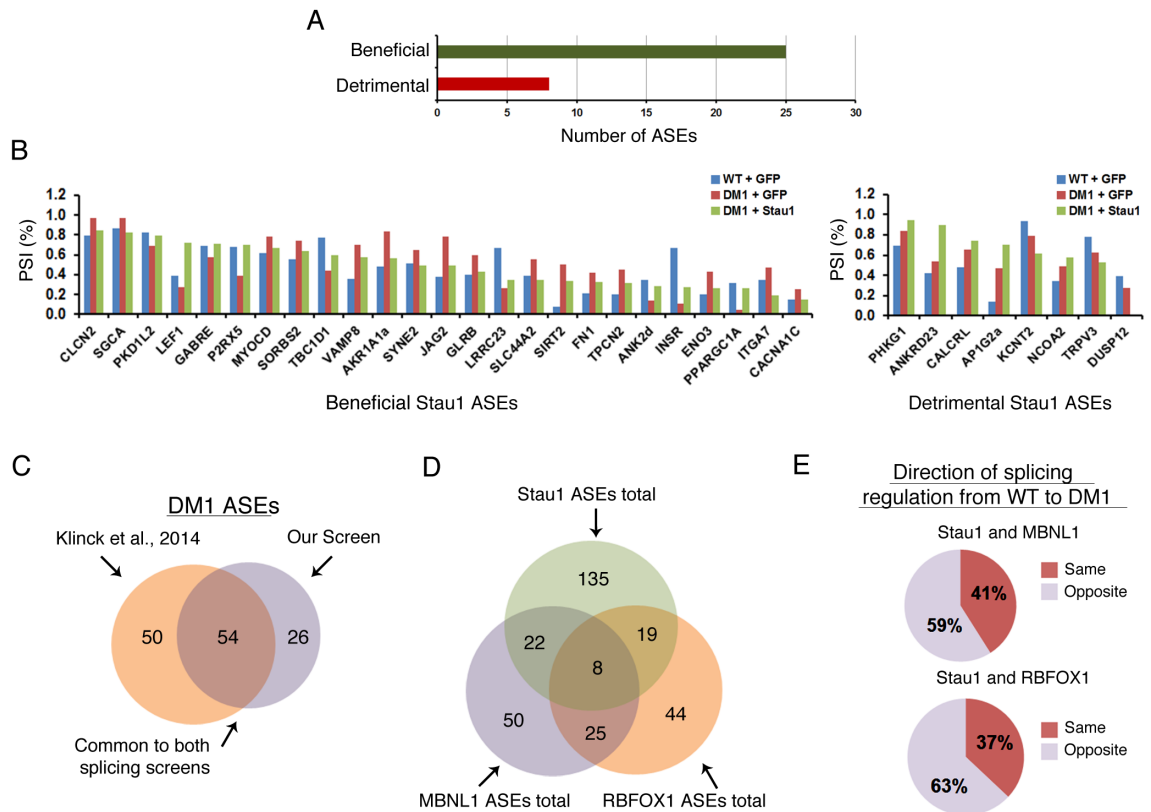


Figure 39. Stau1 overexpression regulates the alternative splicing of numerous ASEs, which are both beneficial and detrimental in DM1 conditions. (A) A total of 33 ASEs were considered for this analysis based on the following criteria. First, ASEs that showed a $\geq 10\%$ Δ PSI from WT to DM1 conditions were selected. From this, ASEs that showed a $\geq 10\%$ Δ PSI 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 ($\geq 5\%$ PSI) to compare the 163 ASEs used in both screens

(Refer to Appendix A Table 3). (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.

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*, *HIF1 α* , *NRG1*, *FN1*, *ACCN3*, *FHL3*, *G6PC3*, *CLCN2* and *CLCN6* (**Figure 40A-D** and **Figure 41 A-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 (**Figure 40A-D** and **Figure 37B,D**). These additional DM1 cell lines were included to investigate the influence of Stau1-HA, which was overexpressed at relatively equal amounts (**Figure 37D**), 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 $\geq 15\%$, independent of the number of CTG repeats (**Figure 40A**). 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.

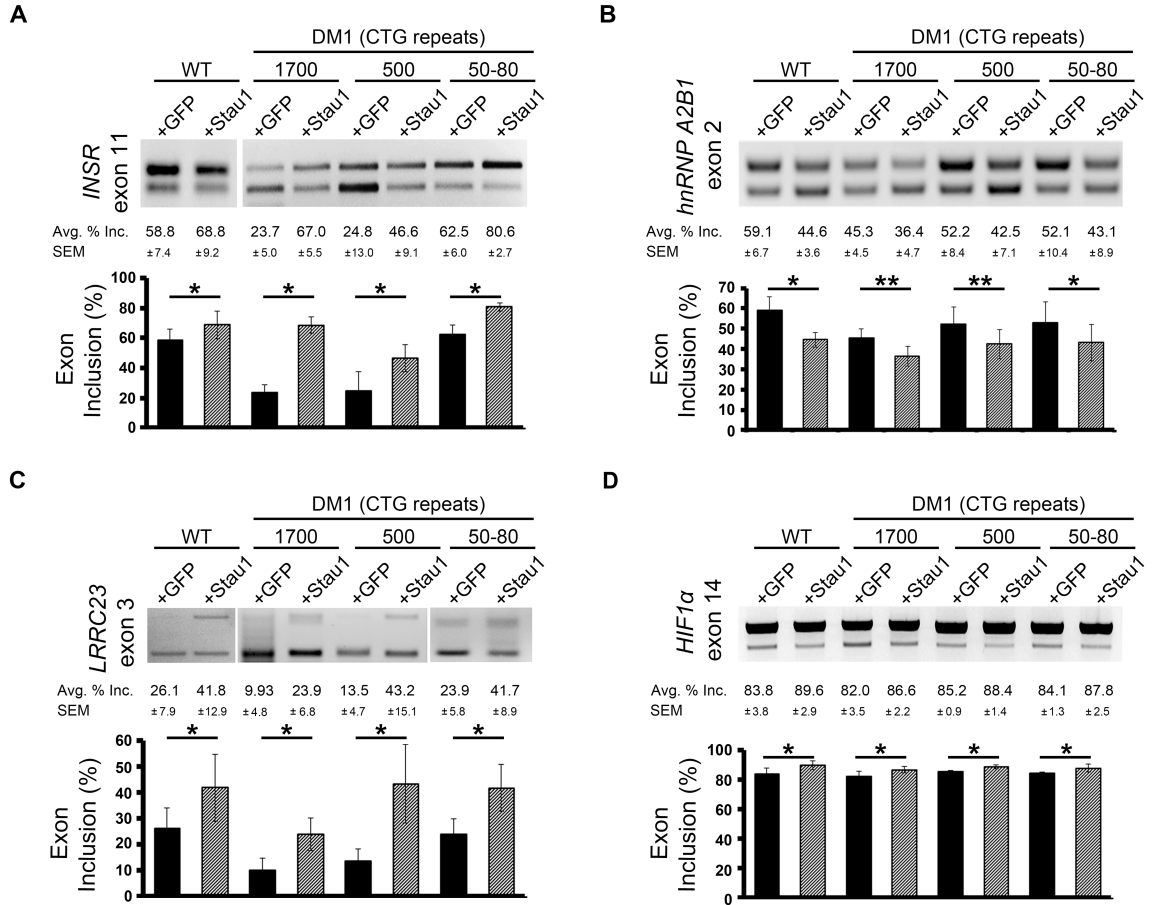


Figure 40. Validation of Stau1-regulated ASEs from RT-PCR splicing screen, in WT and DM1 cell lines. Total RNA was collected from four GM0 cell lines; WT (GM01653) and DM1 cell lines GM03132 (1700 CTG), GM03987 (500 CTG), and GM03991 (50-80 CTG). RT-PCR was performed to determine splicing ratios of ASE in the (A) *INSR* (B) *hnRNP A2B1*, (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 * = $p < 0.5$, ** = $p < 0.01$.

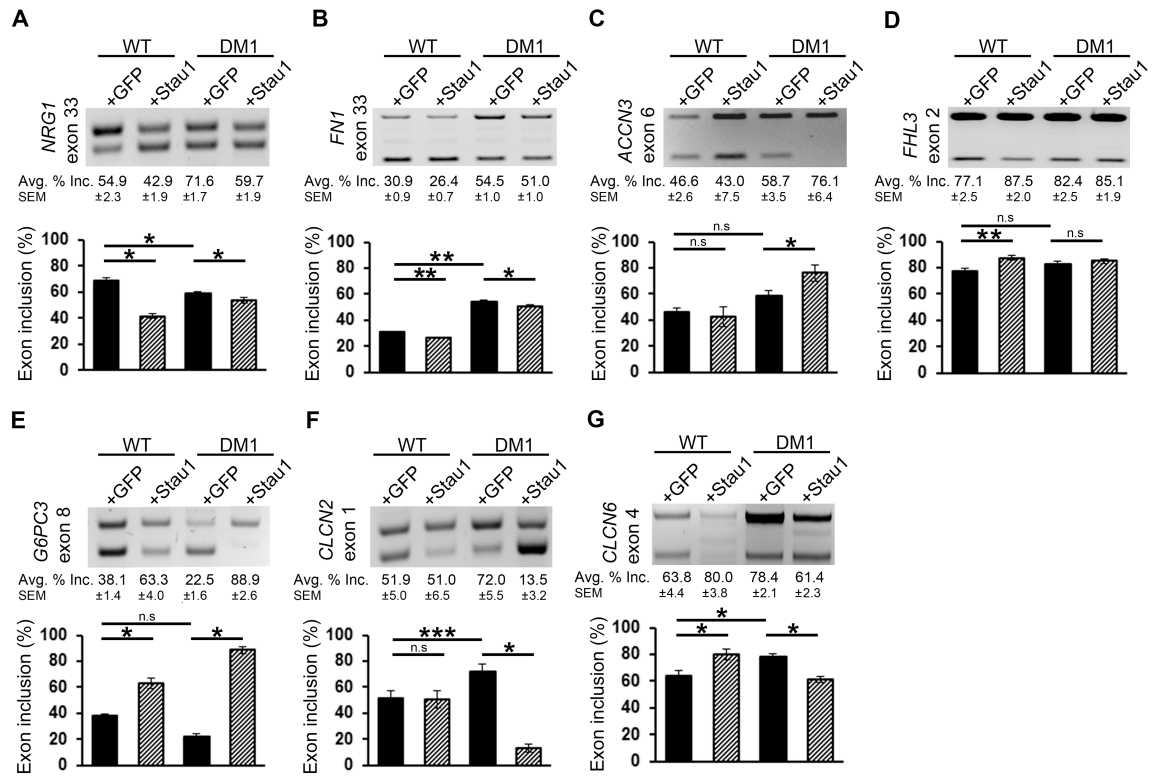


Figure 41. Additional validation of high-throughput RT-PCR splicing screen. (A-G)

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) *FNI*, (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 * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

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 (**Figure 36**), 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). 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 ($\Delta\text{PSI} \geq 15\%$) contained Alu elements. In contrast, only 65.7% of non-Stau1 regulated targets contained Alu elements (**Figure 42A**). 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 (**Figure 42B**). 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 (Kapitonov & Jurka 1996; Deininger 2011). 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; Y. Wang et al. 2012). 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 (**Figure 42B**). 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. 2013), the predicted secondary 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* (**Figure 43A-C**). These may serve as SBS allowing Stau1 to regulate the splicing of these pre-mRNAs when no Alu elements are present.

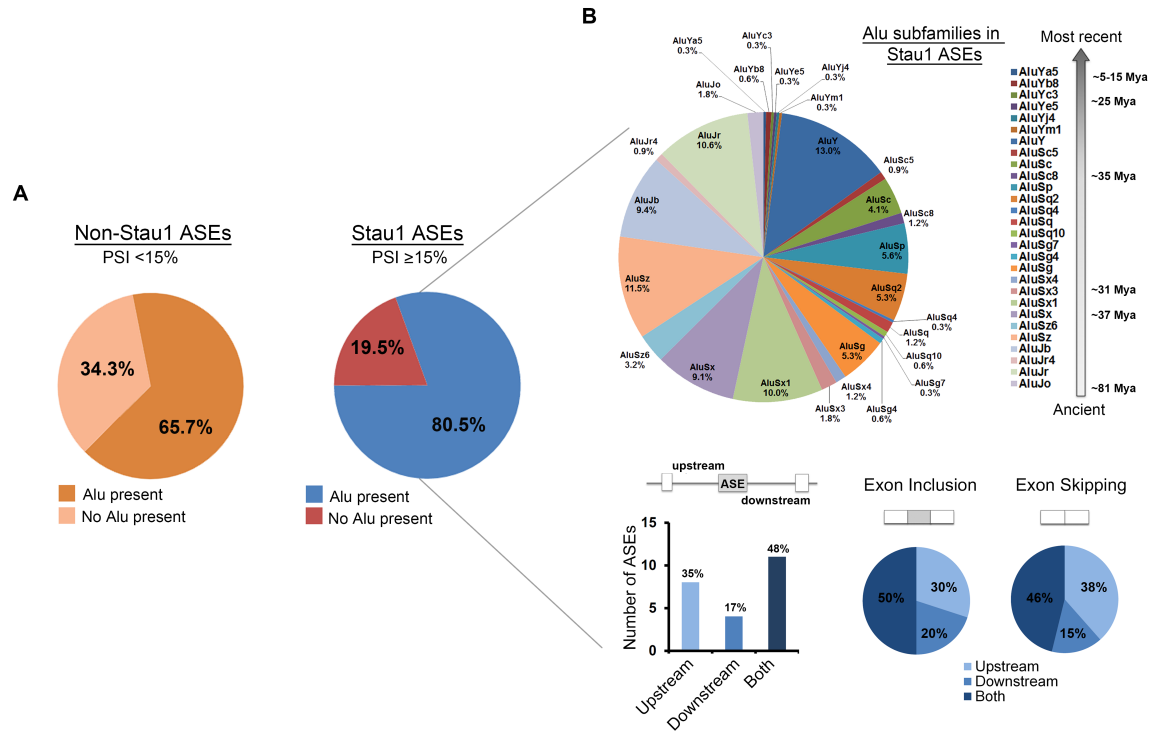


Figure 42. Alu elements in Stau1 and non-Stau1 regulated ASEs. (A) All ASEs were considered for this analysis with the exception of any that had a blank PSI, as described above. Stau1 ($\geq 15\%$ PSI) and non-Stau1 regulated ASEs targets were searched using RepeatMasker software v 4.0.5, to identify 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.

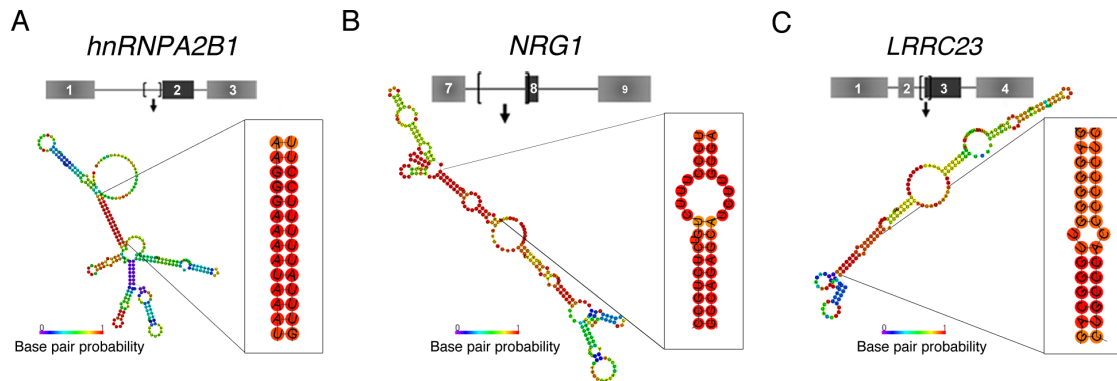


Figure 43. Proposed SBS in validated Stau1-regulated ASEs. The genomic DNA sequence of the human (A) *hnRNPA2B1* (NG_000007.14) (B) *NRG1* (NG_000008.11) and (C) *LRRC23* (NG_000012.12) was used to assess the possible non-Alu SBS. RNA secondary structure of indicated introns was determined by Vienna package RNAfold 2.1.1 and identification of possible SBS were determined following guidelines described in the materials and methods.

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. 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 (52). In a number of cases, binding of trans-acting factors to Alu elements is required to mediate an effect on splicing (46, 53). For example, Zarnack and colleagues demonstrated that, in the absence of hnRNP C, thousands of Alu elements were included as exons in mRNA transcripts (53). 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 **Figure 44**). 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.

Our results show that increased Stau1 levels correlates 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 **Figure 44**, 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 (**Figure 44**). 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 (**Figure 44**). 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.

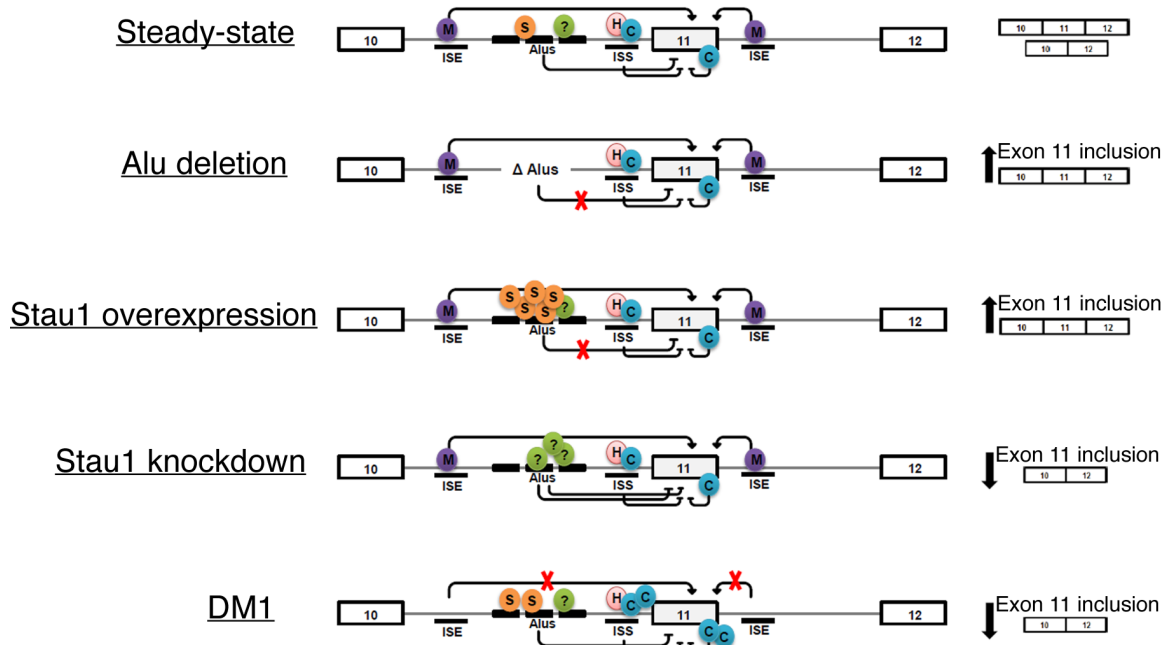


Figure 44. Proposed model of how Stau1 may contribute to *INSR* exon 11 alternative splicing. At steady-state, Stau1's binding to Alu elements participates in the integration of several regulatory mechanisms towards establishing the level of utilisation of exon 11. Introns are denoted as grey lines and 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, full inhibitory effect of the Alu elements would be observed. In DM1,

the combined effects of reduced MBNL1 levels, increased CUGBP1 activity, and moderate increase in Stau1 would result in decreased exon 11 inclusion.

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 **Figure 39D**). 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 (Ricci et al. 2013; Gong & Maquat 2011). 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 (Y. Wang et al. 2012). 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 **Figure 42**). 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 induction of skipping or inclusion. We did not document within this limited dataset Alu 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* (**Figure 43**) 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*, *FNI*, *JAG2*, *NUTF2*, *OGDH*, *SHKBP1*, *SORBP1*, *TBC1D12/13*, and *THRA* (Sugimoto et al. 2015; De Lucas et al. 2014; Ricci et al. 2013).

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 (Timchenko et al. 2004; Ho et al. 2005) 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 to be misregulated with the expression of CUG expansions resulting in altered muscle sarcomere location of these proteins (Llamusi et al. 2012). 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 **Figure 39A,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 & 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.

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 Δ Alus (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, hStau1⁵⁵-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 μ g 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 hStau1⁵⁵-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-LuminataTM 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 2 μ L of collected RNA was assessed on the Take3BioPlate Reader to determine quantity (ng/ μ L) 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 5 μ L (~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 μ L 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 $\Delta\Delta\text{Ct}$ 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 ≥ 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 Appendix A Table 3. 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:

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

Only values with a $\Delta\text{PSI} \geq 10\%$ between conditions were selected for additional analysis:

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

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. 2013) 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: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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Chapter 6: General Discussion

It is well established that transcriptional events mediate the expression of key muscle genes; however, several lines of evidence demonstrate that post-transcriptional events are also crucial during skeletal muscle formation and maintenance. At the forefront of post-transcriptional regulation are multi-functional RBPs. In this context, RBPs are often implicated in neuromuscular diseases such as DM1, SMA, and ALS (Lukong & Fatimy 2012). Recently, we reported that the RBP Staufen1 is regulated during myogenesis, and that it is elevated in DM1 skeletal muscle (Bélanger et al. 2003; Ravel-Chapuis et al. 2012). In fact, the increased expression of Staufen1 initially appeared beneficial for DM1 since further upregulating Staufen1 rescued key aberrant splicing events in DM1 skeletal muscle (Ravel-Chapuis et al. 2012). However, given the multi-functional nature of Staufen1, we more recently hypothesized that Staufen1 likely functions as a disease modifier in DM1. To test this hypothesis, we investigated novel roles for Staufen1 in skeletal muscle biology and disease. Here, we demonstrated that Staufen1 is increased during the early stages of muscle development and that the overexpression of Staufen1 impaired myogenic differentiation through the regulation of c-myc translation (Chapter 2; (Ravel-Chapuis et al. 2014)). The ability of Staufen1 to regulate c-myc, a known oncogene in several cellular systems, suggested a role for Staufen1 in cancer biology and we demonstrated that Staufen1 regulates the tumorigenesis of ERMS and ARMS through differential pathways (Chapter 3). Given Staufen1's role in myogenic differentiation and its increased expression in DM1 (Ravel-Chapuis et al. 2012), we next generated a muscle-specific Staufen1 transgenic mouse model to examine the impact of sustained Staufen1 expression in postnatal skeletal muscle. The Staufen1 transgenic mice developed a myopathy characterized by morphological and functional deficits and the

induction of atrophy-associated genes via PTEN regulation (Chapter 4). In parallel, we also further examined Staufen1's role in the regulation of alternative splicing. Our data demonstrated that Staufen1 regulates numerous ASEs in WT and DM1 muscles both beneficial and detrimental for the pathology (Chapter 5; (Bondy-Chorney et al. 2016)). Collectively, these articles indicate that Staufen1 functions as a disease modifier in DM1 (see model in **Figure 45**).

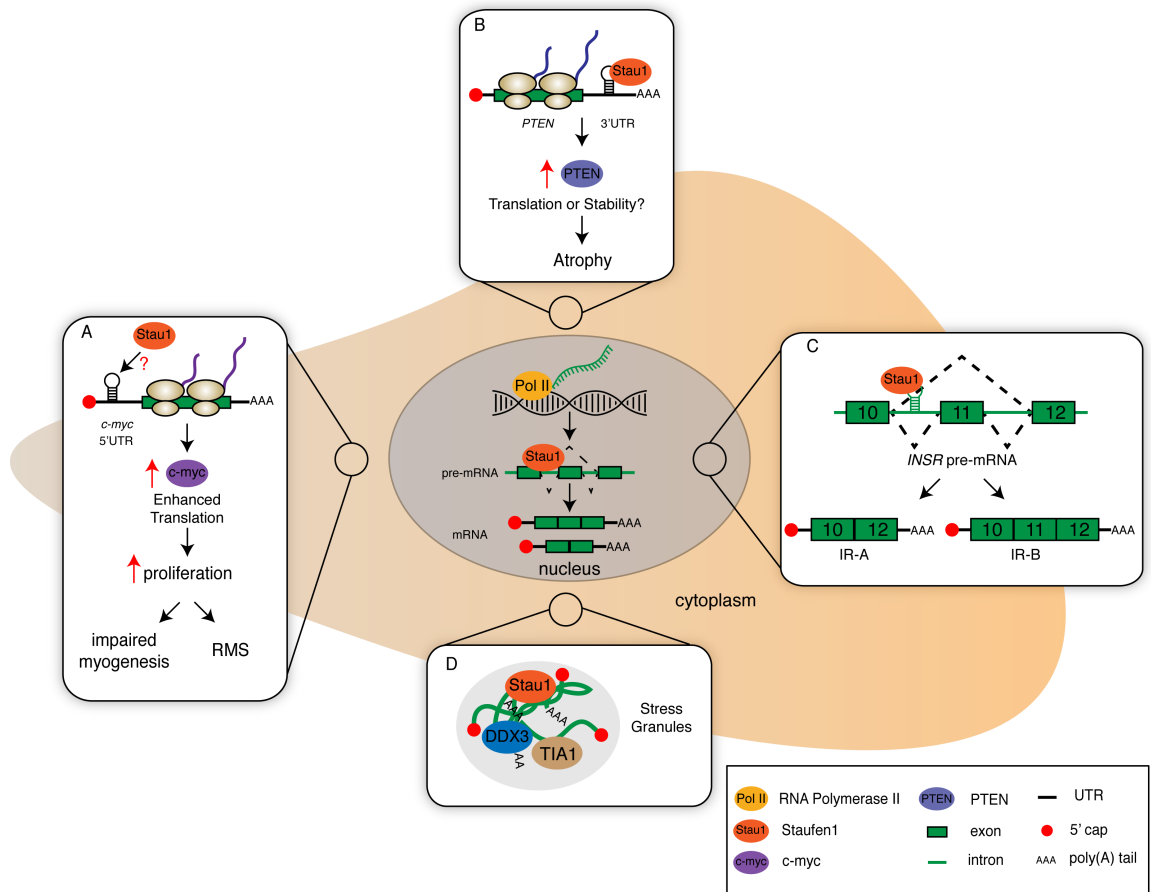


Figure 45. Staufen1-regulated post-transcriptional events in skeletal muscle. (A) Staufen1 controls the translation of *c-myc* mRNAs via the 5'UTR, which directly regulates cell proliferation and is implicated in myogenic differentiation and in Rhabdomyosarcoma (RMS). (B) Staufen1 regulates the expression of PTEN through the 3'UTR, mediated by mRNA stabilization or translational mechanisms. (C) Staufen1 regulates the alternative splicing of several pre-mRNAs in skeletal muscle and specifically enhances the inclusion of the insulin receptor (*INSR*) exon 11 via Alu's located in the upstream flanking intron. (D) Staufen1 is recruited to stress granules in skeletal muscle characterized by its partial co-localization with known stress granule markers, TIA-1 and DDX3.

6.1 Multiple Roles of Staufen1 in Skeletal Muscle

It is well established that the myogenic differentiation program is impaired in DM1 (Furling et al. 2001; Timchenko, Lakova, et al. 2001; Amack & Mahadevan 2001; Amack et al. 2002). The presence of CUG^{exp} mRNAs causes a reduction in MyoD (Amack et al. 2002) and myogenin levels (Amack & Mahadevan 2001) and as a result, impairs myogenesis. The increased expression of Staufen1 in DM1 skeletal muscle (Ravel-Chapuis et al. 2012) and our recent findings that Staufen1 overexpression impairs myogenesis (Chapter 2; (Ravel-Chapuis et al. 2014)) supports the notion that Staufen1 contributes to the delayed differentiation program observed in DM1. The myogenic program is also impaired in several other neuromuscular disorders such as SMA and ALS (Boyer et al. 2014; Martini et al. 2015). For example, the loss of *Smn* in mice disrupts several myogenic factors and impairs the differentiation of primary myoblasts (Boyer et al. 2014), while the expression of the ALS-linked superoxide dismutase 1 (*SOD1*) mutant inhibits myogenic differentiation in cell culture (Martini et al. 2015). Taken together, it is possible that Staufen1 is also misregulated in SMA and ALS and may contribute to the impaired differentiation program.

Progressive muscular atrophy characterized by the induction of atrogene expression and muscle weakness is a key hallmark of DM1 (Vignaud et al. 2010; Seznec et al. 2001; Timchenko 2013). It is well established that the misregulation of CUGBP1 contributes to the atrophic response in DM1 muscle since multiple CUGBP1 transgenic mouse models develop muscle atrophy (Timchenko et al. 2004; Ho et al. 2005; Ward et al. 2010). Here, we demonstrated that sustained Staufen1 expression in postnatal skeletal muscle causes progressive muscle atrophy (Chapter 4) and therefore, these data indicate that Staufen1

also mediates the atrophic response in DM1 muscles. Further, we propose that Staufen1-regulated PTEN expression controls the atrophic phenotype in Staufen1 transgenic mice and interestingly, PTEN is implicated in SMA and ALS pathologies (Ning et al. 2010; Little et al. 2015; Kirby et al. 2011; Yang et al. 2014). Specifically, the knockdown of PTEN promotes the survival of both SMA and ALS motor neurons (Ning et al. 2010; Little et al. 2015; Kirby et al. 2011; Yang et al. 2014). Although no link has been made for PTEN in SMA and ALS skeletal muscles, Staufen1 has a prominent role in the regulation of mRNAs in neurons (Kiebler et al. 1999; Lebeau et al. 2008; Köhrmann et al. 1999; Tang et al. 2001; Krichevsky & Kosik 2001; Marión et al. 1999) and therefore may also regulate PTEN expression in neurons.

In the current studies, we demonstrated that Staufen1 is expressed at high levels during embryonic muscle development and in regenerating skeletal muscle (Chapter 2; (Ravel-Chapuis et al. 2014)). It has been proposed that some ASEs in DM1 muscle are secondary to muscle regeneration (Orengo et al. 2011) and thus are common across several neuromuscular disorders. Similarly, RMS gene expression recapitulates an embryonic muscle state (Tapscott et al. 1993; Tonin et al. 1991). Therefore, the increased expression of Staufen1 in DM1 and RMS may result as a consequence of fetal programming and we speculate that decreasing Staufen1 to basal levels may promote an adult muscle phenotype. Altogether, these findings support the idea that Staufen1 is a disease modifier in DM1 skeletal muscle and highlight its potential involvement in the pathogenesis of other neuromuscular disorders such as SMA and ALS.

6.2 Novel Functions of Staufen1 in Cancer Biology

In addition to muscle deficits, DM1 patients have an increased risk for developing cancer (Win et al. 2012; Mueller et al. 2009). Moreover, several lines of evidence suggest that cellular events common among muscular dystrophies can promote the formation of sarcomas, including RMS (Fanzani et al. 2013). For example, decreased expression of α -dystroglycan, a component of the dystrophin-associated protein complex (Ibraghimov-Beskrovnaya et al. 1992) is described in ERMS and ARMS (Martin et al. 2007). Thus, the increased expression of Staufen1 in DM1 may contribute to the increased risk of cancer. Although little is known about Staufen1's expression in other muscular dystrophies such as Duchenne Muscular Dystrophy (DMD), Staufen1 transgenic mice display muscle deficits consistent with several forms of neuromuscular disorders and therefore may also be involved in the development of sarcomas in these diseases. Collectively, Staufen1 regulates several important aspects of the DM1 pathology and may function as a disease modifier in other relevant neuromuscular disorders.

Alternative splicing is also implicated in cancer biology. Given Staufen1's direct role in mediating RMS tumorigenesis, there is potential for Staufen1 to regulate alternative splicing in this context. As discussed in Chapter 3, the comparison of altered genetic profiles in RMS with identified Staufen1 targets will aid in the investigation into Staufen1-mediated mechanisms in RMS. Of particular interest, the alternative splicing of the human oncogene *MDM2* has been implicated in several cancers including ERMS and ARMS (Bartel et al. 2001; Bartel et al. 2002). In addition, *MDM2* contains a SBS that forms between the coding sequence and the 3'UTR and is classified as a long range duplex of > 500 nt, similar to the SBS observed in the *PTEN* 3'UTR described in chapter

4 (Sugimoto et al. 2015). At least six novel *MDM2* splice variants were identified in RMS (Bartel et al. 2001; Bartel et al. 2002) with the *MDM2-ALT1* variant being expressed in over 70% and 85% of ERMS and ARMS tumours, respectively (Jacob et al. 2013). It is possible that the SBS that forms between the CDS and 3'UTR represents a cis-regulatory element and therefore increased expression of Staufen1 in RMS may enhance the splicing of the *MDM2-ALT1* variant.

Recently, SRSF1 (also called SFRS1), a splicing factor that has a major role in the splicing regulation of several cancer-related genes (Karni et al. 2007), was identified as a regulator of *MDM2* splicing (Comiskey et al. 2015). SRSF1 protein expression is increased in primary RMS tumours and its expression correlates with the *MDM2-ALT1* splice variant (Comiskey et al. 2015). Interestingly, the SRSF1 protein is present in Staufen1 RNPs (Milev et al. 2012) and it has been shown that c-myc regulates the transcription of *SRSF1* (Das et al. 2012). Remarkably, *SRSF1* contains 10 SBSs located within the 3'UTR (Sugimoto et al. 2015). Taken together, with Staufen1's ability to regulate alternative splicing (Chapter 5; (Bondy-Chorney et al. 2016) and (Ravel-Chapuis et al. 2012; Sugimoto et al. 2015)), it seems reasonable to hypothesize that Staufen1 may regulate the alternative splicing of *MDM2* either directly through binding cis-regulatory elements or indirectly through regulation of c-myc and SRSF1 expression. Moreover, given the broad impact of *MDM2* alternative splicing, c-myc and SRSF1 in cancer biology (Karni et al. 2007; Bartel et al. 2001; Bartel et al. 2002), we speculate that Staufen1-regulated events are implicated in other forms of cancer.

6.3 Staufen1-regulated mRNA Localization in Skeletal Muscle

Given the role of Staufen1 in the regulation of mRNA localization and translation in oocytes and neurons (St Johnston et al. 1991; Li et al. 1997; Schuldt et al. 1998; Breitwieser et al. 1996), we must also consider a similar role for Staufen1 in skeletal muscle. Post-transcriptional events that mediate mRNA localization and local translation are particularly important in synapse formation and plasticity in the CNS and at the neuromuscular junction (NMJ) (Martin & Zukin 2006; Chakkalakal & Jasmin 2003). In addition, mRNA localization is also critical in the development and maintenance of myotendinous junctions (MTJ) (Dix & Eisenberg 1990). The 3'UTR of mRNAs play a critical role in the localization and translation of transcripts within the cell (Andreassi & Riccio 2009). Recently, it was identified that MBNL binding sites were preferentially located in the 3'UTRs of target mRNAs and it was demonstrated that MBNL proteins regulate mRNA localization, stability and protein expression in skeletal muscle and the brain (E. T. Wang et al. 2012; Masuda et al. 2012). Similarly, several SBS are mapped to the 3'UTR of mRNAs with a high prevalence in extended 3'UTRs (Ricci et al. 2013; Sugimoto et al. 2015). Moreover, the SBS located in 3'UTRs are highly structured allowing for Staufen1 multimerization and it is possible that Staufen1 regulates both mRNA localization, stability and/or translation of these target transcripts (Ricci et al. 2013; Sugimoto et al. 2015). The potential roles of MBNL proteins and Staufen1 in mediating mRNA localization in skeletal muscle has potential implications at the NMJ of DM1 muscles.

Two separate DM1 mouse models display diaphragmatic NMJ degeneration (Panaite et al. 2008; Panaite et al. 2013) and motor neurons isolated from DM1 embryonic stem

cells are unable to form mature neuromuscular connections (Marteyn et al. 2011). Interestingly, MBNL mutant mice showed moderate defects in NMJ formation (K. Y. Lee et al. 2013). In addition, *Caenorhabditis elegans* carrying a mutated form of the MBNL1 homolog, mbl-1, have selective loss of distal NMJs, however, this was rescued by the neuron-specific and not muscle-specific expression of mbl-1 (Spilker et al. 2012). These findings indicate that MBNL proteins may be responsible for the pre-synaptic formation of the NMJ and in particular mRNA localization at the nerve terminal rather than at the post-synaptic membrane of the NMJ.

In contrast, we previously showed that Staufen1 and Staufen2 accumulate at the postsynaptic sarcoplasm of muscle fibers, at newly formed ectopic synapses and in denervated skeletal muscles (Bélanger et al. 2003). Along those lines, it was recently demonstrated that Staufen1 associates with the muscle nicotinic acetylcholine receptor (AChR) $\alpha 1$ subunit (*Chrna1*) mRNAs in an agrin-dependent manner and defects in AChR clustering was observed upon Staufen1 knockdown in C2C12 myoblast cells (Chang et al. 2012). In agreement with these findings, Staufen is localized at the post-synaptic compartment of the invertebrate NMJ in *Drosophila* where it regulates the synaptic localization and translation of *coracle* mRNAs (Gardiol & St Johnston 2014). Interestingly, Staufen mutant larvae, which contain mutated dsRBD5 demonstrated that although *coracle* mRNAs localize to the NMJ, its translation is decreased and as a result there is a reduction in the number of synaptic boutons (Gardiol & St Johnston 2014). These studies clearly highlight the importance of Staufen proteins in synapse plasticity and maintenance at the NMJ and combined with elevated Staufen1 in embryonic and denervated muscles, it is possible that Staufen1 also regulates mRNA localization outside

of synaptic regions. This level of regulation is likely implicated in mRNA localization in DM1 skeletal muscle and in other neuromuscular disorders.

Furthermore, it is well established that nuclear pre-mRNA splicing can influence the downstream metabolism of spliced mRNAs (Tange et al. 2004). The exon junction complex (EJC), which is formed by splicing-specific proteins located 20-24 nts upstream of an exon-exon junction, regulates several post-transcriptional events (Le Hir et al. 2000; Tange et al. 2004). The EJC appears to impact the subcellular localization and translation of mRNAs (Tange et al. 2004). In particular, *Drosophila* EJC protein homologs of Y14, Magoh and eIF4AIII are crucial for *oskar* mRNA localization in oocytes (Palacios et al. 2004; Mohr et al. 2001; Hachet & Ephrussi 2011). Although *oskar* mRNAs are the only known target of EJC mediated mRNA localization, it is interesting given the importance of Staufén in the regulation of *oskar* mRNA localization (Breitwieser et al. 1996; Johnston et al. 1991). It is attractive to imagine that Staufén1 may use the EJCs as a means to recognize and target specific mRNAs to subcellular compartments. However, extensive research is required to determine if this level of regulation is specific to *oskar* mRNAs or if it represents a common function of the EJC.

6.4 Potential Impact for Staufén1 in the DM1 Brain and Heart

Although major regulators of alternative splicing in DM1 skeletal muscle are known, the mechanisms that control ASEs in the DM1 brain are less defined. However, several aberrant ASEs are described in DM1 brain tissues including the NMDA NR1 (*NMDAR1*) receptor, amyloid beta precursor protein (*APP*) and microtubule-associated protein tau (*MAPT*) mRNAs (Suenaga et al. 2012; Jiang et al. 2004; Dhaenens et al. 2008; Leroy et al. 2006; Sergeant et al. 2001). For example, the fetal isoform of *MAPT* is expressed in

DM1 brain tissue as a result of exon 2, 3 and 10 exclusion (Dhaenens et al. 2008; Jiang et al. 2004), an event regulated by the combined functions of MBNL1 and MBNL2, indicating that the loss of MBNL in DM1 contributes to ASEs in the brain (Carpentier et al. 2014; Goodwin et al. 2015; Dhaenens et al. 2008; Jiang et al. 2004). In contrast to DM1 skeletal muscle where MBNL proteins play a major role in the regulation of numerous ASEs, initial studies demonstrate that MBNL1 has modest effects on ASEs in the DM1 brain (Suenaga et al. 2012). In addition, RNA-sequencing of control and MBNL mutant mice showed the least amount of change in the PSI of mRNAs in the brain as compared to skeletal muscle and heart tissues (E. T. Wang et al. 2012). These studies indicate that although MBNL proteins are important for alternative splicing regulation in the DM1 brain, other splicing regulatory factors are also required. Moreover, MBNL proteins are multi-functional and likely contribute to DM1 cognitive deficits through alternate mechanisms.

As described above, mRNA localization and translation via the 3'UTR of target transcripts are extremely important during synapse formation and for synaptic plasticity in the CNS. Therefore, as indicated for the NMJ, MBNL and Staufen proteins may also have prominent functions in mRNA localization and translation in the CNS. Specifically, Staufen1 and Staufen2 have prominent roles in mRNA localization and translation in the CNS that are fundamental to learning and memory (Lebeau et al. 2008; Köhrmann et al. 1999; Kiebler et al. 1999; Tang et al. 2001; Krichevsky & Kosik 2001). Mice harbouring truncated Staufen1 protein lacking a functional dsRBD3 are viable with no obvious phenotypes affecting development, fertility, health and overall brain morphology; however, hippocampal neurons display a significant reduction in dendritic tree size as

well as an inefficiency in Staufen1-containing RNP delivery to the dendrites (Vessey et al. 2008). Therefore, Staufen1 and possibly Staufen2 may regulate relevant target mRNAs in the brain and their misregulation could contribute to the cognitive impairments observed in DM1 patients.

In addition to muscle and brain deficits, approximately 80% of DM1 patients develop cardiac abnormalities, which represent the second leading cause of death in DM1 (Vinereanu et al. 2004). Specifically, the most common abnormalities include heart blockage that causes conduction defects (Nguyen et al. 1988) and ventricular or atrial arrhythmias (Benhayon et al. 2015). Relatively little is known about the mechanisms regulating cardiac deficits in DM1, however, it was recently demonstrated that MBNL1 and CUGBP1 have prominent roles in the heart (Kalsotra, Ravi K. Singh, et al. 2014; Kalsotra et al. 2008; Kalsotra et al. 2010). In fact, mimicking the embryonic expression patterns of CUGBP1 and MBNL1 reproduced several ASEs present in DM1 heart tissue (Kalsotra et al. 2008). Specifically, the transition from embryonic to adult gene expression in the heart is regulated by miRNAs with miR-23a/b required for the postnatal decrease of CUGBP1 (Kalsotra et al. 2010). Remarkably, the expression of CUG^{exp} mRNAs reverts heart tissue back to an embryonic state (Kalsotra, Ravi K. Singh, et al. 2014). Further analysis demonstrated that there is a loss of MEF2A and MEF2C function in DM1 heart tissue and that the misregulation of mRNAs and miRNAs was rescued by MEF2C expression (Kalsotra, Ravi K. Singh, et al. 2014). Interestingly, we demonstrated in Chapter 2 that Staufen1 overexpression in C2C12 cells decreased MEF2A and MEF2C expression (Ravel-Chapuis et al. 2014). Although the function of Staufen1 in the heart is unknown, it is possible that it is also elevated in DM1 heart tissues. Based on the multiple

roles that Staufen1 has in RNA metabolism, it is conceivable to speculate that Staufen1 regulates several mRNAs implicated in the DM1 heart.

6.5 Preliminary Results

Given the emerging role of Staufen1 as a disease modifier in DM1, there is a clear need to identify pathways involved in regulating Staufen1 expression for the development of future therapies based on Staufen1 modulation. We designed a series of complementary experiments to further investigate the implications of Staufen1 in DM1 skeletal muscle. We first developed two mouse models to evaluate the impact of increased Staufen1 expression in DM1 muscles *in vivo*. The first mouse model is a cross between the Tg-551 Staufen1 transgenic mouse line and the HSA^{LR} mouse model to form mice hemizygous for both transgenes (Tg-551/LR). The second approach uses AAV particles encoding Staufen1-HA, which were injected directly into the TA muscles of adult homozygous HSA^{LR} mice. Together, these mouse models will provide key information regarding Staufen1's role in mild and severe DM1 skeletal muscle, respectively.

In both models, our preliminary results demonstrate that the sustained expression of Staufen1 in a mild or severe HSA^{LR} background induces muscle myopathy characterized by an increase in central nuclei, fiber size variability and an increased frequency of small muscle fibers. In contrast to our findings in cell culture (Bondy-Chorney et al. 2016; Ravel-Chapuis et al. 2012), the overexpression of Staufen1 increases the expression of MBNL1 and CUGBP1 in both conditions. Moreover, the alternative splicing of key DM1 ASEs, *Sercaf* exon 22, *RyR1* ASI and *Tnnt3* F exon are regulated by Staufen1 overexpression. In fact, these ASEs shifted towards DM1 splicing patterns in both models. As a control, *RyR1* ASII, an ASE not regulated in DM1, was examined and our

data show that the splicing is unaffected (**Appendix B**). Collectively, these findings demonstrate that the overexpression of Staufen1 in DM1 skeletal muscle recapitulates several morphological abnormalities observed in MCK-Staufen1-HA mice and are in agreement with our findings that Staufen1 regulates multiple ASEs in DM1 cultured cells further supporting that Staufen1 is a disease modifier.

To complement these preliminary data, a transgenic mouse model homozygous for both MCK-Staufen1-HA and HSA^{LR} transgenes will be developed to better assess Staufen1 overexpression during muscle development in a severe DM1 model. In parallel, an AAV encoding Staufen1-targeting shRNAs will be generated to determine if the reduction of Staufen1 to basal levels rescues the morphological and splicing abnormalities observed in DM1. Finally, to further evaluate the effect of Staufen1 overexpression and/or knockdown in skeletal muscle, WT mice will be infected with the respective AAVs and the above noted analyses will be repeated. Together, these models will serve as tools to better define Staufen1's role as a disease modifier in DM1 muscle and will highlight the therapeutic potential of modulating Staufen1 in DM1.

6.6 Future Directions & Conclusions

As described above, it is also important to examine Staufen1's role in other DM1 affected tissues such as the brain and heart. In addition to investigating Staufen1-regulated ASEs in these tissues, given the abundance of SBSs located within the 3'UTRs of target mRNAs it is important to study Staufen1's role in mRNA localization and translation of relevant transcripts in the DM1 brain. Moreover, the investigation of Staufen1 expression during cardiac development and the identification of DM1 relevant targets will determine if Staufen1 plays a key role in the cardiac abnormalities observed in the pathology.

Altogether, these studies will indicate if Staufen1 functions as a disease modifier in other DM1 tissues and will further highlight the multi-functional nature of Staufen1 across various organs.

The data presented in Chapter 4 suggests that Staufen1 may function as an atrogene since its expression mirrors that of the known atrogens, MuRF1 and MAFbx and the sustained expression of Staufen1 in postnatal muscles induces progressive atrophy. To determine if Staufen1 is a novel atrogene, detailed analyses of Staufen1 expression in various atrophic conditions such as cachexia (Llovera et al. 1994; Baracos et al. 1995; Temparis et al. 1994), diabetes (Price et al. 1996), chronic disuse (Chopard et al. 2009) sarcopenia (Evans & Campbell 1993) and in other neuromuscular disorders is required. It would be informative to develop an inducible muscle-specific Staufen1 knock-out mouse model to perform a series of denervations in order to determine if the loss of Staufen1 prevents denervation-induced atrophy as observed with MuRF1 and MAFbx knock-out mice (Bodine et al. 2001). Together, these studies will examine potential roles for Staufen1 in atrophic conditions and will determine if Staufen1 is a bona fide atrogene.

Finally, future work is required to understand the differential roles of Staufen1 in ERMS and ARMS. A potential experimental approach is to perform high-throughput RNA-sequencing on ERMS and ARMS cells expressing control or Staufen1-shRNA to identify novel Staufen1-regulated targets and ASEs that will provide insight into the mechanisms by which Staufen1 promotes RMS tumorigenesis. A large-scale screen of this nature will also provide key information regarding additional Staufen1 targets that may be highly relevant in other forms of cancer. In this context, it will be informative to

examine the expression of Staufen1 across multiple cancers to determine if Staufen1 has a broad regulatory role in cancer biology.

Overall, the collection of manuscripts presented here describes several novel roles for Staufen1 in skeletal muscle biology. Moreover, we demonstrated that the misregulation of Staufen1 in skeletal muscle is implicated in neuromuscular disease and biology.

Staufen1's role as an atrophy-associated gene makes it an attractive target for future investigations in neuromuscular disorders and conditions characterized by muscle atrophy. Given the multi-functional nature of Staufen1 in neurons and skeletal muscle, it is evident that Staufen1-regulated post-transcriptional events are critical for the development and maintenance of the neuromuscular system. In addition, the importance of Staufen1-regulated events in the RMS pathology highlights the potential of a broad role for Staufen1 in cancer. Future work on Staufen1 should extend beyond skeletal muscle to include the CNS, peripheral nervous system and the heart to gain a clear understanding of the importance for Staufen1-regulated post-transcriptional events and how these contribute to multi-systemic diseases such as DM1, ALS and SMA.

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Appendix A

Due to the extensive length of the data presented in **Table 3**, please refer to the excel file containing the dataset labeled Table S1 at:

<http://journals.plos.org/plosgenetics/article/asset?unique&id=info:doi/10.1371/journal.pgen.1005827.s005>

Table 3. 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 $PSI \geq 5\%$ were considered for any analysis comparing our data with that of Klinck *et al.*, 2014., Tab 6: The subfamilies of Alu elements in Stau1-regulated targets.

Table 4. Validation Genes and Disease.

Gene symbol	Gene name	ASE	Stau1 OE WT	Stau1 OE DM1	Predicted by screen	Variant previously reported	Reported variant name (Δ exon)	Splice variant disease related?
<i>INSR</i>	Insulin Receptor	Exon 11	Inclusion	Inclusion	Y	Y - Savkur et al., 2001	IR-A	Y – DM1 - Savkur et al., 2001
<i>hnRNP A2B1</i>	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
<i>LRRC23</i>	Leucine-rich repeat-containing protein 23	Exon 3	Inclusion	Inclusion	Y	N	-	N
<i>NRG1</i>	Neuregulin 1	Exon 8	Skipping	Skipping	Y	?	?	? – NRG1 dysregulation disrupts synaptic plasticity - Agarwal et al., 2014
<i>HIF1α</i>	Hypoxia-inducible factor 1- α	Exon 14	Inclusion	Inclusion	Y	Y - Gothie et al., 2000	HIF1 α ⁷³⁶ and sHIF1 α	Y- higher expression levels of HIF1 α ⁷³⁶ in OR-negative carcinomas - Dales et al., 2010
<i>FN1</i>	Fibronectin	Exon 33	Skipping	Skipping	Y	Y - Goossens et al., 2009	EDA or EDI	Y – DM1 - Ohsawa et al., 2011
<i>ACCN3</i>	acid sensing (proton gated) ion channel 3	Exon 6	--	Inclusion	Y	?	?	N
<i>CLCN6</i>	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 database (NCBI) however could not find variant in publication; “-” no information

Appendix B

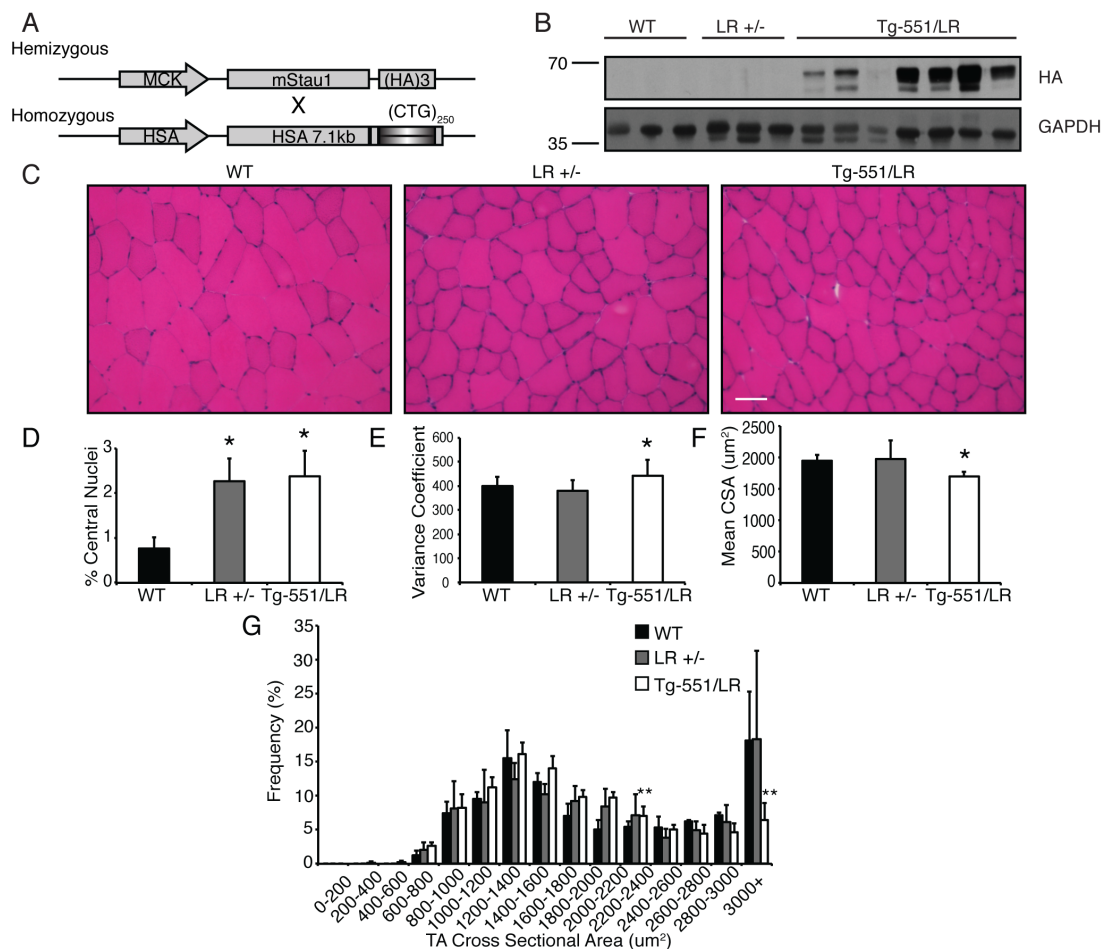


Figure 46. Transgenic overexpression of Staufen1 in HSA^{LR} mice causes muscle fiber variability and central nucleation. (A) Schematic diagram of transgenic cross between MCK-Staufen1-HA line Tg-551 with HSA^{LR} mice (B) Western blotting to confirm transgene expression using HA (1:1000) antibodies with GAPDH (1:100000) as a loading control (C) H&E staining of TA muscle cross sections (10μm) from WT (FVB/N), hemizygous HSA^{LR} (LR+/-) and Tg-551/LR 8 week old mice (D) % central nuclei, (E) Variance coefficient of TA fiber cross sectional area (F) Mean cross sectional area (μm²) (G) TA fiber cross sectional area (μm²) displayed as a frequency distribution (% of Total) of 8 week old mice. WT (n=3), LR+/- (n=3), Tg-551/LR (n=7). Data are means +/- SEM *p<0.05, **p<0.01 Students T-Test.

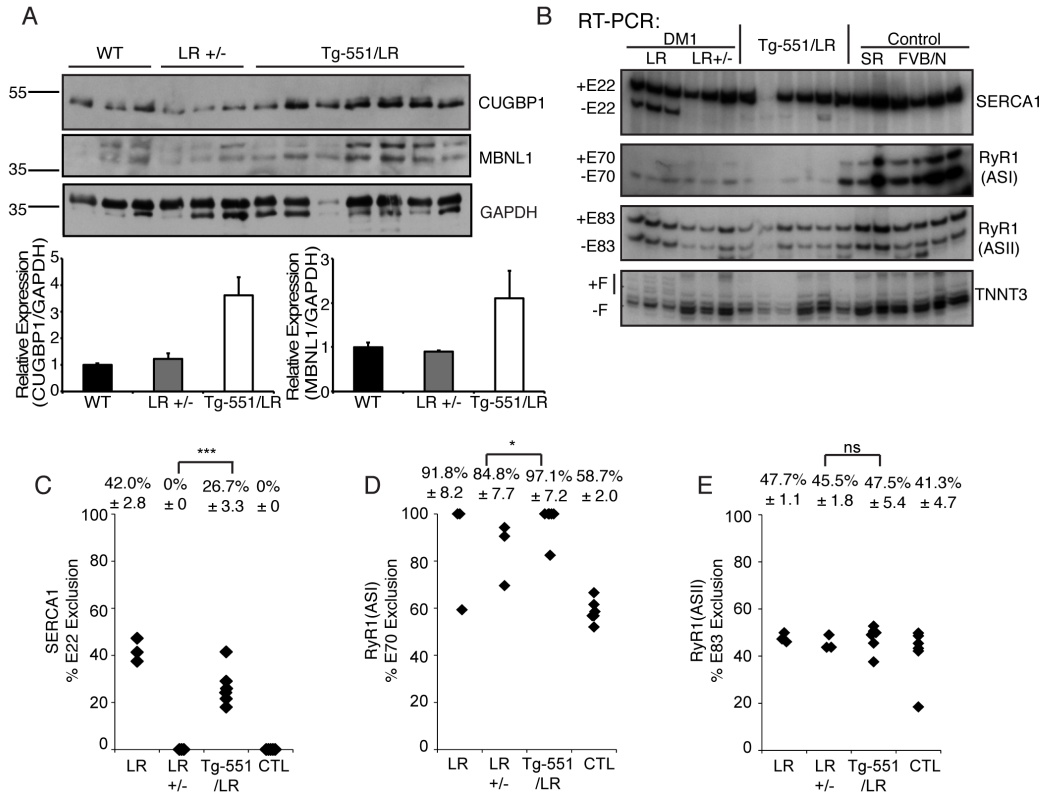


Figure 47. Staufen1 regulates the expression of CUGBP1 and MBNL1 in DM1 skeletal muscle and regulates alternative splicing. (A) Protein was extracted from TA muscles of 8 week-old LR +/-, WT (FVB/N), and Tg-551/LR mice analyzed by Western blot for CUGBP1 (1:500), MBNL1 (1:300) and GAPDH (1:50000) as the loading control (B) Total mRNAs were extracted from TA muscles of 8 week-old LR +/-, WT, and Tg-551/LR mice. Alternative splicing profiles were analyzed by radioactive RT-PCR using specific primers for mRNA targets. Quantification of (C) SERCA1 exon 22 exculsion, (D) RyR1 Exon 70 Exculsion and (E) RyR1 (ASII) Exon 83 exculsion. Data are means +/- SEM **p<0.01, ***p<0.001 Student T-Test.

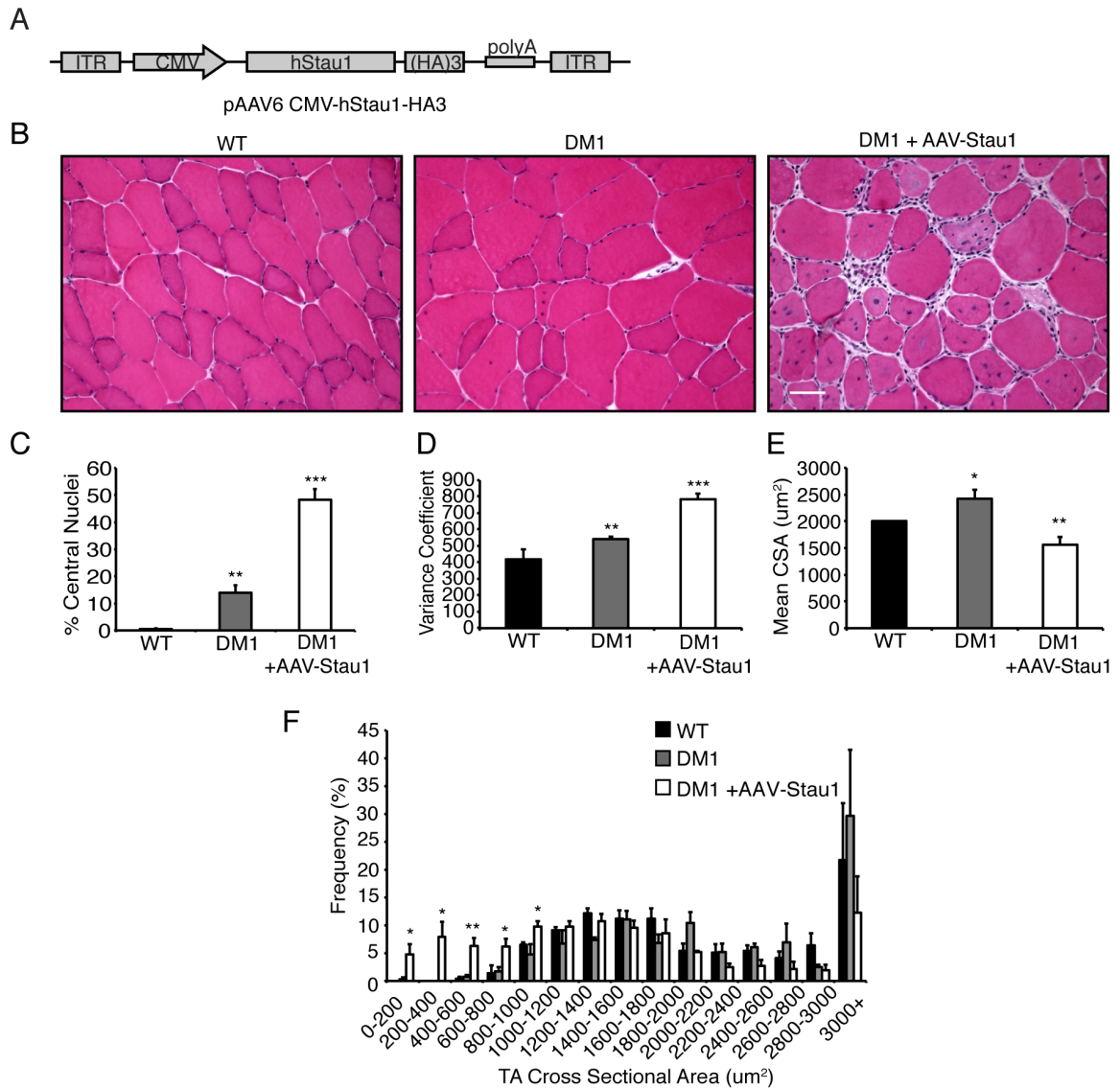


Figure 48. AAV-mediated overexpression of Staufen1 in DM1 negatively impacts skeletal muscle morphology. Adeno-associated virus encoding Staufen1 infected TA muscle of HSA-LR mice and compared with HSA-LR and WT (FvB/N) mice. (A) Schematic of pAAV6-CMV-hStau1-HA3 vector used to infect TA muscle (n=3) (B) H&E staining of TA muscle cross sections (10um) (C) % central nuclei, (D) Variance Coefficient (=1000*(StDev/Mean)) of TA fiber cross sectional area (E) Mean cross sectional area (um²) (F) TA fiber cross sectional area (um²). Data are mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001 Students T-Test.

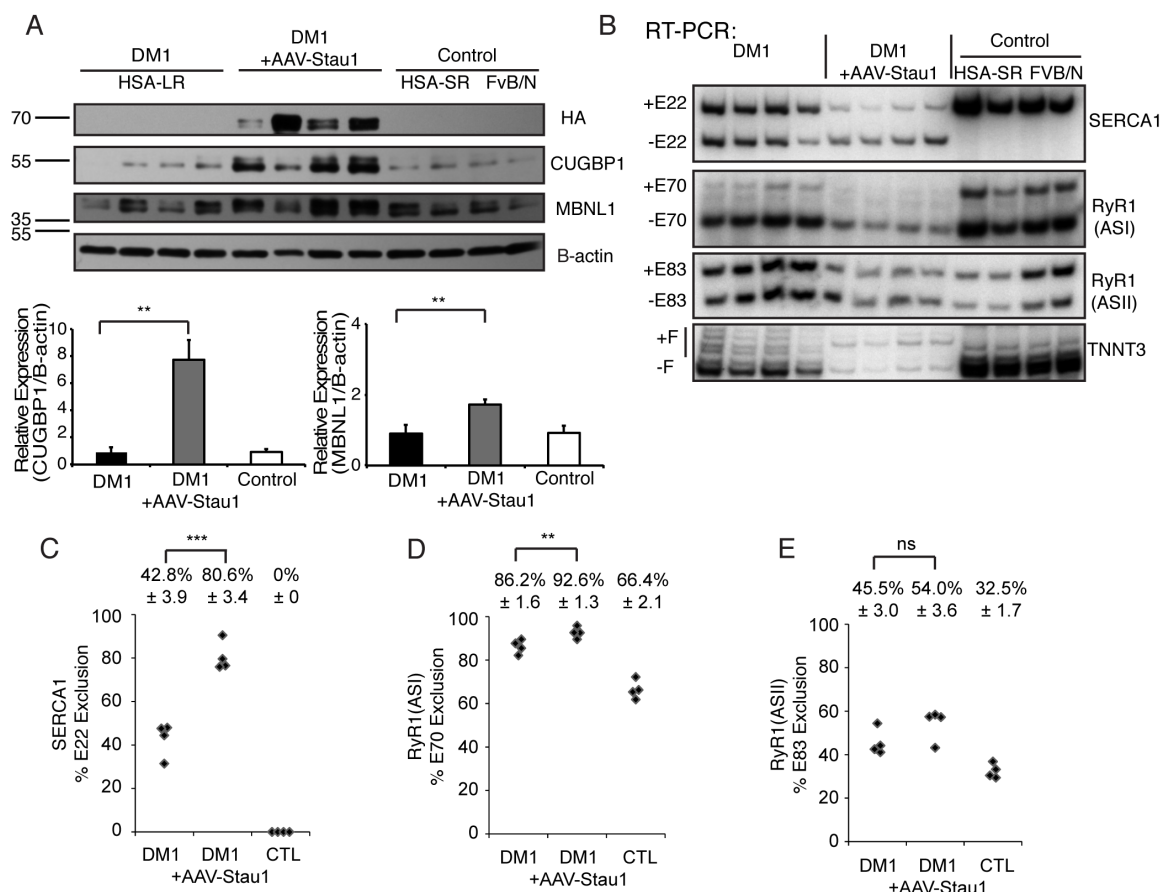


Figure 49. Stau1 regulates the expression of CUGBP1 and MBNL1 in DM1 skeletal muscle and regulates alternative splicing. TA muscle of HSA-LR +AAV-Stau1 are compared with HSA-LR and WT (FvB/N) mice 4 weeks post-infection (**A**) Western blot for HA (1:1000), CUGBP1 (1:500), MBNL1 (1:300) and B-actin (1:10000) as the loading control (**B**) Total mRNAs were extracted from TA muscles and alternative splicing profiles were analyzed by radioactive RT-PCR using specific primers for mRNA targets. Quantification of (**C**) SERCA1 exon 22 exculsion, (**D**) RyR1 Exon 70 Exculsion and (**E**) RyR1 (ASII) Exon 83 exclusion. Data are means $\pm$ SEM ** $p < 0.01$, *** $p < 0.001$ Student T-Test. Note: RT-PCR performed by Christine Peladeau and Aymeric Ravel-Chapuis.

Appendix C

Tara Crawford Parks

1 - Education

Doctorate of Philosophy, Cellular and Molecular Medicine 2011-2016
Faculty of Medicine, University of Ottawa

- Transferred from MSc program in September 2012

**Bachelor of Science Honours in Biomedical Science,
Minor in Chemistry** 2007-2011
Faculty of Science, University of Ottawa

- Cum Laude
- Deans Honours List

2 – Honours and Awards

University of Ottawa Dean's Scholarship \$3 000 2017
University of Ottawa PhD Admission Scholarship \$9 000 2015-2016
Queen Elizabeth II GSST \$15 000 2014-2015
University of Ottawa Excellence Scholarship \$7 000 2014-2015
University of Ottawa PhD Admission Scholarship \$27 000 2012-2014
University of Ottawa Dean's Scholarship \$1500 2012
University of Ottawa Master's Admission Scholarship \$7 500 2011-2012
Top 10 Poster Competition – RiboClub 13th Annual International Meeting 2012
InnoCentive Challenge 9734272 – Nuclear Test Monitoring and
Verification Methods \$3000 2011

- Invented and designed a novel method for verifying underground nuclear explosions as part of an innovation challenge

Science Student's Association Clubs Scholarship \$500 2010
Queen Elizabeth II: Aiming for The Top Scholarship \$100 2007
University of Ottawa Undergraduate Admission Scholarship \$2 500 2007

3 – Teaching and Research Interests

- Cellular and Molecular Biology: gene regulation, mRNA splicing, mRNA transport, mRNA decay, mRNA localization, protein synthesis
- Neuromuscular disorders
- Rare genetic diseases

4 – Teaching Experience

Biotechnology Tutor 2013-2016

- Tutored an Algonquin College student with special needs in the Biotechnology program.

- Course management and ability to effectively communicate subject matter to the student.
- Science Activity Facilitator, **Science Travels** 2013-2014
- Travelled to remote regions of Northern Ontario to teach science in First Nations communities.
 - Organized and taught science lessons aligned with the Ontario curriculum from kindergarten to grade 12 while applying different teaching strategies based on the level of the students.
- Undergraduate Supervisor, **University of Ottawa** 2012-2014
- Direct or co-supervision of two undergraduate students during the course of my PhD.
 - Project management of thesis projects including planning, troubleshooting and monitoring progress.
- Science Activity Facilitator/Demonstrator, **Let's Talk Science** 2011-2016
- Visited local schools and extra-curricular groups to teach science lessons and/or to demonstrate science experiments to students in various grades.
 - Organized and taught science lessons aligned with the Ontario curriculum and applied different teaching strategies based on the level of the students.

5 – Research Experience

- Doctorate of Philosophy Candidate** 2011-2016
 Cellular and Molecular Medicine, University of Ottawa
 Supervisor: Dr. Bernard Jasmin
Thesis Title: Novel Functions for the RNA-binding Protein Staufen1 in Skeletal Muscle Biology and Disease
- Undergraduate Honour's Thesis** 2010-2011
 Regenerative Medicine, Sprott Center for Stem Cell Research
 Ottawa Hospital Research Institute
 Supervisor: Dr. F. Jeffrey Dilworth
Thesis Title: Characterizing the Role of Transmembrane Protein 8c (TMEM8c) in Myogenesis

6 – Publications

6.1 Full Length Manuscripts

Crawford Parks, TE; Marcellus, K; Langill, J; Ravel-Chapuis, A; Michaud, J; Cowan, K; Côté, J; Jasmin, BJ. “Novel Roles for Staufen1 in Rhabdomyosarcoma Tumorigenesis through Regulation of Proliferation and Apoptosis” **Manuscript under revision, Scientific Reports.**

Crawford Parks, TE; Ravel-Chapuis, A; Bondy-Chorney, E; Côté, J; Jasmin, BJ. “Muscle-Specific Expression of the RNA-binding Protein Staufen1 and it's Implications

in Myotonic Dystrophy Type 1” **Manuscript to be re-submitted to Human Molecular Genetics.**

Bondy-Chorney, E; **Crawford Parks, TE**; Ravel-Chapuis, A; Jasmin, BJ; Côté, J. “Staufen1’s role as a splicing factor and a disease modifier in Myotonic Dystrophy Type I” *Rare Diseases*. 2016

Ravel-Chapuis, A; Klein Gunneweik, A; Bélanger, G; **Crawford Parks, TE**; Côté, J; Jasmin, BJ. “Staufen1 Impairs Stress Granule Formation in Skeletal Muscle Cells from Myotonic Dystrophy type 1 Patients” *Mol. Biol. Cell*. 27(11), 1728-1739 (2016).

Bondy-Chorney, E; **Crawford Parks, TE**; Ravel-Chapuis, A; Klinck, R; Chabot, B; Jasmin, BJ; Côté, J “Staufen1 Regulates Multiple Alternative Splicing Events either Positively or Negatively in DM1 Indicating Its Role as a Disease Modifier” *Plos Genetics*. 12(1): e1005827 (2016).

Péladeau, C; Ahmed, A; Amirouche, A; **Crawford Parks, TE**; Bronicki, L; Ljubicic, V; Renaud, JM; Jasmin, BJ. “Combinatorial Therapeutic Activation with Heparin and AICAR Stimulates Additive Effects on Utrophin A Expression in Dystrophic Muscles” *Hum. Mol. Genet*. 25(1): 24-43 (2016).

Ravel-Chapuis, A; **Crawford, TE**; Blais-Crepeau, ML; Bélanger, G; Richer, C; Jasmin, BJ. “The RNA-binding protein Staufen1 impairs myogenic differentiation and involves SMD-independent, c-myc dependent events”. *Mol. Biol. Cell*. 25(23), 3765-3778 (2014).

6.2 – Abstracts

Crawford, TE; Ravel-Chapuis, A; Côté, J; Jasmin, BJ (2015) Muscle-specific expression of the RNA-binding protein Staufen1 induces skeletal muscle atrophy via regulation of Phosphatase Tensin Homolog. *3rd Ottawa International Neuromuscular Disease Conference, Ottawa, Canada, September 24th-26th*.

Crawford TE, Ravel-Chapuis A, Bélanger G, Jasmin BJ (2014). Defining the Role of Staufen1 in Developing and Mature Skeletal Muscle *In Vivo*. *19th Annual meeting of the RNA Society, Québec City, Québec, Canada, June 6-8*. Poster presentation.

Crawford TE, Ravel-Chapuis A, Blais-Crepeau ML, Bélanger G, Jasmin BJ (2013). The RNA-binding Protein Staufen1 Inhibits Myogenic Differentiation via a c-myc Dependent Pathway. *9th International Myotonic Dystrophy Consortium (IDMC-9), San Sebastian, Spain, Oct 16-19*. Poster presentation.

Ravel-Chapuis A, **Crawford TE**, Blais-Crepeau ML, Bélanger G, Richer C, Jasmin BJ (2013). Regulation of Staufen1 during skeletal muscle development, differentiation, and regeneration. *9th International Myotonic Dystrophy Consortium (IDMC-9), San Sebastian, Spain, Oct 16-19*. Poster presentation.

Crawford TE, Ravel-Chapuis A, Blais ML, Belanger G, Jasmin B (2011). Defining Targets controlled by the RNA-binding protein Stau1 during myogenic differentiation and their implications for DM1. *RiboClub 13th Annual Meeting*, Sherbrooke, QC, Canada, Sept 24-26. Ranked Top 10 in Poster competition.

7 – Volunteer and Extra-Curricular Participation

Editorial Team VP Education –U of O Journal of Medicine	2015-2016
Let's Talk Science Team Leader	2013-2016
Science Travels Team Leader	May 2014
Science Travels Volunteer	2013-2014
Lets Talk Science Volunteer	2011-2016
Big Brothers Big Sisters Ottawa In School Mentor	2008-2013
Bio-X Club Senior Advisor	2011-2012
Bio-X Club President	2010-2011
Bio-X Club VP Communications	2009-2010

8 – Work Experience

Ottawa Hospital Research Institute	May 2010-Aug 2010
Summer Research Student	
Regenerative Medicine, Sprott Center for Stem Cell Research	
Ottawa, Ontario	
National Research Council	May 2009-Aug 2009
Summer Student	
Canada Institute for Scientific and Technical Information	
Ottawa, Ontario	
Aim Health Group – Elgin Family Medical Centre	2008-2009
Receptionist	
Ottawa, Ontario	