ANALYSIS AND MODULATION OF PACT, DICER AND MBNL1 IN THE CONTEXT OF MYOTONIC DYSTROPHY TYPE I

by

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ABSTRACT

Myotonic Dystrophy Type I (DM1) is a multi-systemic genetic neuromuscular degenerative disease, has a prevalence in most populations of about 1:8000 and is caused by the nuclear retention of pathogenically expanded *DMPK* mRNA. A previous DM1 RNAi-kinome screen in our lab has identified kinases that reduced both count and area of *DMPK* mRNA foci *in vitro.* One such discovered kinase is PACT, which has showed to decrease foci count and area in DM1 fibroblasts by 30-50%. This study explored PACT as well as binding partner DICER involved in cellular RNA processing machinery, to highlight potential therapeutic targets in DM1. DM1 fibroblasts treated with PACT siRNA showed a non-significant trend of upregulation in MBNL1 mRNA and protein expression. PACT knockdown also showed trend of missplicing normalization in SERCA-1, more prominently seen in DM1-2000 human fibroblasts, whereas IR (insulin receptor) splicing remained unaffected. On the other hand, DICER knockdown did not have profound affect on foci integrity as well as MBNL1 RNA and protein expressions in DM1 fibroblasts. SERCA-1 splicing in DICER siRNA treated samples also remained unchanged. We report here our findings in pursuit of potential therapeutic targets for the treatment of DM1.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
ASO	Antisense oligonucleotide
cDNA	Complementary DNA
CELF1	CUG-BP1 and ETR-3-like factors
Ctnt	Cardiac troponin T
CUG-BP	CUG binding protein
DM1	Myotonic Dystrophy type I
DM2	Myotonic Dystrophy type 2
DMEM	Dulbecco's modified eagle's medium
DMPK	Myotonic Dystrophy Protein Kinase
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FISH	Fluorescence in situ hybridization
GAPDH	Glyceralaldehyde-3-phosphate-dehydrogenase
HPRT1	Hypoxanthine phosphoribosyltransferase I
Hrs	Hours
HSA^{LR}	Human skeletal actin-gene (large repeat)
IR	Insulin receptor
Kb	Kilobase
kDa	Kilodalton
MBNL	Muscle-blind like protein

miRNA	Micro RNA
mRNA	Messenger RNA
NHF	Normal human fibroblast
PACT	Protein activator of PKR
PBS	Phosphate buffered saline
PKC	Protein kinase C
PKR	Interferon-induced double stranded RNA-activated protein kinase
PMSF	Phenylmethylsulfonyl fluoride
PRKRA	Protein kinase, interferon-induced double stranded RNA dependent activator
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
SEM	Standard error of the mean
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
TRBP	TAR-RNA binding protein
UTR	Untranslated region
WT	Wild type
µg	Micrograms
µL	Microliter
µM	Micromolar

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CHAPTER 1

1. INTRODUCTION

1.1 Myotonic Dystrophy Type I

1.1.1 General Introduction and Background

Myotonic Dystrophy Type I (DM1), also known as Steinert's disease, is a multi-systemic neuromuscular disease and is the most common form of adult muscular dystrophy with a global prevalence of 1:8000 (Vignaud et al., 2010). Myotonic dystrophy is one of over 30 neurological disorders known to be caused by tandem DNA repeat expansions above a threshold length (Gatchel & Zoghbi, 2005). In general, through consecutive generations, the repeats exceed a certain size threshold and thus become pathogenic; often displaying inter-individual variable tissue-specific somatic mosaicism in patients (Cho & Tapscott, 2007). The expansion of a DNA repeat sequence has been found to be the basis of an increasing number of human diseases, including fragile x-syndrome, FraX (CGG repeat in 5'-untranslated region), spinal and bulbar muscular atrophy, SBMA (CAG repeat in coding region), and in Huntington disease (CAG repeat in coding region) (Morell, 1993).

DM1 is inherited in an autosomal dominant fashion and is caused by a trinucleotide CTG expansion mapped to chromosome 19q13.3. The amplified trinucleotide CTG repeat is located in the 3'-UTR (3'-untranslated region) of a protein kinase gene named *DMPK* (myotonic dystrophy protein kinase) (Mahadevan et al., 1992). Clinical expression of DM1 is extremely variable, exhibiting a progressive muscular dystrophy that primarily affects distal muscles, and is associated with the inability to relax muscles appropriately (myotonia). Cataracts, cardiac arrhythmia, testicular atrophy and insulin resistance are frequently seen (Harper et al. 2001 and Machuca-Tzili et al. 2005). In unaffected individuals, the DMPK gene contains 5-37 copies of

CTG; mild myotonic symptoms occur in carriers with 50-100 copies of CTG; in patients with severe DM1, the repeat length could reach as much as several kilobases comprising thousands of CTG repeats (J. E. Lee & Cooper, 2009). The onset of DM1 is reciprocally proportional to the length of CTG repeats, with higher repeat length inducing earlier onset, whereas the severity of the disease is directly proportional to the repeat size (greater severity with higher repeat length) (Brook et al., 1992). The propensity of CTG repeats to expand further as they grow longer often leads to a molecular and phenotypic worsening over successive generations, a phenomenon known as anticipation. The transition from asymptomatic carrier with normal genetic fitness to severe congenital DM1 can occur in just a few generations (Harley et al., 1992)

There are currently two known forms of DM: DM1 caused by a CTG repeat expansion in the 3′UTR of the *DMPK* gene, while DM2 results from expanded tetranucleotide repeat CCTG repeat expansion in intron 1 of the zinc finger protein 9 gene (*ZNF9*). Both DM1 and DM2 show childhood and adult onset, but congenital forms only occur for DM1. Although the two genes involved in DM1 and DM2 are functionally different, the syndromes share a similar molecular pathogenic RNA gain-of-function mechanism (Cho & Tapscott, 2007). Indeed, the discovery that these two conditions share clinical phenotypes and expanded RNA in the untranslated region of distinct genes helped establish the case for these expansions as being pathogenic.

1.1.2 DMPK

DMPK is a Ser/Thr kinase homologue and a member of the phylogenetic tree that also includes Rho kinases (ROCKS), cell division control protein 42 (Cdc42)-binding kinases (MRCK) and citron kinases (Riento & Ridley, 2003). Six DMPK isoforms could arise in both humans and mice as a result of alternative splicing. All forms share the N-terminal kinase and

coiled-coil domains, while alternative splicing determines the presence or absence of a 5-amino acid VSGGG motif and the exact nature of the C-terminus (Groenen et al., 2000); the latter determines substrate specificity as well as intracellular localization. DMPK isoforms with hydrophobic C-terminus target to the endoplasmic reticulum (ER), while isoforms with more hydrophilic C-terminus bind to the mitochondrial outer membrane (Wansink et al., 2003). Intriguingly, considering the variety of DMPK isoforms described, Lam et. al using a panel of 16 monoclonal antibodies against several different DMPK epitopes, showed that only a single DMPK protein band of 80 kDa is detected, in skeletal muscle, cardiac muscle, and to a lesser extent, smooth muscle (Lam, Pham, Man, & Morris, 2000). In addition, an autoinhibitory role has been mapped to a domain at the extreme C-terminus of the DMPK; the activity of a C-terminally truncated DMPK is increased by about 3-fold for MBP (Bush, Helmke, Birnbaum, & Perryman, 2000) or 10-fold for MYPT1 (Wansink et al., 2003).

CTG repeat expansions result in a decrease of total *DMPK* mRNA in DM1 patients (Fu et al., 1993). In keeping with mRNA data, DM1 subjects express low levels of DMPK protein in heart and skeletal muscles (Maeda et al., 1995). In skeletal muscle from DM1 patients, *DMPK* mRNA was found to be 50% of that observed in control subjects, but the decrease in protein expression did not correlate with CTG repeat length (Salvatori et al., 2005). In situ hybridization studies have revealed *DMPK* mRNA to be expressed across a range of tissues in adult mice, including skeletal muscle, smooth muscle, cardiac muscle, eye, brain, skin, lung, intestinal epithelium, cartilage and bone. However, *DMPK* is not detected in the ovary, pancreas or kidney (Sarkar, Han, & Reddy, 2004). In the developing mouse embryo, *DMPK* mRNA has been detected in all major muscle groups, including skeletal structures, cardiac muscle and smooth muscle of the lungs (Kaliman et al., 2005).

Although DMPK has been implicated in a variety of functions, including skeletal muscle integrity, cardiac muscle atrioventricular conduction, ion-channel gating and cell metabolism (Kaliman & Llagostera, 2008), the protein's function is not fully understood; indeed the comparatively benign phenotype of knock-out mouse models suggest DMPK to be a non-essential gene (Berul et al., 1999). The ubiquitous expression of DMPK outlined above results in DM1 being a multisystemic disorder (Giagnacovo et al. 2012), however DMPK has shown to mainly be expressed in skeletal and cardiac muscles (Lam et al., 2000). In C2C12 muscle cells, DMPK expression is upregulated via a canonical myogenic pathway (phosphatidylinositol 3-kinase, nuclear factor-B, nitric oxide synthase and p38 mitogen-activated protein kinase) (Carrasco et al. 2002). In addition, data suggests DMPK protein dosage is relevant to skeletal muscle structure and function. DMPK deficient mice develop an adult-onset myopathy phenotype, albeit milder than observed in DM1 patients (Reddy et al., 1996 and Jansen et al., 1996), while DMPK overexpression in C2C12 mice inhibits myoblast differentiation and show skeletal muscle fiber degeneration (Okoli et al. 1998). Taken together, results suggest a role for DMPK in the maintenance of skeletal muscle integrity.

1.1.3 DM1 Molecular Pathogenesis

Several hypotheses have been proposed to explain the molecular pathogenesis of DM1. Initial research targeted the role of DMPK in DM1 pathogenicity. This was supported by studies demonstrating relatively decreased cytoplasmic expression of DMPK protein in DM1 patients (Fu et al., 1993), resulting in DMPK haploinsufficiency. To test the role of DMPK haploinsufficiency in DM1 pathogenesis, *DMPK*-homozygous knockout mice were generated, which displayed comparatively mild adult-onset myopathy (Jansen et al., 1996) and cardiac

conduction defects (Berul et al., 1999); the heterozygous DM1 mice demonstrated no phenotype. In addition, skeletal muscle cells and cardiac myocytes isolated from heterozygous and homozygous DMPK-deficient mice exhibited abnormalities in Na^+ channel-gating and Ca^{2+} cycling similar to DM1 patients, implicating DMPK role in muscle weakness and cardiac dysfunction through its involvement in ion homeostasis (Pall et al. 2003). However more importantly, DMPK-deficient mice did not exhibit myotonia, one of the hallmark characteristic symptoms of DM1. The mild and partial DM1 phenotype observed in the DMPK null and haploinsufficient mice indicated that other models were necessary to fully explain the multisystemic features of this complex disease phenomenon.

Subsequently, interest turned to the CTG repeats themselves and how they might influence expression of adjacent genes. A nuclease resistant region indicating condensed chromatin structure is found downstream of the CTG repeat expansion (Otten & Tapscott, 1995). A homoeodomain-encoding gene named *SIX5* is located in this region and its mRNA level is indeed decreased in DM1 patients (Thornton et al. 1997). However, *SIX5*-knockout mice develop only cataracts and no muscle pathology was observed (Klesert et al., 2000). Therefore, decreased *SIX5* expression is also not the primary cause of DM1.

Eventually, several observations lead to the proposal of the most widely accepted disease model, the toxic RNA gain-of-function hypothesis, which states that the mutant RNA transcribed from the expanded allele is both necessary and sufficient to induce symptoms of the disease. According to Lee et al. (Lee & Cooper, 2009), toxic RNA gain of function hypothesis was based on observations that: i) DMPK loss of function, or decreased expression of surrounding nearby genes did not reproduce major features of DM1 and only contributed mildly to disease phenotype (Jansen et al., 1996), ii) Accumulated discrete nuclear aggregates visible in the nucleoplasm of

DM1 cells, as a result of long CTG repeats transcribing into CUG repeats, suggest a cytoplasm transport failure of some nature (Davis et al. 1997), and iii) Expression of only 3'-*DMPK* UTR with 200 CTG repeats is sufficient to inhibit myogenesis and reciprocate myotonia (Amack, Paguio, & Mahadevan, 1999). Experimental support for the RNA toxic gain-of-function hypothesis was brought forth by the HSA^{LR} mouse model, expressing 250 CTG repeats in the 3'-UTR of the human skeletal α-actin gene. The mice developed myotonia and exhibited muscle histological features similar to that of DM1, such as increased central nuclei and ring fibers (Mankodi et al., 2000). Furthermore, severe muscle wasting was described in an inducible EpA960/HSA-Cre-ER transgenic mice expressing 960 interrupted CTG repeats within the context of the *DMPK* exon 15 (Orengo et al., 2008) and progressive muscle atrophy was observed in mice expressing human *DMPK* mRNA with 550 CUG repeats (Vignaud et al., 2010). Taken together, these studies combined with the clinical features shared between DM1 and DM2, provided strong experimental support for a key role of expanded RNA repeats in DM1 pathogenesis.

1.1.4 Toxic RNA Gain-of-Function

In healthy individuals, *DMPK* is transcribed, processed and transported to the cytoplasm where mRNA transcripts are translated into functional DMPK protein. In contrast, mutant DM1 *DMPK* transcripts form stable clusters that are tightly associated with the nuclear matrix. The retention of mutant transcript is not due to disruption of mRNA processing, as *DMPK* mRNAs are spliced and polyadenylated (Davis et al., 1997), but instead the nuclear retention of expanded CUG repeats. DM1 is thus characterized by the retention of expanded CUG-repeat RNAs in the nucleus – forming discrete aggregates (foci), detectable by fluorescence in situ hybridization

(FISH) (Taneja et al., 1995). These foci have not been detected in control fibroblasts or muscle biopsies.

Within the nuclei, the mutant *DMPK* transcript foci are localized to the periphery of structures enriched in splicing snRNPs and spliceosome assembly factors, known as nuclear speckles; this juxtaposition suggests an early block in nucleoplasmic export (Holt et al., 2007). Within the nucleoplasm, expanded CUG repeats form stable hairpin loop structures, defined by Watson-Crick G-C base-pairing, separated by a periodic U-U mismatch (Michalowski et al., 1999). The hairpin loop structures are believed to be central to the foci structures as well as having a central role in a gain-of-function pathogenicity. This model was further supported by experiments which showed the nuclear export of artificial CUG repeat RNAs by inclusion of Woodchuck post-transcriptional regulatory element reduces DM1 defects (Mastroyiannopoulos et al., 2005), and that production of foci in the cytoplasm does not induce DM1 phenotype in mice (Dansithong et al., 2008).

The pathogenic gain-of-function conferred by the CUG-repeat in *DMPK* mRNA in DM1 is believed to be the misregulation of alternatively spliced pre-mRNAs. This missplicing is different from aberrant splicing brought about by mutations in regulatory splicing sites that lead to expression of aberrant RNA underlying other disorders (Klein, Gasnier, & Furling, 2011). In contrast, DM1 missplicing events results from inappropriate regulation of splicing factors primarily MBNL1 and CELF1. Both factors are developmental regulators of splicing, specifically during fetal to adult transition, and alterations to their activity leads to fetal expression patterns in adult tissues, which result in the characteristic DM1 phenotype (Kalsotra et al., 2008).

1.1.5 MBNL1 Loss-of-Function and CUG-BP1 Gain-of-Function

CUG-binding protein (CUG-BP1) is an RNA-binding protein, which belongs to the CELF1 (CUG-BP1 and ETR-3-like factors) "family of 6" highly homologous proteins (Barreau et al., 2006). Although CUG-BP1 binds to single-strand CUG repeats, it does not co-localize or sequester with the nuclear foci in DM1 cells (Timchenko et al., 2001). The levels of CELF 1 have shown to be increased in DM1 tissues leading to an upregulation of CELF1 activity. This increase is attributed to the inappropriate activation of Protein Kinase C (PKC) which results in hyper-phosphorylation and stabilization of the CELF1 proteins (Wang et al., 2009 and Kuyumcu-Martinez, Wang, & Cooper, 2007). Transgenic mice overexpressing CELF1 have shown to reproduce DM1-like missplicing and muscle features. Furthermore, elevated CELF1 activity is found in DM1 mouse models expressing 960 CTG repeats, which recapitulates muscle wasting as well as splicing defects (Ward et al., 2010)

Muscleblind-like proteins (MBNL) are the other splicing factors also affected in DM1. Unlike CELF1 however, MBNL proteins bind directly to the double-stranded CUG RNA hairpins in a length-dependent manner, and form ribonucleoprotein complexes (foci) (Miller et al., 2000). In DM1, all three isoforms of MBNL proteins (MBNL1, MBNL2, MBNL3) are recruited to the mutant RNA foci, diverting them from their normal cellular functions. MBNL proteins all contain 4 CCCH zinc-finger protein domains structured in pairs that act as RNA-binding domains (Pascual et al., 2006). MBNL1 is the main paralog that functions in primary roles in most tissues, except the brain where MBNL2 is predominantly expressed (Konieczny, Stepniak-Konieczna, & Sobczak, 2014). Expression of MBNL3 is more restricted, with minor reported functions in muscle cell differentiation and regeneration (K.-S. Lee et al., 2010 and Poulos et al., 2013). The sequestration of MBNL1 into nuclear foci and depletion of its activity

within the nucleoplasm has become the prime candidate role for the pathogenic effects of mutant RNA in DM1 pathology. This is substantiated by the fact that MBNL1-deficient mice developed myotonia, cataracts and splicing defects (Kanadia et al., 2003). Moreover, the majority of missplicing events observed in HSA^{LR} mice can be attributed to loss-function of MBNL1 splicing factor. In addition, missplicing events in DM1 can be reversed by MBNL1 overexpression in skeletal muscles (Kanadia et al., 2006). Recently, there have been reports exploring the aggregation of CUG repeat RNAs into foci, and their relation to MBNL1. Interestingly, depletion of MBNL1 protein by RNAi has shown to decrease the aggregation of CUG repeat RNAs suggesting the protein may be structurally important (Dansithong et al., 2005). Foci formation tends to be very fluid, characterized by constantly forming and disassociating structures, with MBNL1 proposed to have direct role involved in the aggregation process (Querido et al., 2011). This is significant from a therapeutic point of view, as small molecules could theoretically be utilized to disrupt the interaction between MBNL1 and CUG repeat RNAs and increase active MBNL1 protein concentrations within the nucleoplasm. This was supported by experiments from the lab of Charles Thornton (Rochester), which showed that by using an antisense oligonucleotide (ASO) – CAG25 – which binds CUG repeat RNA and blocks interaction with MBNL1, is sufficient to reverse DM1 pathological phenotype (Wheeler et al., 2009). Taken together, these reports support a loss of MBNL1 splicing and gain of CELF1 function leading to a pathogenic spliceopathy resulting in the DM1 phenotype (Figure 1).

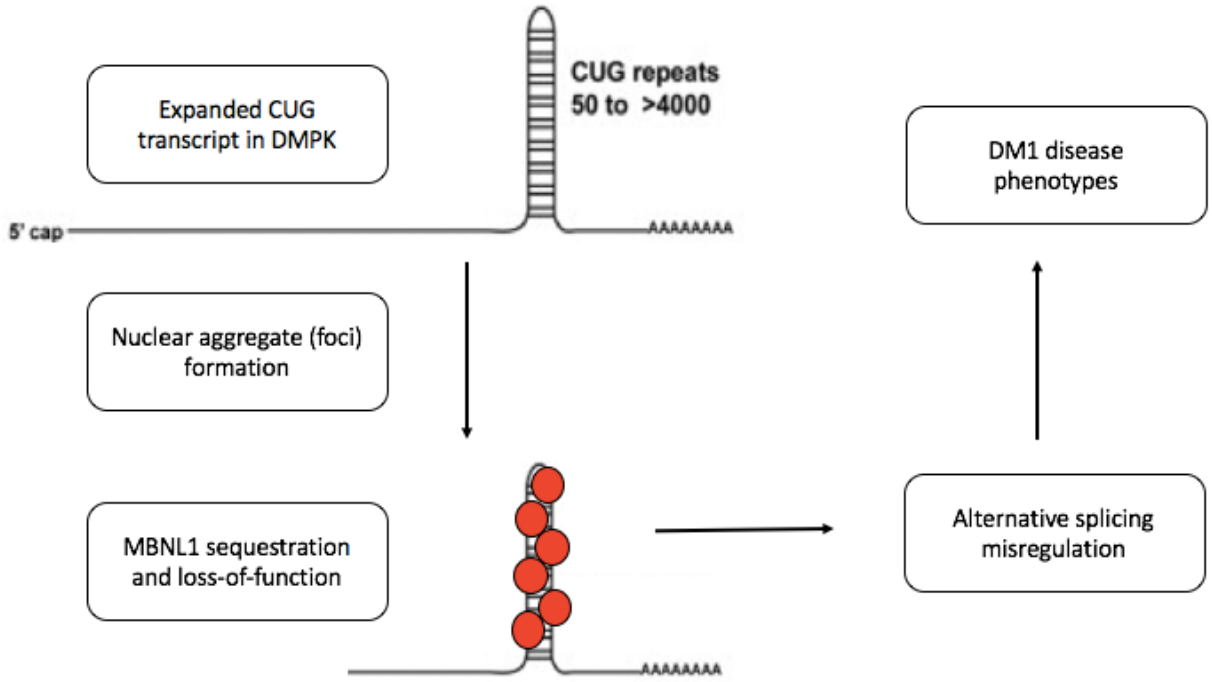


Figure 1 – Simplified schematic diagram for the mechanism of RNA gain-of-function in DM1.
Expansion of CUG repeats in the 3'UTR of DMPK results in the nuclear retention of transcript, which sequesters and alters MBNL1 (circles) activity via nuclear aggregate foci formation. Modulation of these RNA-binding proteins leads to MBNL1 loss-of-function, resulting in misregulation of alternative splicing of specific pre-mRNAs.
Source: Adopted from Klein et al. 2011

1.1.6 DM1 Alternative Splicing

Alternative splicing occurs in about 40-60% of genes in the human genome (Black, 2003). This provides one of the regulatory mechanisms the cells use to control the expression of tissue-specific protein isoforms. RNA-binding proteins such as MBNL1 that bind to specific sequences in pre-mRNAs regulate the inclusion or repression of alternative exons.

The first alternative splicing in DM1, the inclusion of exon 5 in cardiac troponin T (cTNT), was identified in 1998 (Philips, Timchenko, & Cooper, 1998). To date, more than 20 alternatively spliced transcripts have been identified in DM1 due to MBNL1 reduced activity, with more being identified over time (Osborne & Thornton, 2006) (Table 1). Although any of the misspliced genes could theoretically contribute to pathogenicity, only a few have been directly linked to specific symptoms (Klein et al., 2011). For example, missplicing of CLCN1, IR, BIN1 and CACNA1S result in myotonia, insulin resistance, delayed muscle development and muscle weakness respectively, all hallmark findings and symptoms of DM1 (Fugier et al., 2011, Kino et al., 2009, Savkur, Philips, & Cooper, 2001 and Tang et al., 2012). More recently, comparative mRNA expression analysis of HSA^{LR} (250 CTG repeats) mice and MBNL1-deficient mice has revealed 80-90% similarity of alternative splicing events between the two models (H. Du et al., 2010); over 200 splicing events regulated by MBNL1 have been identified in these mice. In addition, Wang et al. used CLIP-Seq analysis on various mouse tissues revealing a total of 900 predicted splicing events and 500 alternative 3'UTRs resulting from MBNL1 deficiency (E. T. Wang et al., 2012).

MBNL1 can interact with a variety of transcripts containing YGCY sequence motifs, but MBNL1 splicing function is contingent on relative exon-binding position on target pre-mRNA. Generally, MBNL1 binding to the alternative exon and upstream intronic regions facilitates exon

skipping, while binding to downstream intronic regions promotes exon inclusion (Witten & Ule, 2011). Furthermore, MBNL1 and CUG-BP1 have opposing functions, with MBNL1 promoting a switch to adult isoform RNAs, while CUG-BP1 promotes retention of embryonic isoforms (Ho et al., 2004). With that being said, MBNL and CUG-BP1 do not directly compete with one another, as their expression levels are independently regulated, and they engage distinct RNA-binding sites on their target pre-mRNAs (Dansithong et al., 2005). For the purpose of this study, the main focus will be on MBNL1 and how MBNL1 modulation could be utilized to reverse the alternative splicing events in DM1. In particular, two genes known to be alternatively spliced in DM1 will be examined in detail. Normalized relative expressions of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA-1) SERCA-1a to total SERCA-1 and insulin receptor (IR) IR-B to total IR will be used to evaluate the effect of treatment conditions on DM1 alternative splicing.

Table 1 – Alternatively spliced pre-mRNAs in DM1.
Source: (Furling, 2012)

Tisues	Pre-mRNA	exon/intron deregulation	Inclusion/exclusion	Ref.
Skeletal Muscle	Insulin receptor (INSR)	Exon 11	Exclusion	Savkur & al., 2001
	Chloride channel (CLCN1)	Intron 2	Inclusion	Charlet & al., 2002 Mankodi & al., 2002
		Exon 7A	Inclusion	Lueck & al., 2006
	BIN1 (Amphyphisine 2)	Exon 11	Exclusion	Fugier & al., 2011
	Calcium channel (Ca (V)1.1)	Exon 29	Exclusion	Tang & al., 2012
	Skeletal Troponin T (TNNT3)	Exon fœtal	Inclusion	Kanadia & al., 2003
	Ryanodine recptor (RyR1)	Exon 70	Exclusion	Kimura & al., 2005
	Sarcoplasmic/endoplasmic reticulum Ca2 + ATPase 1 (SERCA1)	Exon 22	Exclusion	
	Sarcoplasmic/endoplasmic réticulum Ca2 + ATPase 2 (SERCA2)	Intron 19	Inclusion	
	LIM domain inding 3 (LB, ZASP)	11	Inclusion	Lin & al., 2006
	Titin (TTN)	Zr4	Inclusion	
		Zr5	Inclusion	
	Nebulin-related anchoring protein (NRAP)	12	Inclusion	
	Calpaïn 3 (CAPN3)	16	Exclusion	
	Attractin-like (ATRNL1, ALP)	5a et 5b	Inclusion	
	Forming homology 2 domain containing 1 (FHOD1)	11a	Exclusion	
	Glutamine-fructose-6-phosphate transaminase 1 (GFPT1)	10	Exclusion	
	MBNL1	7	Inclusion	
	MBNL2	7	Inclusion	
	SET and MYND domain containing 1 (SMYD1)	39	Inclusion	Du & al., 2010
	Sperm associated antigen 9	39	Inclusion	
	Myotubularin-related protein 1 (MTMR1)	Exon 2.1	Exclusion	Buj-Bello & al., 2002 Ho & al., 2005
		Exon 2.3	Exclusion	
	Alpha-dystrobrevin (DTNA)	Exon 11a	Inclusion	Nakamori & al., 2008
		Exon 12	Inclusion	
Brain	Tau (MAPT)	Exon 2	Exclusion	Sergean & al., 2001
		Exon 3	Exclusion	
		Exon 6	Exclusion exon 6c Inclusion exon 6d	Leroy & al., 2006
		Exon 10	Exclusion	Sergeant & al., 2001 Jiang & al., 2004
	Récepteur-N-methyl-D-aspartate (NMDAR1)	Exon 5	Inclusion	Jiang & al., 2004
	Amyloid precursor protein (APP)	Exon 7	Exclusion	
	MBNL1	Exon 5	Inclusion	Dhaenens & al., 2010

1.1.6.1 SERCA-1

SERCAs are transmembrane Ca^{2+} pumps which replenish the sarcoplasmic or endoplasmic reticula with Ca^{2+} via ATP hydrolysis (Ernő Zádor & Kósa, 2015). As a result, this lowers cytoplasmic calcium levels leading to relaxation of skeletal, heart and smooth muscle. During muscle contraction/relaxation cycles, Ca^{2+} is released into the cytoplasm from the sarcoplasmic reticulum, inducing muscle contractions. This is followed by Ca^{2+} subsequently being pumped back into the sarcoplasmic reticulum by SERCA to allow for relaxation of muscles (Kimura et al., 2005). The SERCA protein family is comprised of SERCA1, SERCA2 and SERCA3 encoded by atp2a1, atp2a2 and atp2a3 genes respectively (Vangheluwe et al., 2009). Alternative splicing of SERCA transcripts leads to protein isoforms that may have different ATPase activity, Ca^{2+} affinity or tissue specificity. The SERCA-1 gene has two specific isoforms exclusively expressed in muscles. SERCA-1a, which is comprised of all exons, is expressed in adult fast type fibers (Talmadge et al., 1996 and Zádor et al., 1998), while SERCA-1b, formed by the excision of exon 22, is expressed in neonatal fibers (Brandl et al., 1986), myoblasts and developing myotubes (Ernő Zádor, Vangheluwe, & Wuytack, 2007). Kimura et al. described the shifting from the adult-form SERCA-1a isoform to neonatal isoform SERCA-1b in DM1. As a result, the finely regulated muscle contraction/relaxation cycle is disrupted, which is believed to underlie both myotonia and muscle wasting in DM1 (Kimura et al., 2005)

1.1.6.2 Insulin Receptor (IR)

Insulin receptor (IR) is a $(\alpha\beta)_2$-type receptor tyrosine kinase that plays a pivotal role in insulin-mediated regulation of cellular metabolism and growth (Tatulian, 2015). The heterodimer $\alpha\beta$ consists of an α-subunit which mediates extracellular hormone-binding and a transmembrane

β-subunit which acts as an intracellular tyrosine kinase domain. The subunits are linked by a disulfide bond and the two protomers which are connected via 2-4 disulfide bonds between the two α-subunits. Insulin binds to the alpha-subunit, which results in the autophosphorylation of the beta-subunit (Savkur et al., 2001).

Alternative splicing of exon 11 of *IR* results in IR-A (lacking exon 11) and IR-B (including exon 11) (Moller et al., 1989 and Seino et al., 1989). IR-B is primarily expressed in insulin-responsive tissues responsible for glucose homeostasis such as adipose tissue, liver and skeletal muscle. In DM1 patients, there is inappropriate expression of the IR-A isoform in skeletal muscle as a result of alternative splicing (Savkur et al., 2001), and this loss of muscle-specific splicing of IR in DM1 patients correlates with reduced insulin responsiveness, leading to overall lower signaling capacity as well as lower tyrosine kinase activity. This is supported by studies that show a switch from IR-A to IR-B induced by dexamethasone correlated with increased insulin sensitivity (Kosaki & Webster, 1993). In addition, studies have shown that glucose disposal in DM1 patients is reduced by 15-25% following insulin infusion as well as a 70% reduction in insulin sensitivity is observed in skeletal muscles (Moxley et al., 1984). Taken together, although the mechanism of insulin resistance in DM1 is unknown and DM1 patients do not have clinical symptoms of type 2 diabetes, missplicing of IR in specific tissues and resulting altered receptor function provides an explanation for the insulin resistance phenotype observed in DM1 patients.

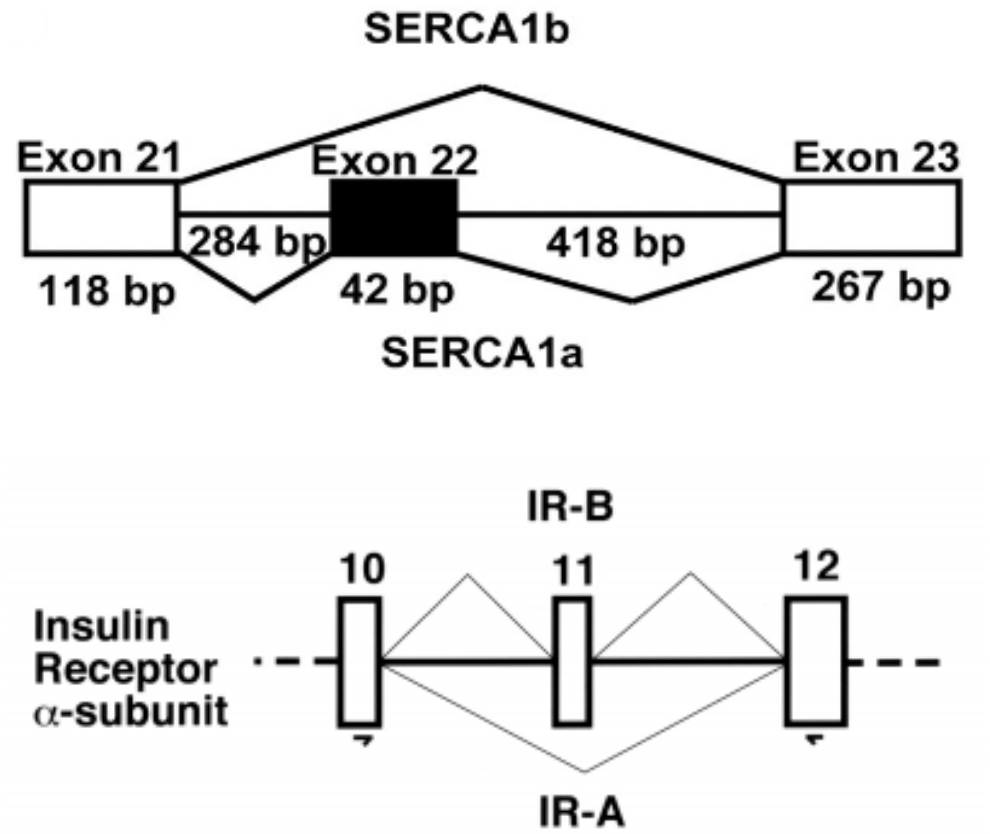


Figure 2 – Schematic diagram of SERCA-1 and IR alternative splicing in DM1
Top) Exclusion of exon 22 by alternative splicing of SERCA-1 yields SERCA-1b (- exon 22) isoform, which is detected at a higher concentration relative to wild type SERCA-1a (+ exon 22) isoform in DM1 patients. Source: Hino et al. 2007. Bottom) Exclusion of exon 11 by alternative splicing of IR yields IR-A (- exon 11) isoform, whereas normal splicing includes exon 11 to yield IR-B (+ exon 11). The former isoform is upregulated in DM1. Source: Savkur et al. 2001

1.2 RISC

RNA-induced silencing complex (RISC) describes the group of molecular complexes that are used to target genes for silencing. Generally, this silencing is initiated by the presence of dsRNA in the cytoplasm which are processed into smaller RNAs of 20-30 nucleotides that assemble themselves into RISC, guiding the complex towards target RNAs via complementary base-pair interactions (Pratt & MacRae, 2009). RISC silencing of targeted genes can take place via three mechanisms: i) Inhibition of protein synthesis by repression of translation of target RNA, ii) Inhibition of target RNA by degradation of corresponding mRNA and iii) Inhibition of transcription of target RNA by formation of heterochromatin or elimination of DNA (Pratt & MacRae, 2009). The small regulatory RNAs that are incorporated into RISC and guide the complex have been termed various names, mainly based on their biosynthetic pathways, including: siRNA, miRNA, piRNA, rasiRNA, tasiRNA, tncRNA, hcRNA, and scnRNA (Tolia & Joshua-Tor, 2007). However, once bound in RISC, they all function the same way to guide RISC and silence target genes. At its core, every RISC contains a member of the Argonaute (AGO) family of proteins that bind to small RNAs. In addition, in every RISC, the small-bound RNA acts as a guide for the silencing of target RNA. Argonaute functions to bind the small RNA sequences within RISC and position in order to facilitate target recognition.

1.2.1 miRNA Processing

One of the most predominant modes of gene silencing by RISC in mammalian systems is the repression of targeted mRNAs guided by miRNAs. miRNAs are a class of regulatory RNAs found in plants and animals, with a length of a ~22 nucleotides (Kim, Han, & Siomi, 2009). It is currently believed that more than 2000 miRNA species are encoded in the human genome

globally impacting gene expression (Agarwal et al., 2015). miRNA genes are initially transcribed by RNA polymerase II (RNA Pol II) as primary miRNAs (pri-miRNAs), containing short double-stranded hairpin loop structures (Y. Lee et al., 2002). Next, the hairpins in pri-miRNAs are recognized in the nucleus by a complex called Microprocessor (consisting of an RNase III enzyme Drosha, and a double-stranded binding protein DGCR8). Microprocessor cleaves off the hairpin at their stem regions to generate ~25 nucleotide precursor miRNAs (pre-miRNAs) (Y. Lee et al., 2003). Pre-miRNAs are then exported into the cytoplasm by a nuclear export machinery consisting of Exportin-5 and Ran small GTPase (Yi et al., 2003). Lastly, the stem regions of pre-miRNAs are cleaved at their terminal loop by RNase III enzyme DICER in cooperation with TRBP (Y. Lee et al., 2002 and Chendrimada et al., 2005). The resultant ~22 nucleotide dsRNA, called miRNA are assembled with AGO proteins. Another protein PACT is also integral to the function of RISC and critical for efficient miRNA processing, as it's depletion results in decreased mature miRNA levels and target gene silencing (Y. Lee et al., 2006). To assemble RISC, the loaded ds-miRNA species are cleaved to yield a guide strand and a passenger strand. Guide strands are retained within and function in RISC for recognition of target RNAs, while passenger strands are ejected from AGO proteins (Maniataki & Mourelatos, 2005). Computational estimates suggest each miRNA can target anywhere between 100-200 transcripts, usually within their 3'UTRs (Lewis, Burge, & Bartel, 2005). For the purpose of this study, proteins involved in RISC assembly and function, specifically DICER and PACT have been subjected to modulation, in order to analyze and assess the role of these proteins in the context of DM1.

1.2.2 DICER Role in RISC

DICER is a ~220 kDa endonuclease, which carries two RNase III domains forming an intramolecular dimer; it frequently acts in RNAi pathways to produce small RNAs. DICER is encoded by a single gene and is responsible for cleaving substrates into small RNAs, which can act in both miRNA and RNAi pathways (Svobodova, Kubikova, & Svoboda, 2016). In general, during RNAi, DICER cleaves long dsRNAs into short segments of ~20-23 nucleotide RNAs and then mediates their loading onto an AGO protein. In this study, DICER modulation was assessed within the context of DM1 and whether fundamental disruption of small RNA fragment production could modulate DM1 disease phenotypes.

The full DICER crystal structure has not yet been solved; current structural information is based on biochemical studies of recombinant DICER and individual domains (Ma et al., 2008), studies of mammalian DICER fragments (Z. Du et al., 2008) and cryoelectron microscopy (cryo-EM) studies of DICER complexed with other proteins (Lau et al., 2009). Cryo-EM studies in particular have helped shed light on the structure of DICER. The overall shape of DICER resembles an L, with the shape divided into a head, a body and a base. The head of the protein contains the PAZ domain, which is an RNA binding module that recognizes the 3'end of short RNA segments. The body contains the RNase III domains and the helicase domain constitutes the base (Lau et al., 2009).

The principal role of DICER in mammals is miRNA biogenesis. Pre-miRNAs are cleaved within the RNase III domain and are released as a single small RNA for AGO loading. The PAZ domain of DICER accesses the 3' overhang of pre-miRNAs generated by the nuclear microprocessor complex (Feng et al., 2012). This is important because not only does the recognition of the overhang lead to higher substrate processing, but also is critical for the

accuracy of miRNA biogenesis, as a single nucleotide shift at the 5' end of miRNAs redefines their target sites (Park et al., 2011). The other structural domain of DICER is the N-terminus helicase, contiguous to the RNase III domains and positioned to bind the stem loops of pre-miRNAs. However, the helicase domain appears to have limited miRNA processing activity and instead enables substrate selectivity, favoring pre-miRNAs as substrates (Ma et al., 2008).

1.2.3 PACT Role in RISC

PACT (Protein kinase RNA (PKR) activator) was initially known as a protein activator of PKR through its interaction with PKR via it's dsRBD1 and dsRBD2 domains (Peters et al., 2001). PKR is a dsRNA-dependent serine/threonine protein kinase, which causes general reduction of translation by phosphorylation of eIF2a and Ser51 (Taylor, Haste, & Ghosh, 2005). In 2006, Lee et al. expanded the role of PACT as another dsRNA binding protein that functions as a component of the RISC (Y. Lee et al., 2006). This was significant because PACT had been shown to be structurally similar to TRBP which also interacts with PKR in a similar fashion to PACT, albeit as an inhibitory role (Gupta, Huang, & Patel, 2003); PACT dsRNA binding domain role was further investigated.

Depletion of PACT by RNAi results in a reduction of mature miRNAs (Y. Lee et al., 2006). This reduction does not affect pre-miRNAs, indicating that role of PACT in RISC does not take place at the pre-miRNA cleavage step. Due to the similarities between PACT and TRBP, Lee et al. investigated the possibility that the roles of dsRBD-containing DICER cofactors in RISC may be partially redundant, as RNAi experiments indicated that both PACT and TRBP could be involved in miRNA generation and siRNA-induced RNAi. This idea is further supported by experiments that revealed both TRBP and PACT share the common DICER

binding regions (246-584 amino acids) as well as containing the dsRBD3 that is required for binding to DICER. In addition, gel filtration and co-immunoprecipitation experiments in HEK293T cells revealed that AGO, DICER, TRBP, and PACT interact with one another to form a RISC of ~500 kDa (Y. Lee et al., 2006). Interestingly, a competition between the RNAi pathway and the PKR pathway may exist, because the dsRBD3 domain of both PACT and TRBP interacts with DICER as well as regulates PKR. This leads to the possibility of a crosstalk between the RNA silencing and PKR pathways. For the purpose of this study, the PACT role in DM1 phenotype modulation was explored from the perspective of PACT human RISC modulatory function.

1.3 DM1 Therapeutics

Currently there is no effective therapy for DM1. Most work to date involved in patient therapies have mainly focused on symptom management and supportive care (Konieczny et al., 2014). The most common causes of death in DM1 patients remains respiratory failure, cardiovascular disease, and sudden death due to arrhythmia or neoplasms (de Die-Smulders et al., 1998). In addition, roughly 50% of adult onset DM1 patients were either partially or completely wheelchair bound at end of life, reflecting a significant unmet medical needs for DM1 patients.

1.3.1 Symptom Management

Active DM1 patient management involves monitoring for the complications of the disease. The following description highlights some of the efforts placed in treating the various symptoms arising in DM1 such as muscular issues, insulin resistance, cardiac and respiratory difficulties.

Strength training and exercise programs may increase muscle and cardiorespiratory function and prevent additional atrophy in DM1 patients (Voet et al., 2010). However, this needs to be conducted judiciously, as over exertion may cause more rapid disease progression. A study of strength training effect on 36 DM1 patients showed no significant difference between training and non-training groups. Having said that, given that there does not seem to be any harm associated with moderate intensity strength training, DM1 patients should be encouraged to partake in such activities.

Muscle weakness in DM1 may also be associated with a low circulating levels of DHEA (dehydroepiandrosterone). A pilot study initially suggested improvements to myotonia and

muscle weakness by oral prescription of DHEA in DM1 patients, by examining the the change in the manual muscle testing scores. However, within the 12 week course treatment, no evidence suggested that the use of DHEA improved muscle power (Pénisson-Besnier et al., 2008). In addition, certain drugs such as sodium channel blockers (mexiletine, phenytoin and procainamide), tricyclic antidepressive drugs, benzodiazepines, calcium antagonists, taurine and prednisolone have all undergone clinical trials for the treatment of myotonia with little success (Trip et al., 2006)

iPLEX (a combination of recombinant IGF1 and binding partner BP3) is another drug approved by FDA in 2005 for the treatment of children with growth failure due to IGF1 deficiency. In early studies, there was some hint that iPLEX may improve insulin insensitivity and function in DM1 myoblasts (Furling et al., 1999 and Winn et al., 2002). An early phase 2 safety study of daily iPLEX use has suggested promising results with an increase in muscle mass as well as improvements in other systems. The trial is currently in the process of completion (Turner & Hilton-Jones, 2010).

Cardiac arrhythmias may be responsible for ~30% of all fatalities in DM1 (Mathieu, Allard, Potvin, Prévost, & Bégin, 1999). Not all cardiac arrests can be prevented by pacemaker insertions, suggesting that many DM1 patients may die from malignant tachyarrhythmia. The need for permanent pacemakers is currently under investigation to evaluate arrhythmic risk in 540 DM1 patients which will shed some light on the uncertainties surrounding risk minimization of cardiac diseases in DM1 (Turner & Hilton-Jones, 2010).

Lastly, sleep disorders caused by central or obstructive sleep apnea may contribute to patient death. Patients with excessive fatigue/sleepiness require overnight sleep studies as a minimum investigative standard (Turner & Hilton-Jones, 2010). For patients that have breathing

difficulties, non-invasive ventilation is offered. If sleep disorder treatment of is unsuccessful in addressing breathing difficulties, then central nervous system stimulants such as modafinil, dexamphetamine and methylphenidate can be administered. So far, analyses of psychostimulant treatment of DM1 have been inconclusive and further trials are needed.

1.3.2 Current Therapeutic Approaches

As noted, DM1 symptom management remains the most effective way of combating the symptoms associated with this disease. As a result, there is increasing interest in tackling DM1 at its root cause, which is the presence of pathogenic RNA transcripts. Recent advances in understanding the molecular mechanisms of pathology in DM1 has given way to more specific and effective treatment strategies. The reduction of *DMPK* mRNA transcripts in DM1 cells as well as inhibition of the CUG-repeat MBNL1 interaction are currently two of the most promising therapeutic mechanisms in development. Both approaches aim to boost the nucleoplasmic pool of available MBNL for interaction with pre-mRNA targets.

Small molecules directed at CUG repeats either dislodging MBNL1 or blocking it's sequestration to the repeats represents a credible therapeutic strategy; a number have been identified by Gareiss et al. utilizing a resin-bound dynamic combinatorial chemistry screen (Gareiss et al., 2008). Further refinement of these compounds verified their ability to restore MBNL1-dependent alternative splicing in HSA^{LR} mice (Ofori et al., 2012). Similarly, an additional screen conducted with small compounds known to target CUG repeats specifically highlighted the small molecule pentamidine (Warf et al., 2009). Initial work suggested that pentamidine frees MBNL1 from CUG repeats by binding to the transcripts, however follow-up studies have disputed this by showing that CUG transcripts expression is significantly reduced in

pentamidine treatment conditions. This suggests that the mode of action is not by directly blocking MBNL1-CUG repeat interactions but rather inhibiting expression and/or increasing breakdown of mutant *DMPK* mRNA (Coonrod et al., 2013). Another example of utilizing small molecules in DM1 therapy is D-amino acid hexapeptide (ABP1), which not only reduced foci formation and muscle degeneration in DM1 flies, but also reversed muscle histopathology and spliceopathy in a DM1 mouse model (García-López et al., 2011). In vitro analysis indicated that ABP1 did not prevent CUG repeat MBNL1 interactions directly, but instead induced conformational changes in CUG RNA secondary structures which prevented MBNL1 sequestration. In addition to the above mentioned studies, there are numerous other works carried out over the past decade exploring the use of small compounds in the treatment of DM1.

By employing alternative therapeutic strategies, Langlois et al. were among the first groups to demonstrate that hammerhead ribozyme mediated the destruction of toxic RNA inclusions in DM1 myoblasts leading to a reduction in mutant DMPK transcripts, as well as rescue of IR splicing (Langlois et al. 2003). However, more recent efforts have targeted CUG repeat expansions using small antisense oligonucleotides (ASO). These sequences target CUG repeat RNAs and inhibit toxicity in DM1 mouse models (Mulders et al., 2009 and Wheeler et al., 2009). A specific type of ASOs called CAG25 morpholinos in DM1 mice resulted in the release of MBNL1 from foci and the subsequent transport of CUG repeat RNAs into the cytoplasm where they were degraded. These strategies suggested sufficient degradation of foci can normalize alternatively spliced genes and thus alleviate DM1 clinical severity. The mechanism of action of ASOs are not fully understood, although they are believed to either interrupt MBNL1 sequestration to CUG repeats or prevent the formation of foci by targeting CUG repeats for degradation or possibly both (Klein et al., 2011). This approach has also paved the way for

cellular therapy models of HD and SCA3, which indicates that although not fully mechanistically understood, the targeting of expanded repeats is a promising therapeutic approach (Hu et al., 2009 and Gagnon et al., 2010)

Our lab is focused in uncovering therapeutic targets in DM1 that are druggable by small compounds, particularly those drugs that are FDA approved. Our approach parallels that of small molecule therapies where a small molecule could be utilized to modulate the expression of our gene of interest. The purpose of this study is to evaluate the proteins PACT and DICER in their ability to target and reverse key DM1 disease markers, and further screen for drugs that could modulate these proteins.

1.4 Previous Work in Lab

It is clear that CUG-repeat aggregates within the nucleoplasm of DM1 cells is likely the pathogenic driver for DM1 and thus the disruption of the foci may have significant clinical importance. The aim of our research project was to identify factors that modulate foci aggregation thereby identifying potential therapeutic targets in DM1. To embark on this project, a previous high-throughput kinome RNAi screen was developed and performed in our lab in DM1 patient fibroblasts. Since kinases represent crucial signaling checkpoints in various molecular cascades, and also are highly targetable with small compounds/drugs, they represent an appropriate set of targets for drug discovery inquires in DM1. Reduction in foci size and area were determined as final readouts in this screen which lead to the identification of PACT as a novel genetic modifier of nuclear foci in DM1. Considering the role of PACT in PKR activation and implication of PKR in apoptosis, PACT represented an ideal target for further evaluation.

1.5 Project Rationale

A secondary reexamination of PACT knockdown validated the previously observed impact on foci integrity. However, inhibition of PKR via small molecules did not modulate foci as expected. Interestingly, and rather unexpectedly, initial reports also suggested an increase in MBNL1 protein expression in PACT knockdown treatment conditions (one might expect release of MBNL1 with foci reduction but not necessarily a change in total cellular MBNL1). As a result, PACT and it's binding partner DICER in RISC were subjected to knockdown experiments in this study to evaluate their effect on MBNL1 mRNA and protein expression as well as an alternative splicing modulation in DM1 fibroblasts.

1.5.1 Hypothesis and Objectives

We hypothesize that PACT kinase and the associated DICER will have an impact on foci formation, MBNL1 expression and gene missplicing. More specifically, PACT identification will allow us to investigate additional pathways that could be involved in the modulation of DM1 disease phenotypes, with the possibility of identifying genes that could be targeted by drugs to be used for DM1 therapy.

To carry out this project, two main objectives have been explored:

i) Analysis of PACT knockdown in NHF and DM1 fibroblasts and assessment of the effect of PACT modulation on MBNL1 mRNA and protein expression, as well as splicing regulation in SERCA-1 and IR.

ii) Analysis of PACT binding partner in RISC – DICER – knockdown in NHF and DM1 fibroblasts, and assessment of DICER modulation on MBNL1 mRNA and protein expression, as well as splicing regulation in SERCA-1.

In addition to the two main objectives listed above, we also ventured into possible reasons behind the unexpected modulation of MBNL1 expression in our PACT knockdown conditions by modifying and analyzing the current protocols in place for protein extraction in DM1 fibroblasts.

CHAPTER 2

2. MATERIALS & METHODS

2.1 Cell Culture

All cell lines were grown in either 10/15 cm plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% (v/v) glutamine, 10% (v/v) fetal calf serum (FCS) (Sigma) and 1% penicillin/streptomycin (v/v) (HyClone). Cells were grown at 37°C in a 5% CO_2 incubator. Culture media was changed every 2-3 days and cells were passaged 1:2 or 1:3 every 3-4 days depending on how well they grew. Cell lines used in this study were all human fibroblasts. Normal healthy control fibroblasts were attained on site and patient (DM1) fibroblasts were attained from Coriell cell repository. The following are the cell lines used in this study:

NHF 1

NHF 2

NHF 3

NHF 4

NHF 5 – (Grandfather of patient DM1-1600 HF) – GM04603

DM1-66 HF (Father of patient DM1-1600 HF) – GM06076

DM1-500 HF – GM03987

DM1-1000 HF – GM04033

DM1-1600 HF – GM04602

DM1-2000 HF – GM03132

DM1-2000 (2) HF – GM03759

DM1-2000 (3) HF – GM03989

2.2 siRNA Transfection

Cells were transfected with siRNA using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific). Initially, siRNAs and RNAiMAX reagent were diluted separately to the desired concentrations. siRNAs were diluted to a final concentration of 20nM in Opti-MEM (serum-free media), and Lipofectamine RNAiMAX was diluted at a concentration of 4μL per well in a 6-well plate. The two preparations of siRNA and RNAiMAX were then amalgamated and incubated at room temperature for a minimum of 20 minutes (As recommended by manufacturer's guidelines) to allow for the siRNA molecules to be integrated into lipid micelles. In the meantime, cells were washed with 1x PBS (phosphor-buffered saline) twice and trypsinized to dislodge cells from the surface of plate. Harvested cells were counted using a haemocytometer. Cells were seeded in 6-well plates at 80,000-100,000 cells per well in 1.6mL media. 400μL of siRNA-Lipofectamin reagent mix was subsequently added to each well for a total volume of 2mL. Plates were incubated at 37°C and 5% CO2 for 72hrs. Falcon 6-well plates were used for tissue culture. Transfection efficiency was assessed by All-STAR Death Control siRNA (Invitrogen), which targets essential ubiquitin genes resulting in apoptosis and visible cell death, thus providing readout of transfection efficiency. PACT, DICER and MBNL1 were subjected to siRNA transfection in this study.

2.3 Protein Extraction

2.3.1 Protein Extraction for PACT and DICER Modulation

Cells in a 6-well plate were washed by 1x PBS and treated with trypsin to dislodge the cells and harvest them. Cells were then pelleted by centrifugation at 300g for 5 minutes, and re-washed again with 1x PBS. Cell pellets were treated with 120μL of RIPA lysis buffer containing 3 protease inhibitors (Aprotinin, Leupeptin, and Phenylmethylsulfonyl (PMSF)) at a concentration of 1:100, for a duration of 30 minutes at 4°C. Following lysis, cells were subjected to 3 rounds of sonication (DiaMed Transonic T460) of 15 seconds and then centrifuged at 14,800g for 40 minutes at 4°C. After centrifugation, the supernatant was separated from the pellet (includes protein extracts) and stored at -20°C for later use. Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad).

2.3.2 Protein Extraction for Serial Fractionation

Cells were washed by 1x PBS and treated with trypsin to dislodge the cells and harvest them. Cells were then pelleted by centrifugation at 300g for 5 minutes, and re-washed again with 1x PBS. Cell pellets were treated with varying volumes of either RIPA or 7M Urea (depending on the number of cells) lysis buffer containing 3 protease inhibitors (Aprotinin, Leupeptin, and Phenylmethylsulfonyl (PMSF)) at a dilution of 1:100, for a duration of 30 minutes at 4°C. Cells were then subjected to 8 rounds of sonication with Bioruptor (Diagenode) of 15 seconds and then centrifuged at 14,800g for 40 minutes at 4°C. After centrifugation, the supernatant was separated from the pellet and kept at 4°C. The remainder cell pellet was subjected to further protein extraction for a total of 3 times using RIPA, followed by a fourth extraction using 7M Urea. Protein concentrations were determined using a 2-D Quant kit (GE Healthcare).

2.4 Western Blot

2.4.1 Western Blot for DICER Modulation

Samples were prepared in 1x Laemmli Buffer containing 0.05% BME (B-mercaptoethanol), 30-35 μg of protein and extra RIPA lysis buffer for a total volume of 50μL and subsequently boiled for 5 minutes at 95°C to denature proteins in sample. Samples were subjected to separation in 11% SDS-PAGE for 2hrs (80V for 30 minutes, 120V for 1.5hrs). Proteins from the gel were then subsequently transferred to a nitrocellulose membrane in a transfer buffer mixture of Tris, glycine and methanol overnight (~16hrs) at 4°C. Following transfer, the membrane was incubated in a milk blocking solution (5% milk in PBS-T) for 1hr at room temperature and then incubated overnight with primary antibody for protein of interest. Membranes were then washed with PBS-T (1x PBS, 0.05% Tween-20) three times of ten minutes each, followed by incubation with secondary antibodies (Cell Signaling) (anti-rabbit at 1:2000 and anti-mouse at 1:5000 concentrations) for 1hr at room temperature. Membranes were washed again following secondary antibody incubation three times for 15 minutes each. Membrane-bound antibody complexes were visualized by autoradiography using X-ray film (Kodak) and Western blotting systems Amersham Hyperfilm ECL (GE Healthcare) for DICER, while Clarity ECL (Bio-Rad) for all other proteins. Quantification of signal was performed by scanning the autoradiographs and determining band intensities by densitometry using Image J software.

2.4.2 Stain-free Western Blot for PACT Modulation and Serial Fractionation

Samples were prepared in 1x Laemmli Buffer containing 0.05% BME (B-mercaptoethanol), 30-35 μg of protein and extra RIPA lysis buffer for a total volume of 50μL

and subsequently boiled for 5 minutes at 95°C to denature proteins. Samples were subjected to separation in 10% TGX Stain-Free FastCast gels (Bio-Rad) for 1hr at 200-250V. Proteins from the gel were then subsequently transferred to a low fluorescence PVDF (polyvinylidene difluoride) membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). The resulting blot was then imaged using the Chemidoc Touch Imaging System (Bio-Rad) which is used for total protein quantification. Following transfer, the membrane was incubated in a milk blocking solution (5% milk in PBS-T) for 1hr at room temperature and then incubated overnight with primary antibody for protein of interest. Membranes were then washed with PBS-T (1x PBS, 0.05% Tween-20) three times of ten minutes each, followed by incubation with secondary antibodies (Cell Signaling) (anti-rabbit at 1:2000 and anti-mouse at 1:5000 concentrations) for 1hr at room temperature. Membranes were washed again following secondary antibody incubation three times for 15 minutes each. Membrane-bound antibody complexes were visualized by Chemidoc imaging system and image analysis done using Bio-Rad image software.

2.5 RNA Extraction and cDNA Synthesis

Total RNA isolated from cells according to manufacturer's guidelines using the RNeasy Micro extraction kit (Qiagen). Isolated RNA was then quantified using Nanodrop ND1000 Spectrophotometer. For qPCR experiments, RNA was reverse transcribed to cDNA using the provided primer mix from the iScript cDNA synthesis kit (Bio-Rad).

2.6 RT-qPCR

Samples for qPCR were prepared in a 96-well plate using the SYBR green supermix kit (Bio-Rad) for a total reaction volume of 20μL per well. The plate was read using the CFX96 Touch Real-time PCR detection system (Bio-Rad) and results analyzed with Bio-Rad CFX Manager Software. Primer sequences for the gene of interest were designed utilizing Cluster Omega for multiple sequence alignment and Primer3 for selection of primer pair sequences. Primer sequences of interest were further analyzed using IDT oligoAnalyzer 3.1 for physical properties and possible dimerization potential. Final selected primer sets were subjected to BLAST in Ensemble for specificity of oligos to the gene of interest. Primers were used at a concentration of 1μM. The following primer sequences have been used in this study:

DICER:
Forward 5`-AAGGAAGCTGGCAAACAAGA-3`
Reverse 5`-AAAAGGAACCACCAAGTTGC-3`

MBNL1:
Forward 5`-TTGATCTTGGCTTGCAAATG-3`
Reverse 5`-TGATTGTCGGTTTGCTCATC-3`

PACT:
Forward 5`-GCCATGCACATCAGAGAAAG-3`
Reverse 5`-AAGGCCTGTTAGTGCTGTCC-3`

Insulin Receptor B (IR-B) – E11-12:
Forward 5`-AAAACCTCTTCAGGCACTGG-3`
Reverse 5`-CGACTCCTTGTTCACCACCT-3`

Insulin Receptor Total (IR-Total) – E9-10:
Forward 5`-GGCAACATCACCCACTACCT-3`
Reverse 5`-ACTCGAATGGTGGAGACCAG-3`

SERCA-1a – E21-22:
Forward 5`-AGTGGCTCATGGTCCTCAAG-3`
Reverse 5`-ATGGAGGAGGGGGAACAGT-3`

SERCA-1Total – E20-21:
Forward 5`-CTCCATCTGCCTCTCCATGT-3`
Reverse 5`-CTTGAGGACCATGAGCCACT-3`

GAPDH:
Forward 5`-TGCACCACCAACTGCTTAGC-3`
Reverse 5`-GGCATGGACTGTGGTCATGAG-3`

HPRT1:
Forward 5`-TGACACTGGCAAAACAATGCA-3`
Reverse 5`-GGTCCTTTTCACCAGCAAGCT-3`

2.7 Statistical Analysis

For statistical analysis of mRNA/protein expression change, the Student's t-test (2 tailed, unequal variance) was used. All graph error bars represent the standard error of the mean (SEM) as indicated. P-values <0.05 were considered significant (*).

2.8 Workflow for PACT/DICER Results

In order to ensure consistency in results for PACT/DICER RNAi experiments, cells were treated with respective siRNA for 72hrs and once harvested, were separated into two equal halves, one used for RNA extraction, and the other for protein extraction. As a result, RNA/protein levels could be compared in each sample group more accurately, and thus more faithfully reflect the effect of treatment conditions.

CHAPTER 3

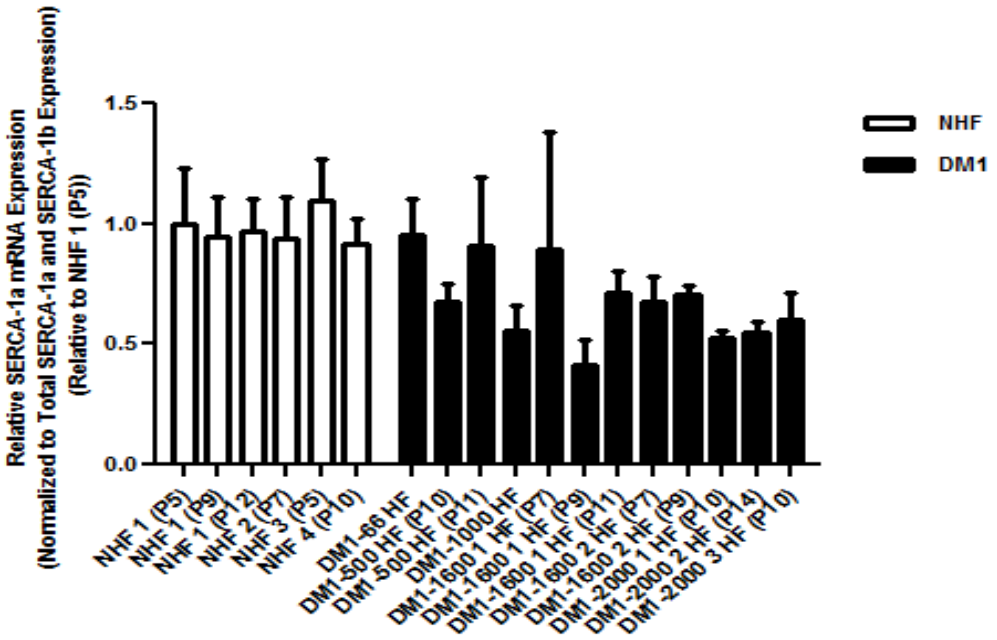
3. RESULTS

3.1 SERCA-1 and IR Expression in NHF vs. DM1

In order to initially validate the primer sequences used in this study and then confirm DM1 related dysregulated splicing, we measured SERCA-1 and IR expression profiles in control and DM1 fibroblasts. Primer sets for SERCA-1 were designed to flank exon 21-22 junction for SERCA-1a and to flank common exon regions 20-21 for total SERCA-1a and SERCA-1b. Real-time qPCR analysis was undertaken to compare relative SERCA-1a expression to total SERCA-1a/SERCA-1b expression using 20ng cDNA and the 1uM primer set. Significant inter-individual variability was seen, more prominently between DM1 patient fibroblasts (Figure 3).

Primer sets for IR were designed to flank exons 11-12 for IR-B expression and to flank common exon regions to both IR-B and IR-A (exons 9-10) for total IR expression. Real-time qPCR analysis done by comparing expression between IR-B expression in NHF vs. DM1, normalized to respective total IR-B and IR-A expressions. Inter-individual IR-B expression variability was seen amongst NHF subjects (Figure 4). There was no discernable difference detected between control vs. DM1 fibroblasts for IR-B expression.

A



B

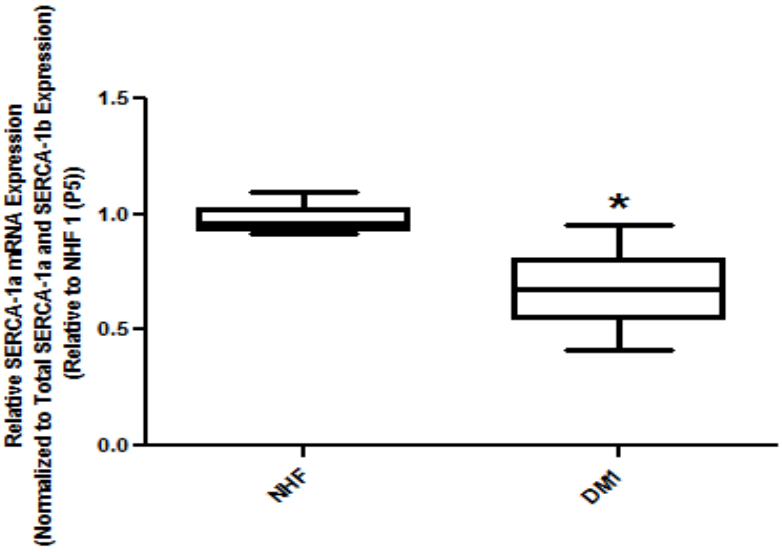
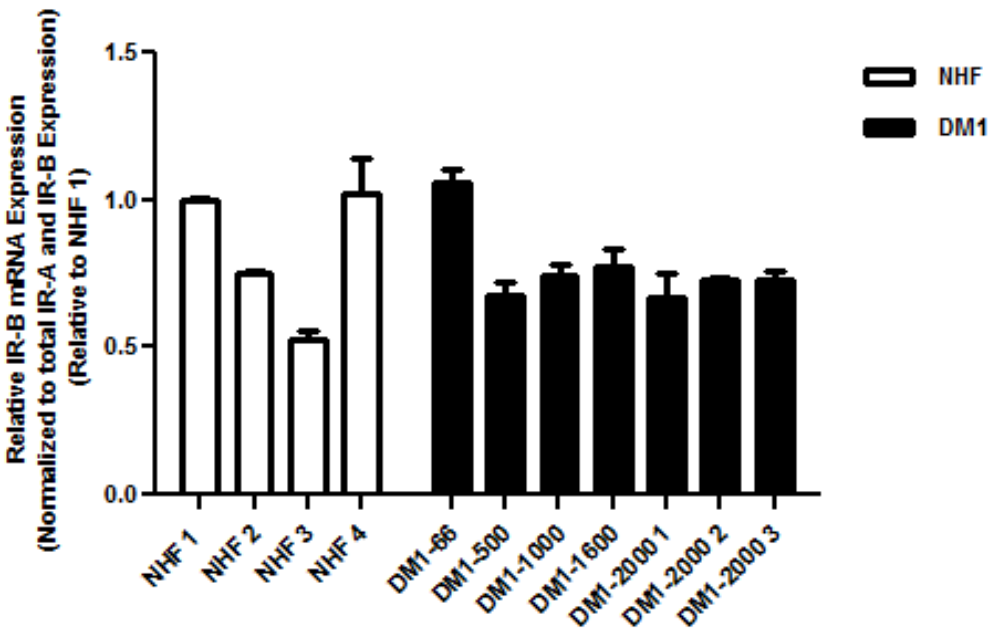


Figure 3 – SERCA-1a mRNA expression in NHF vs. DM fibroblasts via qPCR.
A) Relative SERCA-1a mRNA expression to total SERCA-1a and SERCA-1b (n=2). Reaction based on 20 ng cDNA, 1uM primer. B) Box-plot analysis indicates significantly reduced expression of SERCA-1a in DM1 fibroblasts compared to healthy controls (* DM1 SERCA-1a mRNA expression is relatively lower than in NHF, $P<0.05$)

A



B

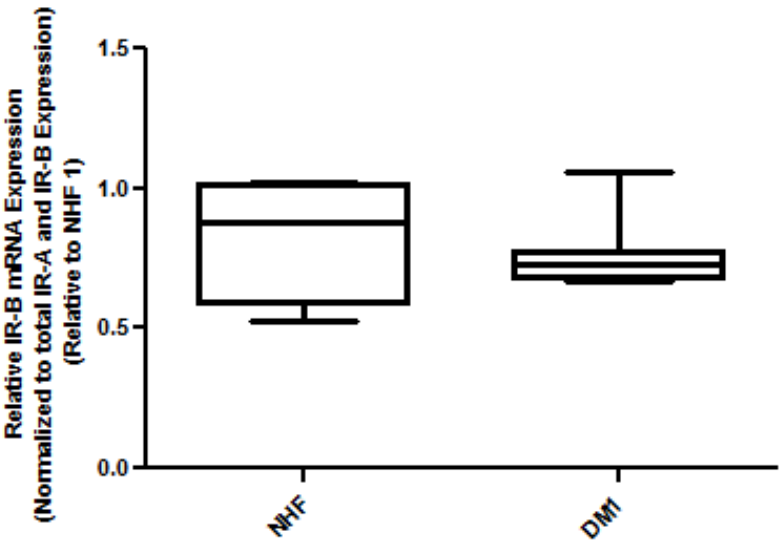


Figure 4 – IR-B mRNA expression in NHF vs. DM fibroblasts via qPCR.
A) Relative IR-B expression to total IR-B and IR-A. Reaction based on 20 ng cDNA, 1uM primer. B) Box-plot analysis indicates no aberrant splicing of IR detected between control and patient fibroblasts (n=2).

3.1.1 MBNL1 Knockdown and Alternative Splicing

We next explored the effect of MBNL1 modulation on alternatively spliced mRNA targets in NHF and DM1. MBNL1 knockdown reduced SERCA-1a expression normalized to total SERCA-1 expression in control as well as DM1 fibroblasts (Figure 5). In addition, MBNL1 reduction also modulates the alternative splicing of IR in fibroblasts (Figures 6). IR-B expression was reduced in response to MBNL1 RNA knockdown in both control as well as DM1 fibroblasts, normalized to total IR expression. These results supported a direct role for MBNL1 in the alternative splicing of SERCA-1 and IR expression, as well as validating the primer sets used in our study. The MBNL1 knockdown was validated by qPCR analysis using MBNL1 primers, encompassing all isoforms of MBNL1 (Figure 7).

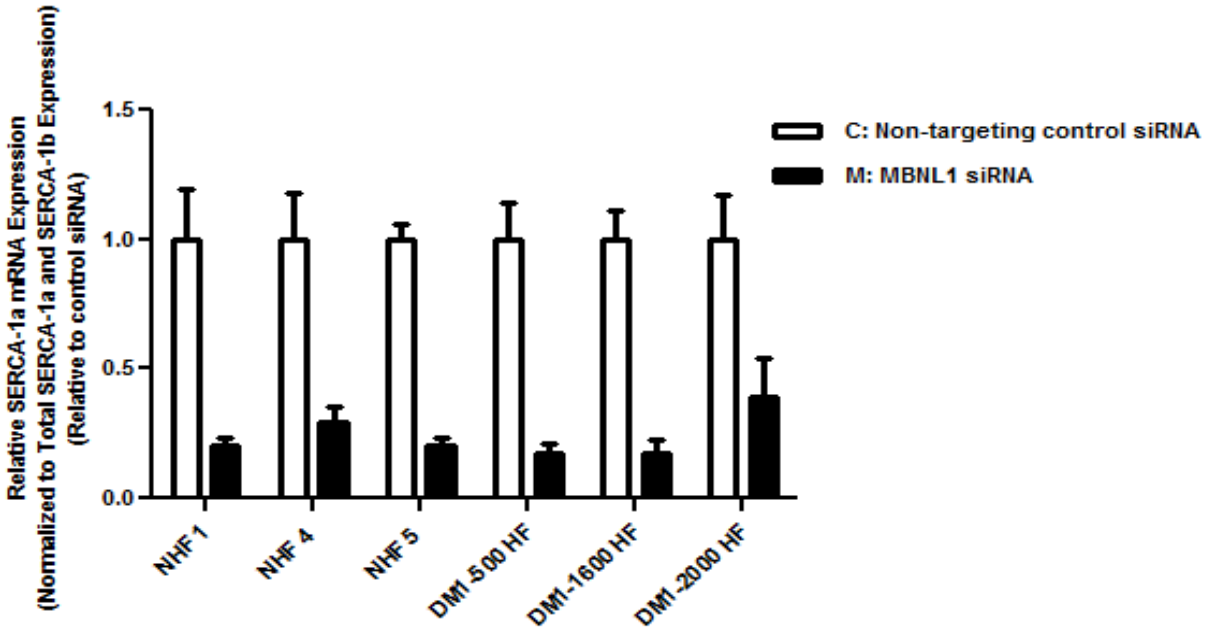


Figure 5 – Knockdown of MBNL1 RNA reduces relative SERCA-1a mRNA expression in healthy and DM fibroblasts.
Treatment of control and DM1 fibroblasts with 20nM MBNL1 siRNA for 72hrs reduces the relative expression of SERCA-1a to total SERCA-1. (n=2)

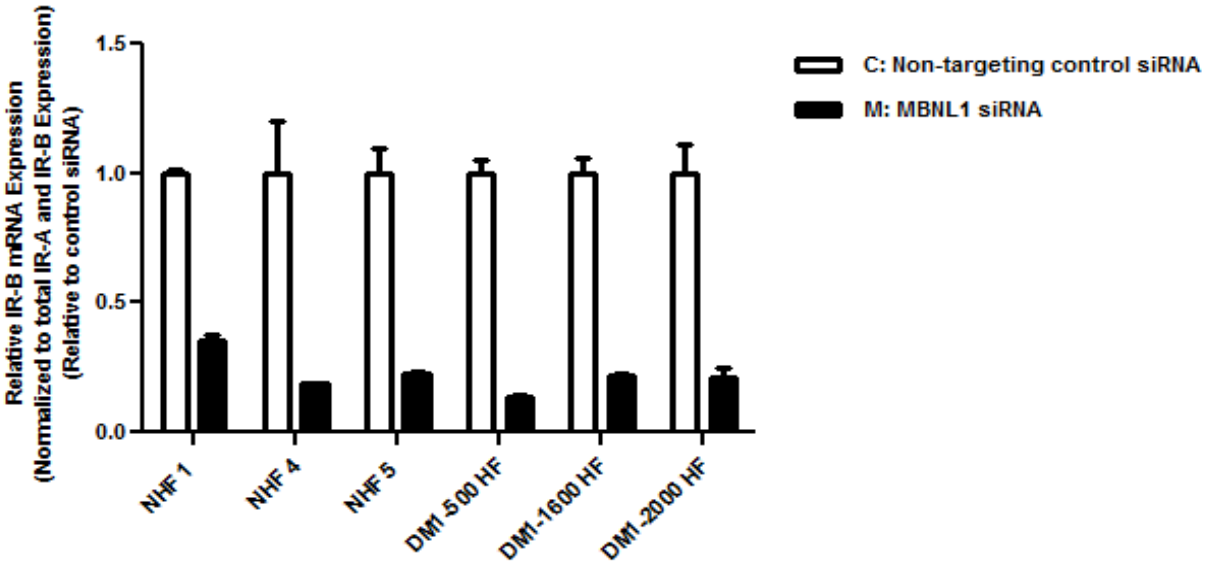


Figure 6 – Knockdown of MBNL1 RNA reduces relative IR-B mRNA expression in healthy and DM fibroblasts.
Treatment of control and DM1 fibroblasts with 20nM MBNL1 siRNA for 72hrs reduces the relative expression of IR-B to total IR. (n=2)

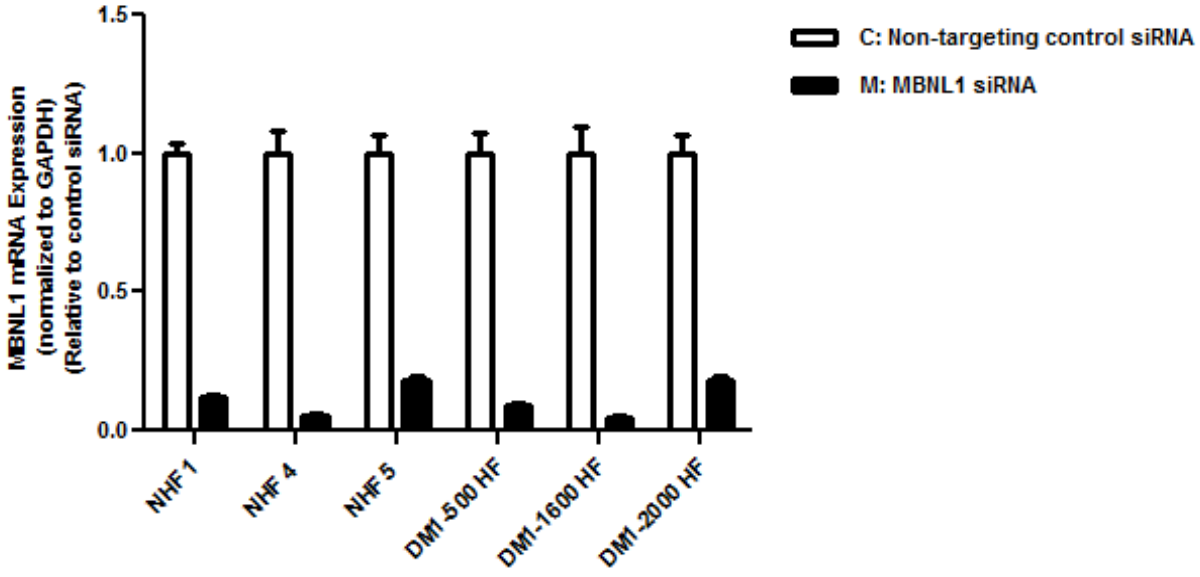


Figure 7 – Validation of MBNL1 knockdown in control and DM1 fibroblasts.
Treatment of control and DM1 fibroblasts with 20nM MBNL1 siRNA for 72hrs reduces the relative expression of MBNL1 mRNA (n=2).

3.2 PACT Knockdown and MBNL1 Expression

In order to explore the relationship between PACT knockdown and MBNL1 expression, NHF 1, DM500 and DM2000 cell lines were next subjected to 20nM PACT siRNA for 72hrs, resulting in a significant reduction of PACT mRNA (Figure 8 – left panel). Further analysis of qPCR data using primers encompassing all MBNL1 isoforms revealed an increase of MBNL1 mRNA in DM1 fibroblasts upon PACT reduction (Figure 8 – right panel). Interestingly, PACT siRNA treated NHF cells also induce MBNL1 mRNA indicating a non-DM1 related mode of action. MBNL1 mRNA is more prominently expressed at higher level in DM2000 fibroblasts than other cell lines.

Using cells from the PACT siRNA treated samples, protein was extracted and relative expression of PACT and MBNL1 proteins quantified. PACT protein was reduced in response to PACT RNAi as expected. MBNL1 protein levels were increased in all three cell lines, although not to the same degree as seen with MBNL1 mRNA expression (Figure 9). MBNL1 siRNA was included for MBNL1 antibody control. Although an induction of MBNL1 mRNA and protein observed in samples, the increase failed to reach the level of statistical significance.

Additional experiments involving PACT siRNA in NHF, DM500 and DM2000 reveals slight upregulation of MBNL1 mRNA in samples in a non-significant fashion. PACT knockdown was validated by qPCR using primer sequences targeting PACT mRNA (Figure 10).

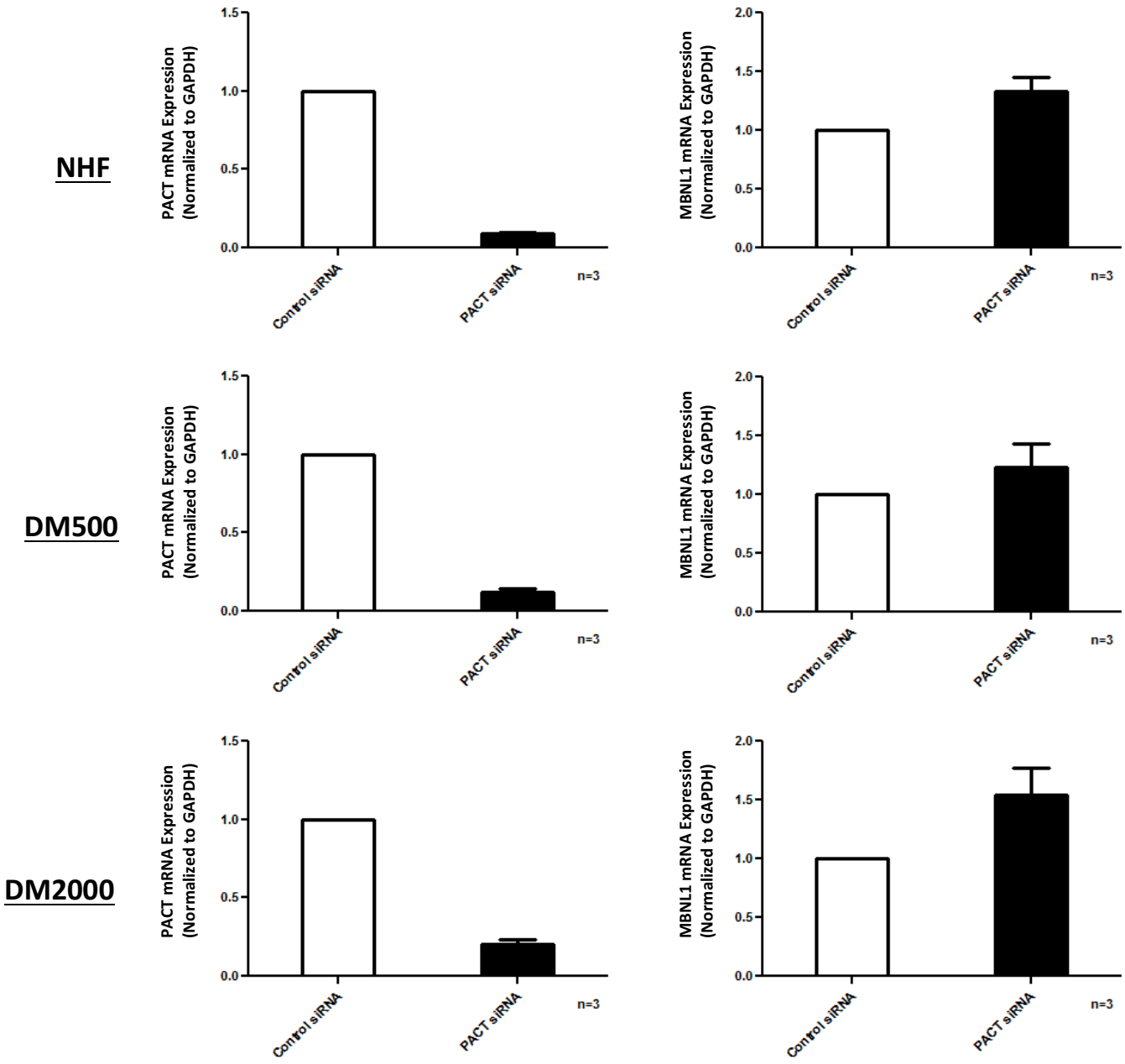


Figure 8 – PACT knockdown in NHF, DM500 and DM2000 induces trend of MBNL1 mRNA upregulation.
Left Panel) NHF, DM500 and DM2000 cells treated with 20nM PACT siRNA for 72hrs demonstrate reduced PACT mRNA expression. Right Panel) MBNL1 mRNA upregulation observed in PACT siRNA treated cells particularly in DM2000 although the upregulation of MBNL1 does not achieve statistical significance, (n=3).

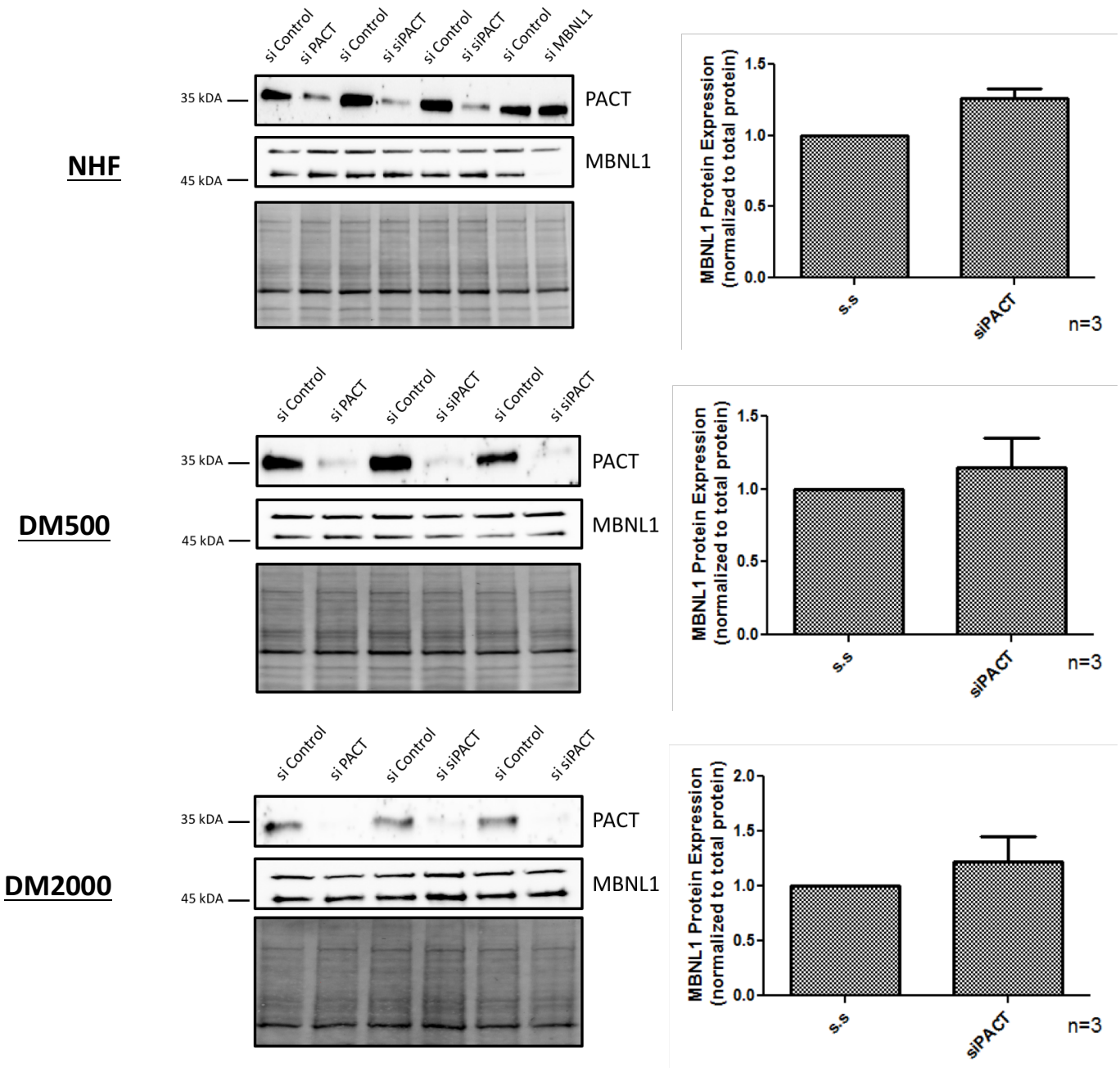
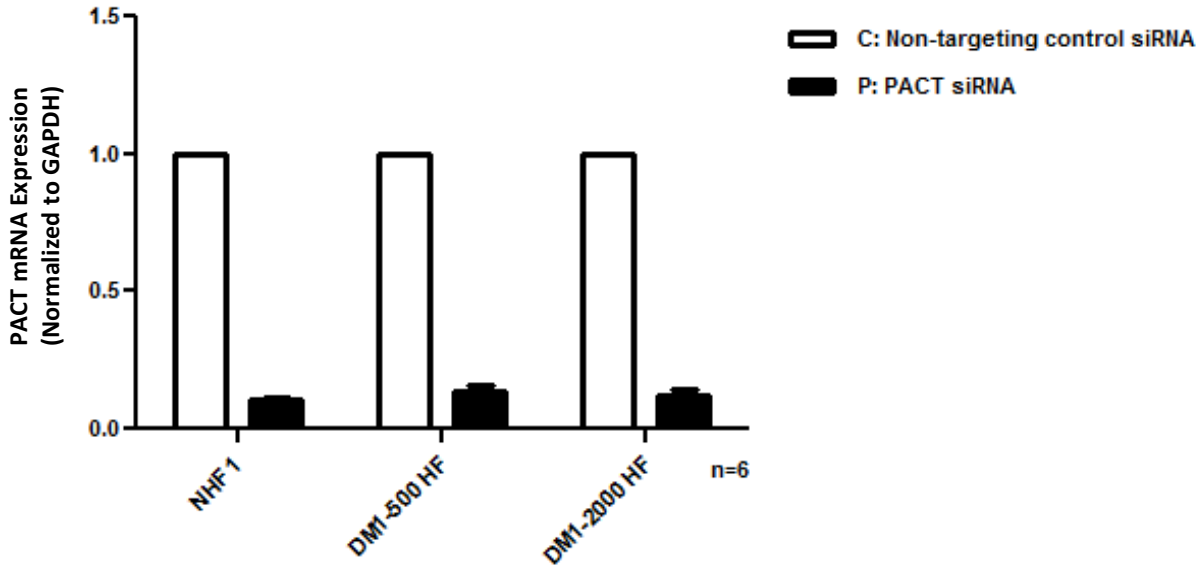


Figure 9 - PACT knockdown in NHF, DM500 and DM2000 induces trend of MBNL1 protein upregulation.
Left Panel) Representative Western Blot analysis of NHF, DM500 and DM2000 treated with 20nM PACT siRNA for 72hrs. The lower band corresponds to MBNL1 (45kDA). Right Panel) Quantification of MBNL1 proteins expression using Bio-Rad Image Lab, normalized to stain-free blot. A slight increase in MBNL1 protein was observed in PACT siRNA treated cells.

A



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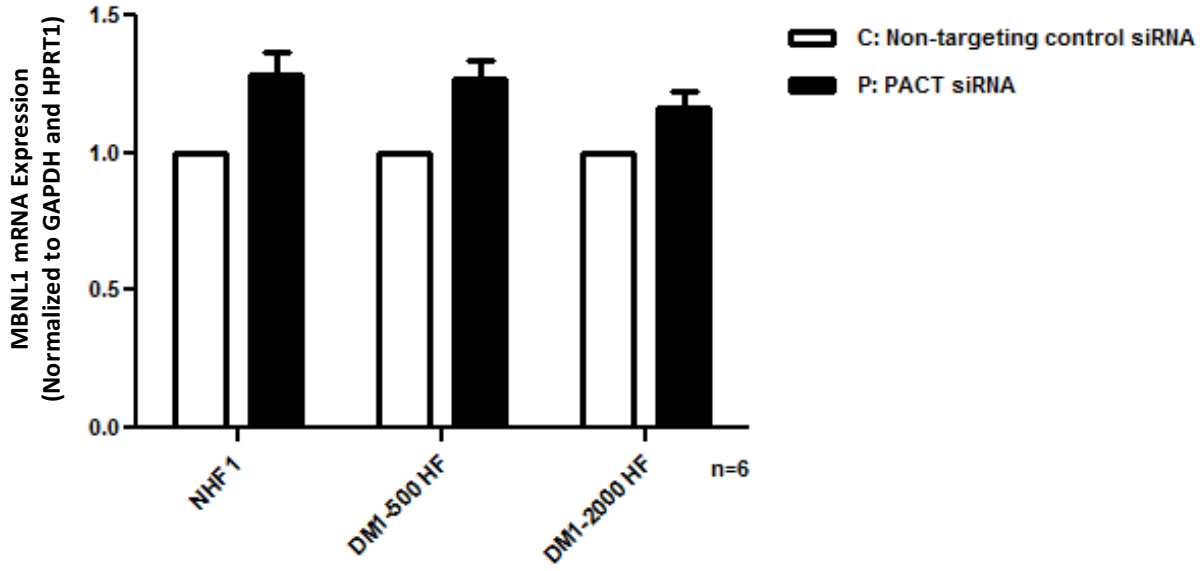


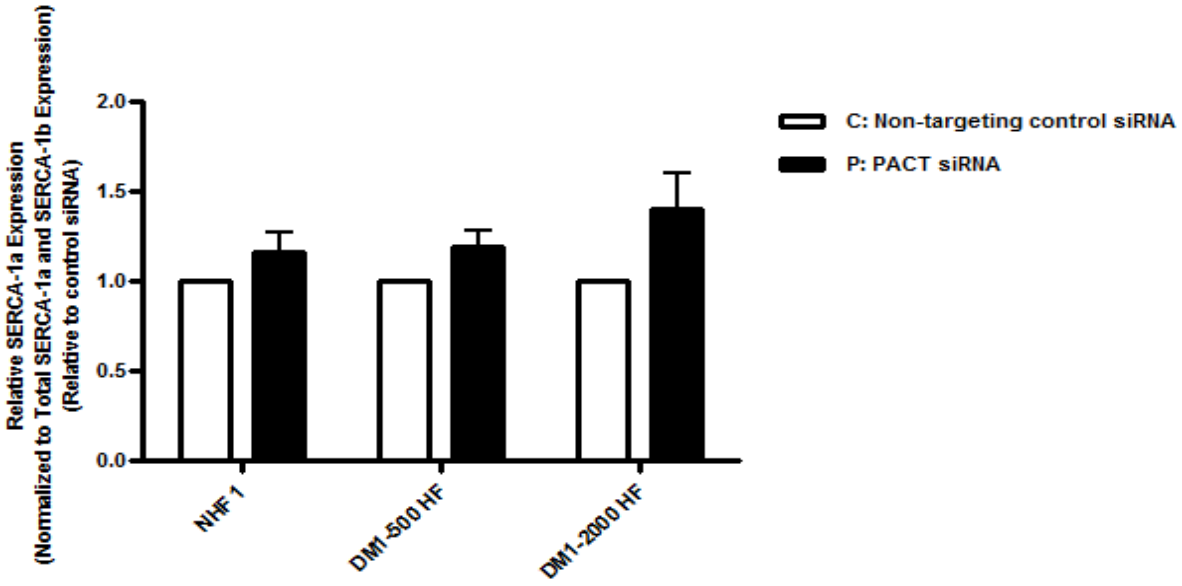
Figure 10 – PACT knockdown induces non-significant trend of MBNL1 mRNA upregulation.
A) Validation of reduced PACT mRNA expression in NHF, DM500, DM2000 treated with 20nM PACT siRNA for 72hrs. B) Statistically non-significant upregulation of MBNL1 mRNA in PACT siRNA treated cells (n=6).

3.2.1 PACT Knockdown and Alternative Splicing

Next, the analysis of NFH 1, DM500 and DM2000 cells treated with 20nM PACT siRNA for 72hrs revealed increased SERCA-1a expression when normalized to total SERCA-1 expression (Figure 11A). PACT RNAi thus resulted in a partial resolution of the dysregulated SECA1 splicing seen in the DM1 cells, although this did not reach the level of statistical significance. The cell population utilized is the same as samples used for MBNL1 mRNA analysis in PACT knockdown conditions.

In contrast, PACT siRNA treated cells do not modulate IR-B mRNA expression in any of NHF1, DM500 and DM2000 cell lines (Figure 11B). IR-B expression is normalized to total IR expression. Modulation of alternative splicing was thus solely seen in SERCA-1 expression of the two genes studied.

A



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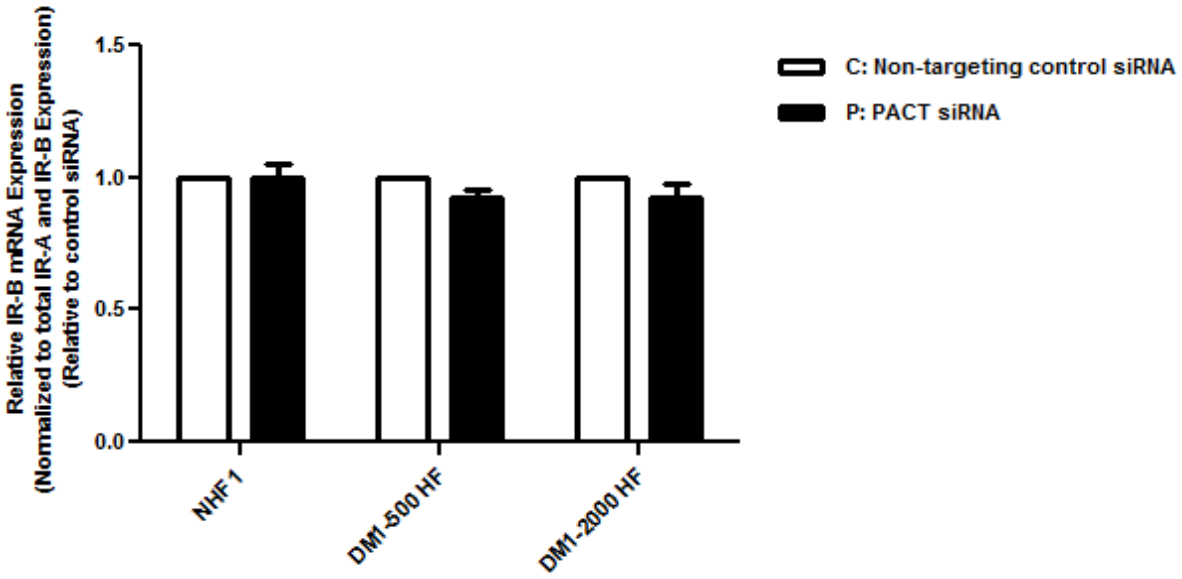


Figure 11 – PACT knockdown modulates SERCA-1 splicing but has not effect on IR splicing.
NHF, DM500 and DM2000 treated with 20nM PACT siRNA for 72hrs. A) PACT knockdown shows non-significant upregulation of SERCA-1a expression relative to total SERCA-1 with the highest levels seen in DM2000. B) PACT knockdown does not affect splicing of IR-B relative to total IR. (n=6)

3.3 DICER Knockdown and MBNL1 Expression

NHF 1, DM500 and DM2000 were next treated with 20nM siDICER for 72hrs. DICER knockdown was validated by qPCR for DICER mRNA expression using DICER specific primers. The analysis of MBNL1 mRNA expression via qPCR indicates no detectible changes in NHF and DM2000 although a slight increase was detected in DM500 (Figure 12). Results based on n=6.

Using cells from the same DICER siRNA treated samples, proteins were extracted and relative expression of DICER and MBNL1 proteins quantified, similar to our approach in the PACT knockdown experiments. Analysis of data by western blot validated DICER protein knockdown. The quantification of MBNL1 protein band by densitometry, normalized to housekeeping gene Vinculin, indicate no detectable modulation in MBNL1 protein expression in NHF and DM2000 (Figure 13). In DM500, a modest increase in MBNL1 protein expression was observed following DICER knockdown. Results based on n=6. We next treated DM500 fibroblasts with 20nM siDICER for 96hrs; no modulation of MBNL1 mRNA and protein levels was observed (n=3, data not included).

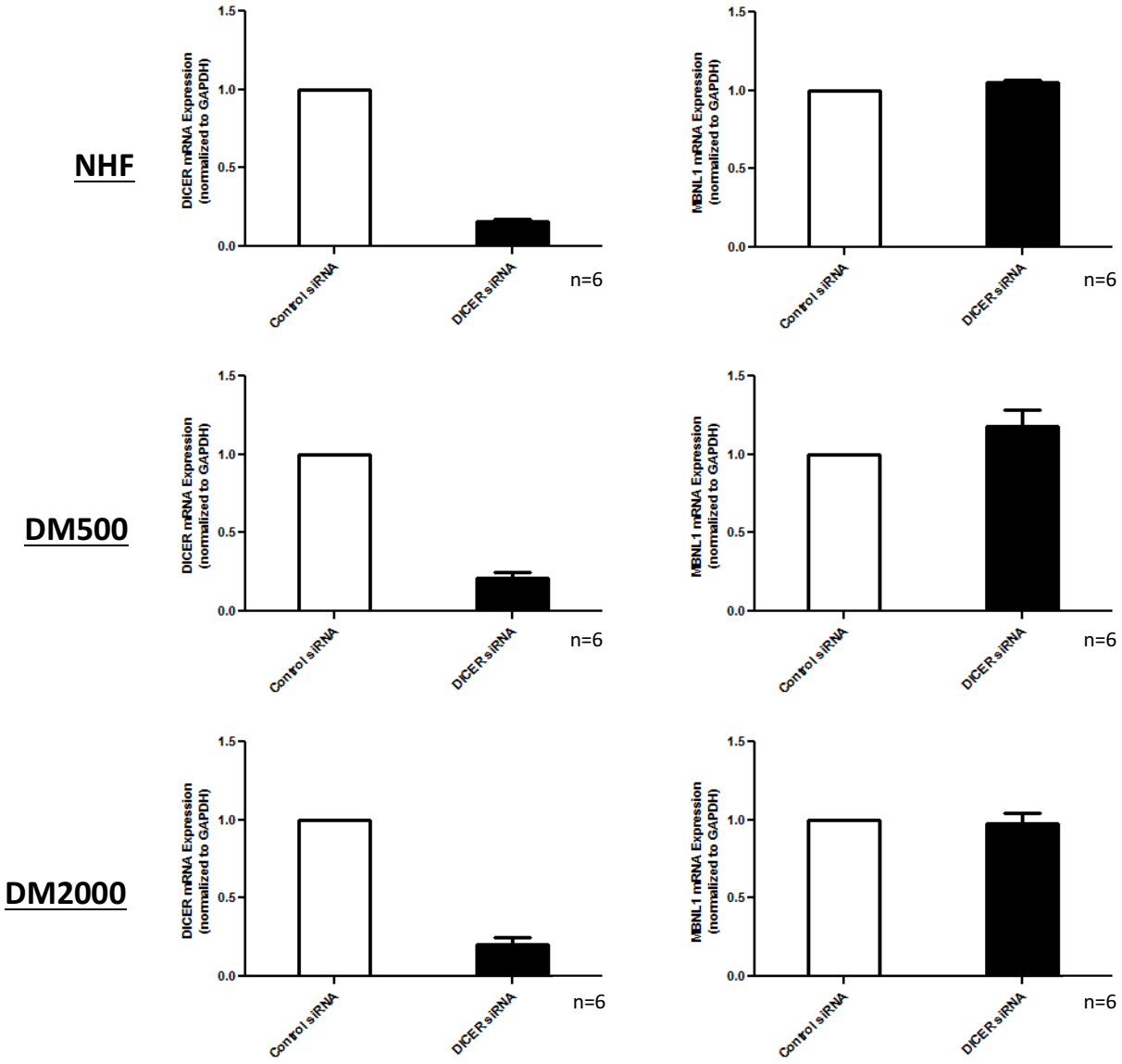


Figure 12 – DICER knockdown in NHF, DM500 and DM2000 does not modulate MBNL1 mRNA expression.
Left Panel) NHF, DM500 and DM2000 cells treated with 20nM DICER siRNA for 72hrs reduces DICER mRNA expression. Right Panel) MBNL1 mRNA expression remains unaffected in siDICER treated samples (n=6).

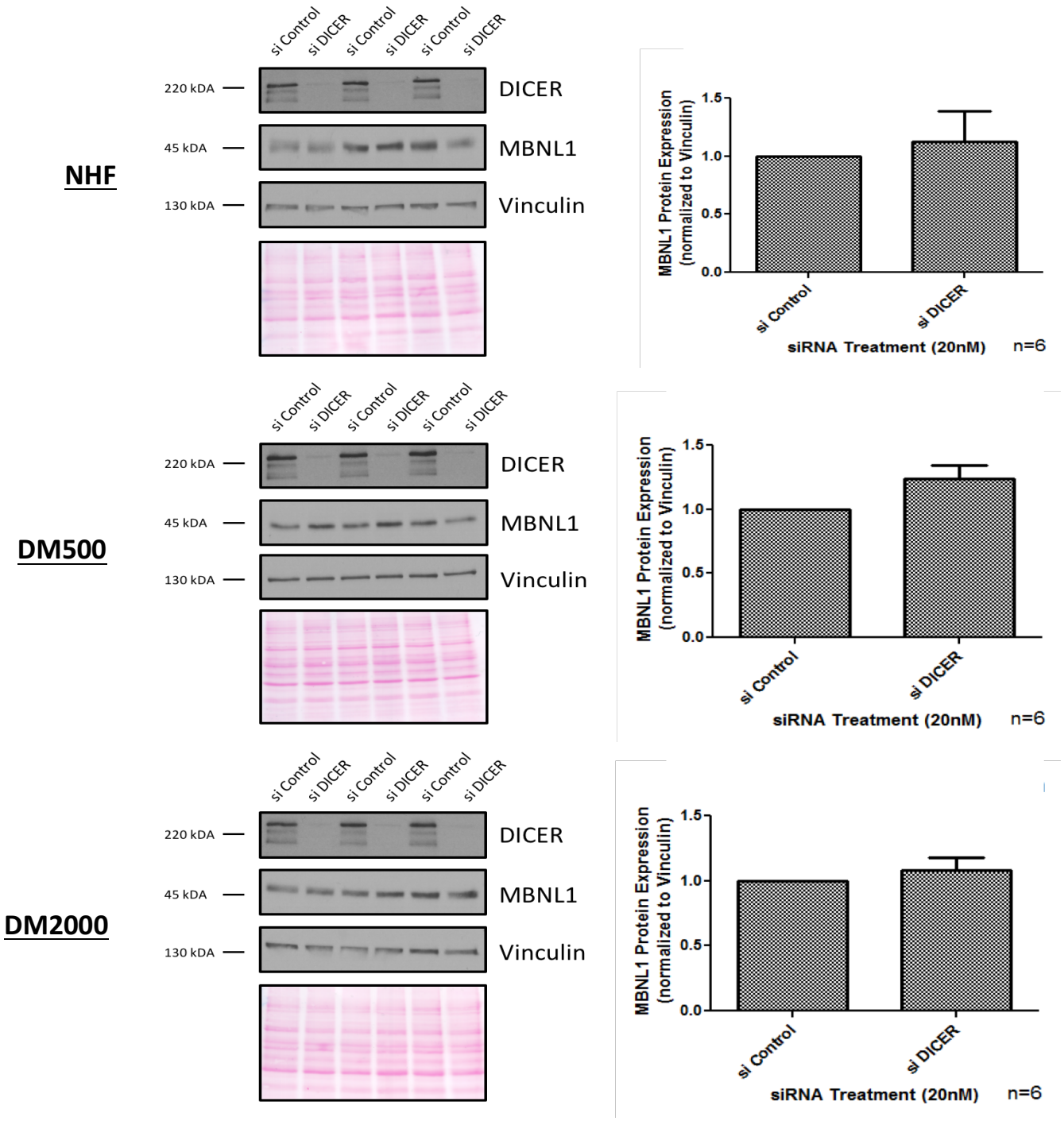


Figure 13 - DICER knockdown in NHF, DM500 and DM2000 does not modulate MBNL1 protein expression.
Left Panel) Representative Western Blot analysis of NHF, DM500 and DM2000 treated with 20nM DICER siRNA for 72hrs. Densitometric quantification of MBNL1 protein expression normalized to Vinculin. Ponceau staining was used as a loading control. Right Panel) Slight MBNL1 protein upregulation was observed in DM500. No changes were detected in NHF and DM2000 (n=6).

3.3.1 DICER Knockdown and Alternative Splicing

In order to explore a possible impact of DICER knockdown in DM1, NFH 1, DM500 and DM2000 cells treated with 20nM DICER siRNA for 72hrs resulted in no change in relative SERCA-1a expression, normalized to total SERCA-1 expression (Figure 14). A variability in SERCA-1a mRNA expression was observed in all three cell lines, particularly in DM2000. The changes were not statistically significant. The cell population was the same as samples used for MBNL1 mRNA and protein analysis in the DICER knockdown experiments (n=6).

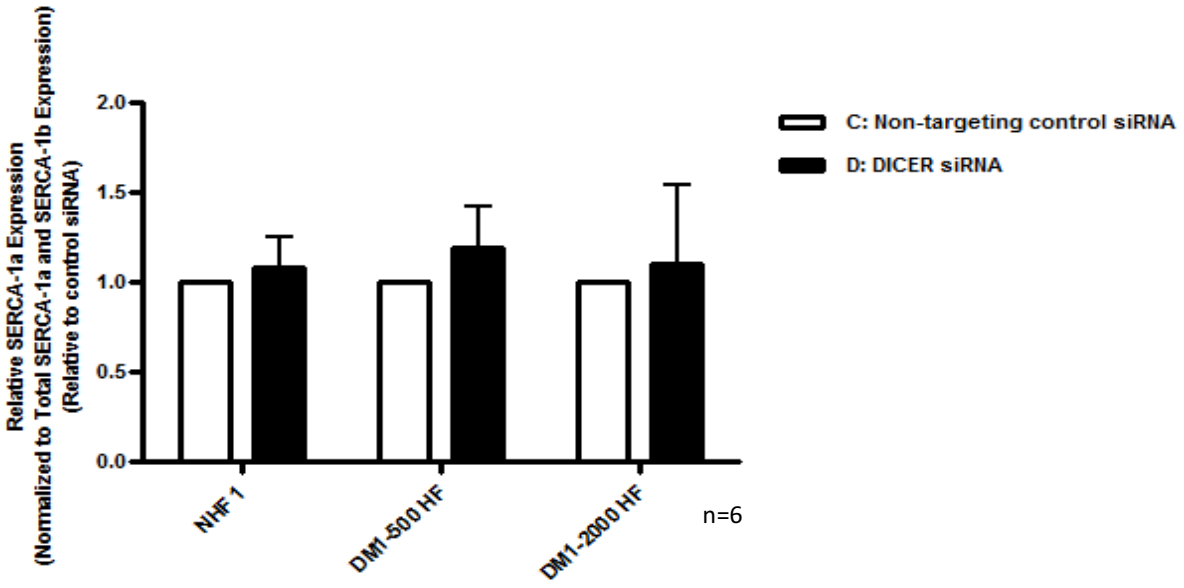


Figure 14 – DICER knockdown does not modulate SERCA-1 splicing.
NHF, DM500 and DM2000 treated with 20nM DICER siRNA for 72hrs. SERCA-1a relative to total SERCA-1 expression shows no change after DICER knockdown (n=6).

3.4 Total MBNL1 Protein Extraction

Experiments exploring MBNL1 levels in control vs. DM1 fibroblasts revealed a discrepancy between mRNA and protein expression levels (Data not shown). Specifically, although MBNL1 mRNA expression was significantly higher in DM2000 compared to NHF 1 and DM500, subsequent analysis of protein extracts from the same cell population revealed no difference in relative MBNL1 protein expression between cell lines. Although this suggests that MBNL1 mRNA and protein levels are not directly correlated, we wondered, given MBNL1 protein extensively complexing with the DMPK mRNA CUG hairpin loops, whether there might be a fraction of the protein not being solubilized by our extraction protocol, and hence potentially skewing our protein results. This led to the idea of modifying the extraction protocol to ensure extraction of total MBNL1 protein. We initially attempted three rounds of RIPA extraction (our standard protocol) followed by a round of 7M Urea treatment. In each phase, cells are lysed with lysis buffer and sonicated to extract potential RIPA-insoluble fractions of MBNL1. Our analysis of protein extracts revealed small amounts of MBNL1 protein extracted in second and third rounds of RIPA lysis, as well as what appears to be a DM1-specific fraction of MBNL1 protein in 7M Urea extracts (Figure 15). To ensure that the extraction has been done accurately, blots were probed for the highly soluble protein GAPDH to rule out incomplete/insufficient extraction of proteins in each phase (Figure 16). Close analysis by oversaturation of the MBNL1 protein signal, revealed a band (circled in red; Figure 17) uniquely present in DM1 fibroblasts and not observed in healthy cells. Although there is no exploration of this phenomenon that we could find in the literature, these results were in agreement with those of my fellow student Nafisa Tasnim, which indicated the presence of 7M Urea-extracted MBNL1 protein correlated with the number of foci per nucleus in DM1 cells (Figure 18).

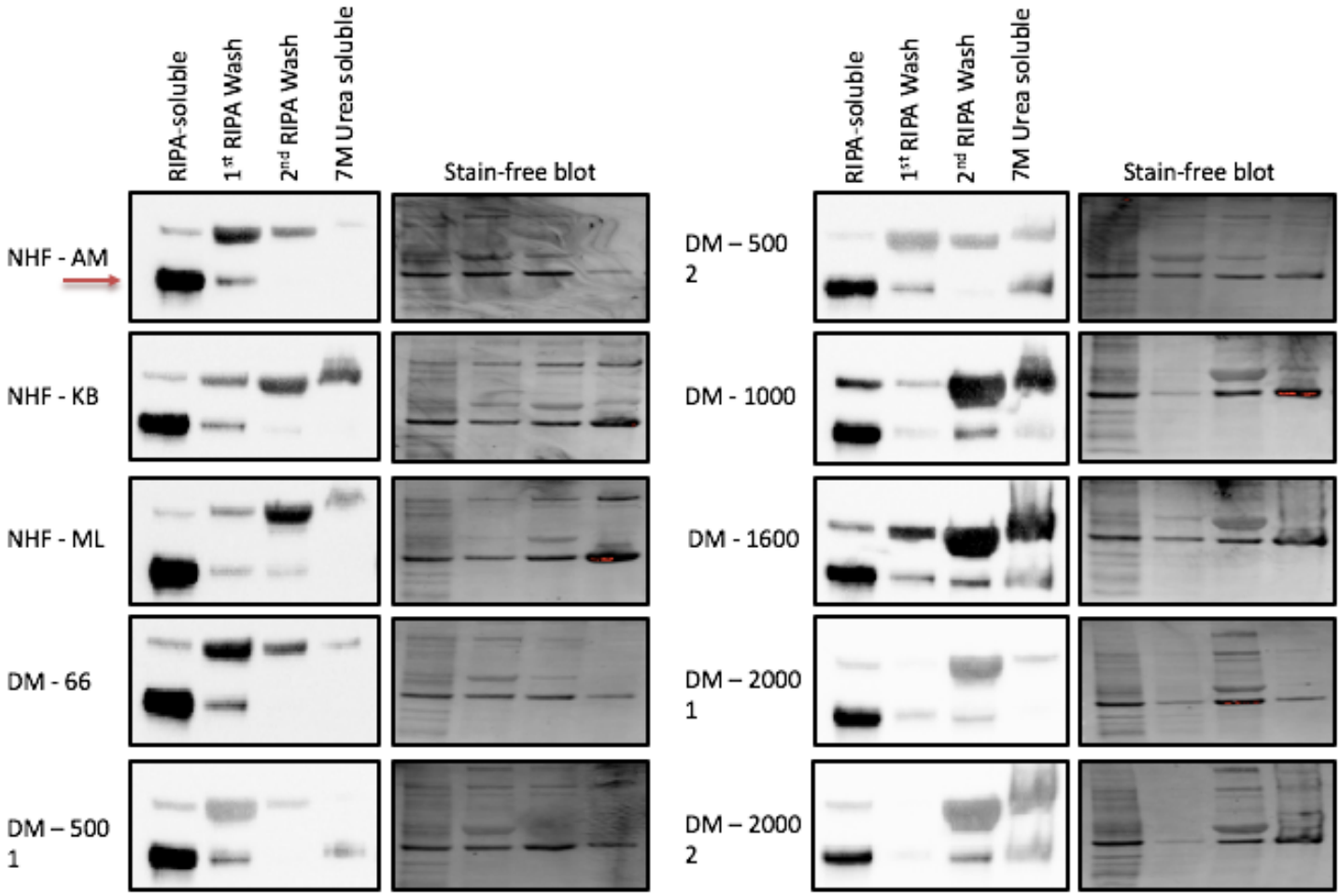


Figure 15 –Total MBNL1 extraction in normal fibroblast requires serial RIPA lysis extractions; DM1 fibroblasts require a round of urea extraction.
Protein from control and patient fibroblasts treated with three rounds of RIPA lysis buffer followed by a final 7M urea extraction. Representative western blots indicate RIPA-resistant MBNL1 fractions in DM1 fibroblasts. Lower band (red arrow) indicates MBNL1 protein. Quantification of MBNL1 band by Bio-Rad Image Lab, normalized to total loaded proteins in stain-free blots.

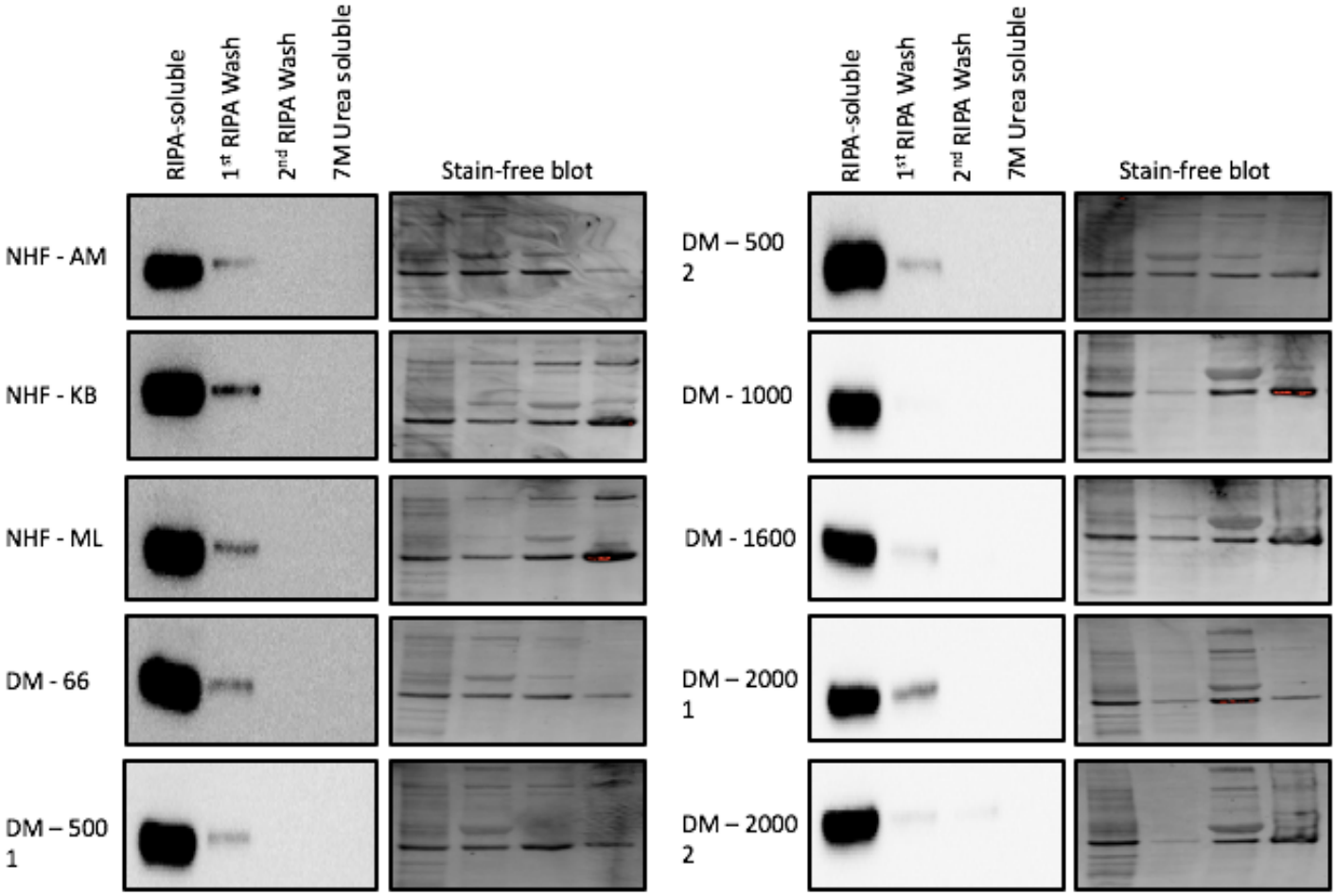


Figure 16 – Total GAPDH protein extraction with RIPA lysis buffer.
Western blots showing GAPDH levels in control and patient fibroblasts treated with three rounds of RIPA lysis buffer followed by a final extraction using 7M urea.

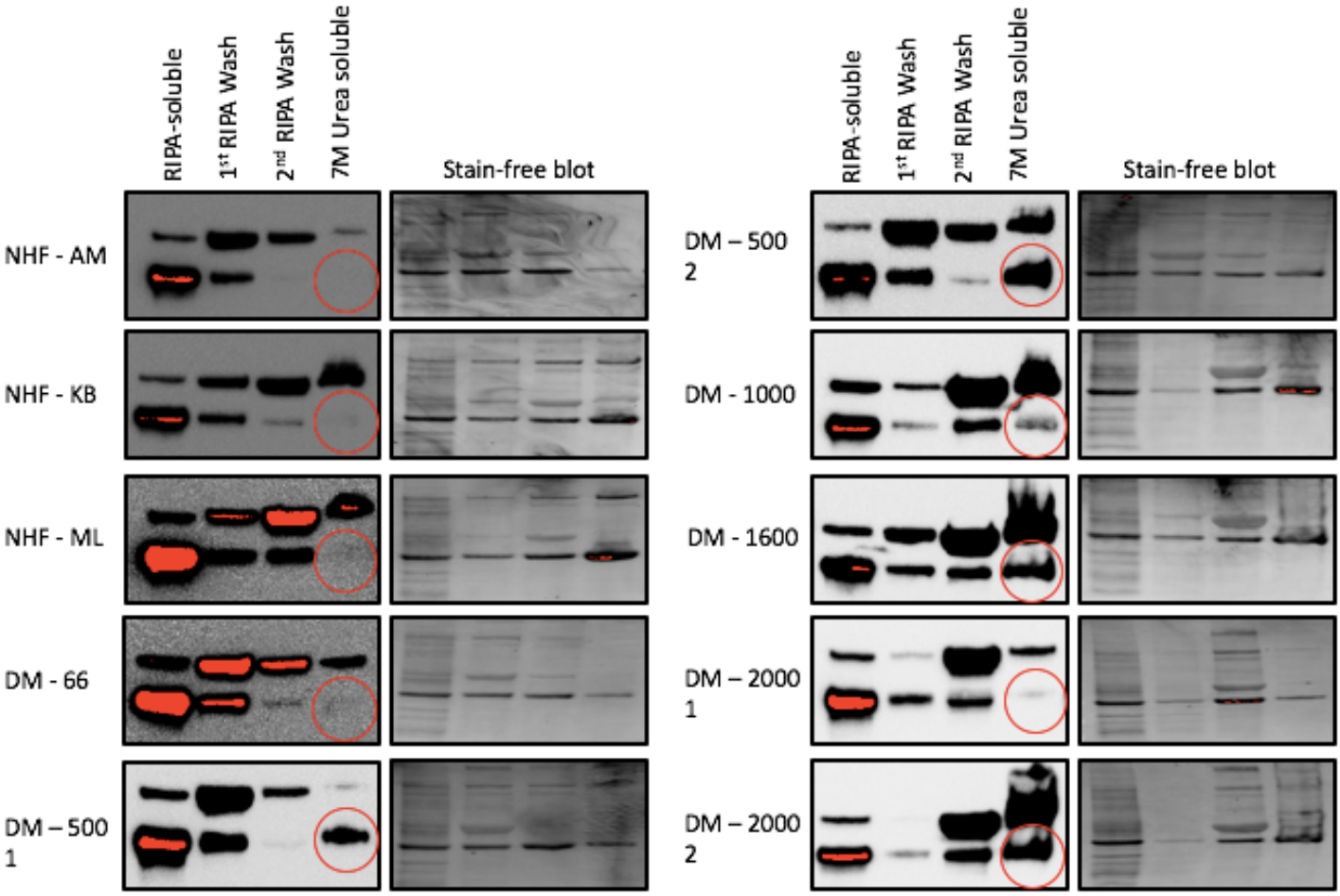
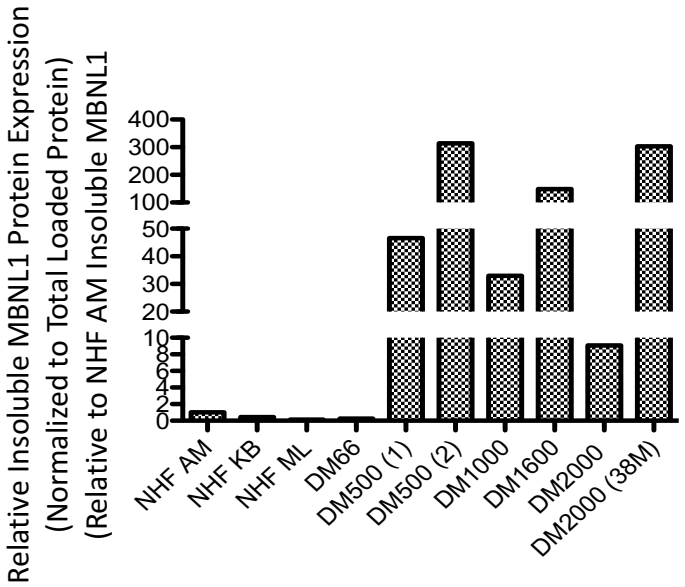


Figure 17 – Oversaturation of western blots reveals DM1-specific RIPA-resistant/7M urea soluble MBNL1 protein fraction.
Protein extracted from control and patient fibroblasts after three rounds of RIPA lysis buffer followed by a final 7M urea extraction. The lower band is MBNL1 protein. A 7M urea soluble MBNL1 fraction (red circle) was detected in DM1 fibroblasts but not in NHFs. Quantification of MBNL1 band by Bio-Rad Image Lab, normalized to total loaded proteins in stain-free blots.

A



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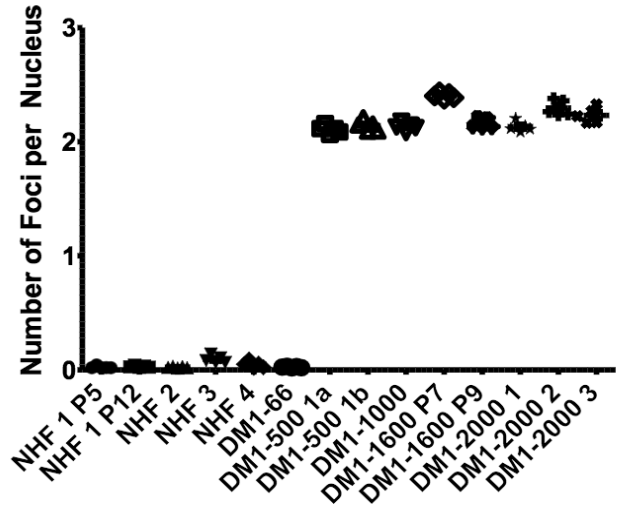


Figure 18 - Insoluble/7M urea soluble MBNL1 protein fraction is significantly higher in DM1 than control fibroblasts.

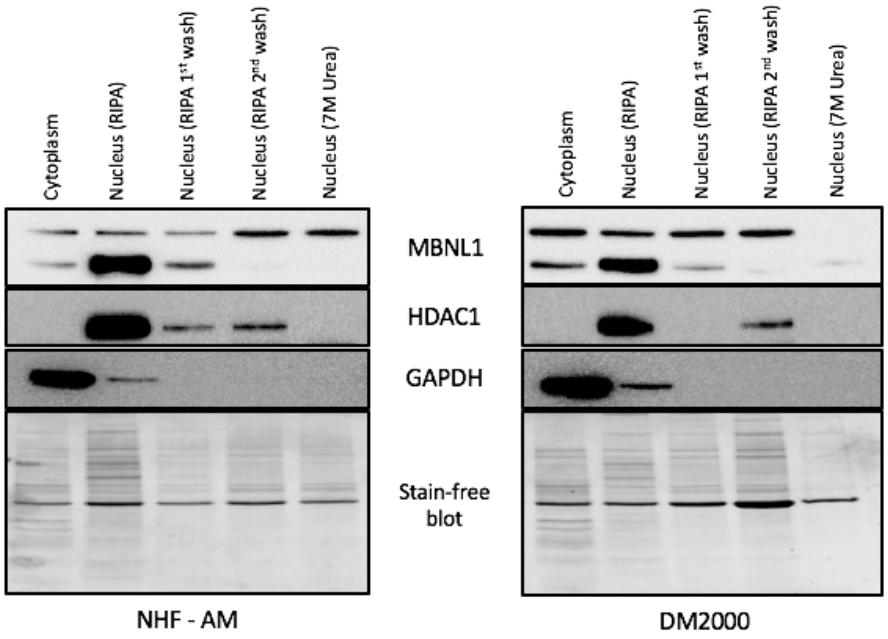
A) DM-specific RIPA-insoluble/7M urea-soluble MBNL1 fraction is variably higher in DM1 fibroblasts compared to healthy control cells. The quantification of RIPA-insoluble MBNL1 fraction was conducted using Bio-Rad Image Lab, normalized to respective total protein loaded in stain-free blots. B) The presence of foci in DM1 fibroblasts correlates well with the presence of RIPA insoluble MBNL1 fractions. Figure done by Nafisa Tasnim (Alex MacKenzie Lab).

3.4.1 Insoluble MBNL1 Protein Localization

In an effort to determine the source of the MBNL1, NHF 1 and DM2000 cell lines were subjected to cytoplasmic/nuclear fractionation. Subsequently, the nuclear fraction was further serially extracted with RIPA lysis reagent followed by a final round of 7M Urea extraction. Most of the MBNL1 protein is localized to the nucleoplasm of fibroblasts as indicated in corresponding western blot analysis (Figure 19A). In contrast, the majority of GAPDH protein (used as a cytoplasmic protein control) is detected in the cytoplasmic fractions of both NHF 1 and DM2000 cell lines (Figure 19A). Conversely most of the HDAC1 (used as a nuclear protein control) is detected in the first RIPA nuclear extract, with a small portion detected in the second and third RIPA-treated fractions (Figure 19A).

Oversaturation of MBNL1 signal revealed a DM1-specific RIPA-resistant/7M Urea soluble MBNL1 fraction localized to the nucleoplasm of DM2000. The expression of MBNL1 in this fraction is significantly higher in the DM2000 extract compared to NHF.

A



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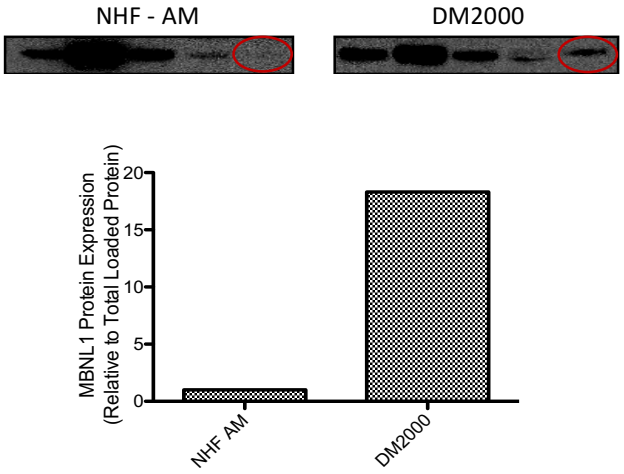

Figure 19 – Cytoplasmic/nuclear fractionation reveals insoluble MBNL1 fraction is localized to the nucleus of DM1 fibroblasts.
A) Cytoplasmic/nuclear fractionation of MBNL1 in fibroblasts. Representative Western Blots of nuclear protein fraction serially extracted with three rounds of RIPA followed by a final round of 7M urea shows that most of the MBNL1 protein is localized to the nucleus. HDAC1 was used as a nuclear extraction control and GAPDH as a cytoplasmic extraction control. B) Oversaturation of the MBNL1 signal reveals a DM-specific RIPA-insoluble/7M urea-soluble band in DM2000. The nuclear insoluble MBNL1 fraction (red circle) is significantly higher in DM2000 compared to NHF - AM.

CHAPTER 4

4. DISCUSSION

4.1 Therapeutic Targets

Currently there is no effective treatment for DM1, a significant chronic disorder with a real level of morbidity and mortality. Disease progression varies depending on age of onset and chiefly the number of *DMPK* CTG repeats. The extent of DM1 disease reversibility is currently unknown. Mouse studies have revealed a reversal in myotonia, as well as a restoration of the normal adult-splicing pattern of pre-mRNAs, by overexpression of MBNL1 protein *in vivo* (Kanadia et al., 2006). This result as well as the regenerative nature of the affected tissues (e.g. muscle) makes one hopeful that inducing similar effects in DM1 patients will confer clinical benefit. In addition to clinical monitoring for benefit (e.g. muscle strength, EMG, insulin sensitivity), it is important to establish specific biomarkers when assessing therapeutic efficacy. Three key areas I believe are important for therapeutic target evaluation when using foci modulating agents are : i) Analyzing the effect on foci size and area, ii) evaluating the relative expression of MBNL proteins, and iii) assessing the multisystemic reversal of alternatively spliced mRNAs. If a target meets the stated conditions, then it could be explored further for it's therapeutic benefits as well as investigated for potential drugs that could modulate the expression of said target. Additionally, the nature of candidate drugs/small compounds would also need to be carefully examined, given that most patients would be required to take the compounds for life. Drugs with significant side effects or toxicity notwithstanding beneficial DM1 impact are clearly not options. The aim of our lab in a broader perspective of rare genetic disorders is to identify suitable therapeutic targets that could be modulated with safe doses of small compounds/drugs for clinical improvement. For the purpose of DM1 research, our target for analysis has been the newly identified PACT kinase and interacting partner DICER in the RISC.

4.2 Alternative Splicing in Fibroblasts

Two known DM1 alternatively spliced genes (SERCA-1 and IR) were examined to assess impact of our interventions on alternative splicing in DM1 fibroblasts. These were chosen because of their expression in fibroblasts, as well as their postulated roles in DM1 symptomatology (myotonia, myopathy, insulin resistance). Previous studies of these alternatively spliced genes have utilized PCR techniques with radiolabeled primer probes. This method involves radiolabeled primer sets for each subset of alternatively spliced mRNA, followed by quantification of the relative ratio between the two isoforms. Although this method has shown to be very effective, it is a very long procedure with exposure times of more than a month at a time, rendering it a comparatively inefficient approach. As a result, we decided to utilize qPCR and primers sets that encompass regions common to both mRNA sets.

4.2.1 SERCA-1 and IR Expression in Fibroblasts

Initially, six normal healthy fibroblast cell lines as well as 12 DM1 fibroblast cell lines were chosen to assess the expression of SERCA-1 (n=2) (Figure 3A). Expression of SERCA-1a (WT copy) normalized to total SERCA-1 expression was conducted in normal vs. DM1 fibroblasts to assess whether these primer sets would yield the expected outcome as been shown in literature. As can be seen in Figure 1, there is inter-individual variability of relative SERCA-1a expression amongst fibroblasts of healthy donors as well as DM1 patients. DM1 expression of SERCA-1a is more widely variable than in normal cells, potentially due to the differing nature of disease in these different patients as well as different number of CTG repeats correlating with disease severity. However, there does not seem to be correlation between the number of repeats and the expression of SERCA-1a in DM1 fibroblasts. It should be noted that SERCA-1 levels are

low in fibroblasts detected late in qPCR cycles, with CT values of ~29-31. This might affect our results by skewing the relative expression of SERCA-1a to total SERCA1. As a result, had the following been conducted in myoblasts instead of fibroblasts, where SERCA-1 expression is higher, it might be more indicative of SERCA-1a expression differences between NHF and DM1 cells. Having said that, box-plot analysis of SERCA-1a expression normalized to total SERCA-1 is significantly lower in DM1 than NHF cells (Figure 3B), suggesting that the primer sets used in this experiment, as well as the qPCR method, could be used to accurately assess the effect of our treatment conditions on DM1 alternative splicing *in vitro*. To further support this idea, siMBNL1-treated cells were assessed for SERCA-1a expression, showing a significant downregulation of SERCA-1a expression upon knockdown of MBNL1 (Figures 5 and 7). This experiment supports findings in literature concerning the role of MBNL1 in alternative splicing of DM1-associated mRNAs, as well validating our qPCR with designed primer sets for evaluating expression of SERCA-1 in these cell lines.

IR expression in fibroblasts of healthy individuals and DM1 patients was assessed in a fashion similar to SERCA-1 experiments. Primers for IR-B and total IR amplification were designed and utilized in qPCR to determine the relative expression of IR in these cell lines. The expression of IR-B of 4 healthy fibroblast cell lines was compared to 7 DM1 fibroblast cell lines (Figure 4A). Data indicate no discernible difference of IR-B expression between NHF vs. DM1 cells, mainly due to the variable expression of IR in these cell lines. In healthy fibroblasts, the expression of IR tends to be more variable compared to DM1, which could be due to non-DM1 related genetic factors and lifestyle of these individuals. Furthermore, knockdown of MBNL1 in these cells also reduces IR-B expression non-discriminately, indicating the association between alternative splicing of IR and MBNL1 expression (Figures 6 and 7). These results suggest that

although expression of IR is affected upon treatment with siMBNL1 in these cells, IR expression in fibroblasts might not be indicative of alternatively spliced IR mRNAs in other tissues of these individuals. This is important to keep in mind while assessing the effect of treatment conditions on IR splicing in fibroblasts specifically. Ideally, the expression of IR would be compared between various cell types of the same individuals tested to uncover the most suitable cell line with aberrant IR expression.

The experiments are limited in their ability to detect alternatively spliced RNAs in fibroblasts, mainly due to low relative RNA expression (in the case of SERCA-1), as well as very small differences in alternative isoform expression between healthy and DM1 fibroblasts (in the case of IR). MBNL1 expression rescue experiments would analyze the effect of MBNL1 on SERCA-1 and IR expression, while ruling out non-specific modulation. There may also be a role for more quantitative measures to be used, such as TaqMan probe assays more accurately measuring the expression of both sets of isoforms for alternatively spliced genes. Additionally, employing other cell lines that are more representative of disease conditions, such as myoblasts may yield a better readout on the effectiveness of treatment conditions.

4.3 PACT as DM1 Biomarker Modulator

Previous work in our lab had indicated PACT as a foci modulator in DM1 fibroblasts. Reduction of PACT protein by siRNA in 72hrs reduced foci area by ~30-50% (data not shown), indicating that the reduction in foci area could be therapeutically relevant. Additionally, PACT kinase substrate PKR was also investigated for PACT RNAi effects. The chemical inhibition of PKR revealed no effect on foci integrity as well as no modulation of MBNL1 expression and so was deduced not to be relevant to PACT modulation activity in DM1 (data not shown). Preliminary work had also detected an increase in MBNL1 protein expression upon reduction of PACT protein in these cell lines.

We investigated the effects of PACT knockdown on MBNL1 expression, specifically in three fibroblast cell lines (control, DM500 and DM2000). Fibroblasts treated with PACT siRNA for 72hrs reduced PACT mRNA expression as expected (Figure 8). MBNL1 mRNA expression was moderately induced as a result of PACT knockdown in all three cell lines, albeit non-significantly; yet more prominently detected in DM2000, with an induction of ~1.5 fold. Interestingly, MBNL1 mRNA induction was also observed in NHFs, indicating a non-DM1 mechanism might be responsible for this induction. This finding also highlights the possibility of PACT modulating foci and MBNL1 expression via 2 separate molecular pathways. Protein extraction of the same cell populations displayed downregulation of PACT protein in PACT siRNA-treated cells (Figure 9). Examining the expression of MBNL1 protein suggested a statistically-insignificant minor induction in all three cell lines, relatively less so than the corresponding mRNA expression. MBNL1 siRNA was used as a positive control for the antibody being used in these experiments. Two MBNL1 bands were detected in our western blot analysis, of which only the lower band corresponded to the predicted detectible size of 45kDa for

MBNL1 according to manufacturer's guidelines. The same band also displayed downregulation of MBNL1 protein once treated with siMBNL1 (Figure 9).

However, it is important to note that the mechanism of foci reduction and MBNL1 induction is currently unknown. The slight induction of MBNL1 in PACT siRNA-treated cells was unexpected, given that although a reduction in foci would theoretically dislodge and free up MBNL1 from the foci within the nucleoplasm, this should not be affecting the total cellular MBNL1 levels. Thus, changes in MBNL1 expression may not be accurately reflected, there may be a shift in internal pools away from foci bound (undetectable by our method of MBNL1 quantification) to more bioavailable nucleoplasmic MBNL1 (detectable by our method of MBNL1 quantification).

Next, the effect of PACT knockdown on alternative splicing was investigated (Figure 10). SERCA-1b expression was slightly induced in NHF and DM500 cells, whereas SERCA-1b expression in DM2000 cell lines were induced by an even greater amount, potentially highlighting the therapeutic benefits of PACT downregulation in DM1. On the other hand, IR expression in siPACT treated cells remained unchanged in all three cell lines. Although we had established earlier that PACT modulates MBNL1 expression (Figure 8 and 9), and that MBNL1 expression is linked to IR expression (Figure 6), it is somewhat puzzling that PACT knockdown had no effect on the splicing of IR. One possible reason for this could be that IR activity is low in fibroblasts, and that the relatively low modulation of MBNL1 is not sufficient to modulate IR-B expression to the point of detection.

Considering the effect of PACT knockdown on DM1 biomarkers and reversing key DM1 phenotypes such as foci reduction, MBNL1 induction and alternative splicing normalization (SERCA-1); makes PACT a very good candidate for further research. Assessing the effect of

PACT knockdown on other alternatively spliced mRNAs would validate the benefits of PACT as a therapeutic target. PACT knockdown could also be investigated in fibroblasts to assess the effect on intra-nuclear MBNL1 protein concentration as a result of foci reduction by utilizing immunofluorescence (IF) techniques to quantify the relative expression of MBNL1 within the nucleoplasm. Combined with Western blot analysis, IF experiments would provide a direct quantitative measure of the shift of internal pools from the amount of MBNL1 present in the nucleus that could contribute to therapeutic benefits.

4.4 DICER Role in DM1

Given that previous work ruled out PKR activity in DM1-associated modulation of foci integrity and MBNL1 expression, we decided to look for other pathways associated with PACT that may subserve this role. Previous research suggested a role for PACT in RNAi pathways as forming a component of the silencing complexes. Together with DICER, PACT enhances miRNA processing and thus modulates gene expression. We therefore focused on DICER, a major protein in the RISC, analyzing the effect of DICER on DM1 biomarkers. A time-course of DICER Knockdown in DM1fibroblasts did not have a profound effect on foci integrity (data not shown), with a minor reduction in foci area observed at 72hrs. As a result, as with the PACT knockdown experiments, NHF, DM500 and DM2000 cell lines were subjected to DICER siRNA for 72hrs at 20nM concentration. DICER mRNA was found to be downregulated significantly as a result of siRNA transfection (Figure 12). mRNA data from the same samples indicated no change in MBNL1 mRNA expression in NHF and DM2000, while a non-statistically-significant minor induction of MBNL1 mRNA was observed in DM500 RNA extracts. Protein expression of DICER and MBNL1 was evaluated following siDICER treatment conditions in NHF, DM500 and DM2000 (Figure 13). DICER protein was significantly reduced in siDICER treatment conditions, while MBNL1 protein expression was largely unchanged except in DM500 fibroblasts where MBNL1 protein was induced by ~1.3 fold, although the increase was found to also not be statistically significant. Given the results observed in DM500 with siDICER treatment at 72hrs, we decided to carryout knockdown experiments in DM500 with siDICER at 96hrs as well; however, no change in MBNL1 mRNA and protein expression was observed (data not shown).

Samples from the same cell populations were also analyzed for SERCA-1b expression in

siDICER treatment conditions, with no noticeable change in the expression of SERCA-1b between control and siRNA treated samples detected (Figure 14). This may be partially due to the variability of relative SERCA-1b expression in these samples, which resulted in a large margin of error. As a result, we are not able to definitely deduce whether siDICER has an effect on alternative splicing in DM1 fibroblasts *in vitro*. Analysis of DICER knockdown results seem to suggest that DICER does not modulate key DM1 biomarkers and that PACT modulation of these targets could be via other molecular pathways. However, further research would need to be done to assess the role of DICER in DM1, given the vast role DICER plays in gene regulation via miRNA processing. One such approach might involve an experiment similar to the one outlined for PACT, which is to quantify the amount of bioavailable MBNL1 present in the nucleoplasm in siDICER treated samples. This may provide a more faithful reflection of relative MBNL1 protein activity, exploring the relevance of DICER modulation in DM1. Having said that, it is important to note the role of DICER in global gene regulation via miRNA processing. DICER knockdown leads to the destruction of the majority of miRNAs associated with RISC-silencing, some of which have been implicated in DM1 directly. In addition, relatively recent developments in understanding miRNA processing and specific miRNA research have identified numerous examples of miRNA-miRNA antagonistic relationships (Yu et al. 2009). The global modulation of miRNAs via DICER inhibition could conceivably disrupt antagonistic functions of miRNAs making it difficult to trace back precisely the DICER role in any observed alterations in key DM1 biomarkers within the context of DM1. Conceivably, miRNA suppression by dsRNA-binding siRNA silencing proteins, such as modified p19 proteins could be utilized in order to target miRNA species and evaluate their effect in the context of DM1. In a process similar to the outcome of DICER knockdown, the p19 protein from Carnation Italian Ringspot

Virus (CIRV) is able to bind 21-23 nucleotide dsRNAs with nanomolar affinity in a size-dependent and sequence-independent manner (Jin et al. 2010). This type of experiment would, in all likelihood, shed light on the extent of small RNA involvement in DM1.

4.4.1 Role of miRNAs in DM1

miRNAs have recently been implicated in the pathogenesis of many human diseases (Koscianska et al., 2015). MiRNAs mediate biological effects by binding to 3'UTRs of target mRNAs, which results in the decay, translational suppression as well destruction of targeted mRNAs (Bartel, 2009). Recent studies have revealed aberrant expressions in various miRNA species in DM1. miR-1, the most extensively-studied miRNA in the context of DM1, is downregulated in cardiac muscles (Rau et al., 2011); interestingly, the decreased expression of miR-1 in the heart and corresponding increase of its targets in the heart of DM1 patients are mediated by depletion of MBNL1, which affects the processing of pre-miR-1. In addition, according to Rau et al. 2011, analysis with miRanda, a tool used for miRNA target site prediction, revealed the cardiac L-type calcium channel gene (*CACNA1C*) to be a predicted target site of miR-1 (Rau et al., 2011). *CACNA1C*, one of the alternatively spliced genes in DM1, is the main calcium channel in heart tissues, and mutations in this gene could result in arrhythmias and sudden death (Splawski et al., 2004). Rau et al. confirmed *CACNA1C* regulation by miR-1, as co-expression of miR-1 reduced the expression of a luciferase reporter assay plasmid containing part of the 3'UTR of *CACNA1C*. In addition, overexpression of miR-1 in cardiomyoblasts H9C2 reduced endogenous levels of *CACNA1C*, whereas downregulation of miR-1 increased expression of endogenous *CACNA1C* (Rau et al., 2011). More recently, Fernandez-Costa et al. investigated a transgenic DM1 Drosophila model (CTG480 – Drosophila

line carrying 480 CTG repeats), which revealed that miRNA alterations in DM1 were caused directly by CTG repeats. Comparison between the Drosophila model and human skeletal muscle biopsies revealed 20 miRNAs to have differential expression; 19 down-regulated and 1 upregulated; miR-1 was found to be downregulated in both samples examined (Fernandez-Costa et al., 2013). However, this is in direct conflict with another finding from a previous group where in DM1 skeletal muscles, miR-1 was observed to be upregulated compared to control muscles (Perbellini et al., 2011). In addition to miR-1, other miRNAs have been implicated in DM1 and their aberrant expressions documented. A brief table summary of the recently validated miRNA discoveries in the context of DM1 can be found in Table 2.

The field of miRNA research is fairly new and there is much that is unknown. The sheer number of miRNAs expressed in the genome and the numerous functionalities ascribed to them, either synergistically or antagonistically, has opened the field for the exploration of this class of molecules in human disease mechanism. An effective means of identifying miRNAs involved in DM1 would be by carrying out a large-scale miRNA array study to uncover differentially expressed miRNAs in various tissues. These screens may identify among the many miRNAs that are affected in DM1, those that are clinically relevant to the disease. As well useful biomarkers detectable in human peripheral blood plasma may be identified as has been previously established in the diagnosis and prognosis of various other diseases (Koscianska et al., 2015)

Table 2 – Reported Changes in miRNA Expression in DM1
Source: Koscianska et al., 2014

Source of miRNA	Reported Changes	Method	Comments	References
DM1				
Human muscle biopsies from the vastus lateralis	↑ miR-206	RT-qPCR	Seven unrelated patients, aged 30–50 years	Gambardella *et al*. (2010)
		Northern blot	Total RNAs were pooled (two samples analysed vs. one control)	
Human muscle biopsies from the biceps brachii	↑ miR-1, ↑ miR-335, ↓ miR-29b, ↓ miR-29c, ↓ miR-33	RT-qPCR	Nine affected males and six females, age = 38±17 years old	Perbellini *et al*. (2011)
Human heart left ventricles samples	↓ miR-1	RT-qPCR	Eight adults	Rau *et al*. (2011)
		Northern blot		
Human muscle biopsies from the biceps brachii	↑ miR-208a, ↑ miR-381, ↓ miR-193b-3p	Gene Chip Human Exon 1.0 ST Array (Affymetrix)	miRNAs that were validated as significantly deregulated in DM2 (11 miRNAs) were also tested in an age- and sex-matched cohort of DM1 patients	Greco *et al*. (2012)
Drosophila i(CTG)480 transgenic line	1 miRNA up-regulated, 19 down-regulated	SOLiD™ 3 sequencing	Deregulation of miR-1, miR-7 (given their conservation in humans) and miR-1003 (given its miRtronic nature) validated by northern blotting	Fernandez-Costa *et al*. (2012)
Human skeletal muscle biopsies (biceps, vastus and deltoid)	↓ miR-1, ↓ miR-7, ↓ miR-10a	RT-qPCR	Five patients, aged 47±5 years	

4.5 MBNL1 Insolubility in DM Fibroblasts

We have observed an induction in MBNL1 mRNA and protein expression in DM1 fibroblasts via knockdown of PACT. The increase in MBNL1 expression also coincided with an increase in relative SERCA-1a expression, implicating normalization of aberrant splicing in DM1. We had hypothesized that PACT-mediated reduction of foci in DM1 fibroblasts would lead to an increase in bioavailable MBNL1, which if recapitulated *in vivo* might moderate disease progression. However, the amount of MBNL1 required for the *in vitro* correction of DM1 spliceopathy is currently unknown. As a result, we measured *MBNL1* mRNA and protein levels in normal and DM1 fibroblasts. Preliminary results suggested a relative increase in *MBNL1* mRNA expression in DM2000 compared to normal healthy fibroblasts, whereas DM500 did not elicit any difference (data not shown). This may reflect a compensatory mechanism used in DM1 cells to offset reduced nuclear MBNL1 bioavailability. By extracting protein from the same samples, we compared MBNL1 protein expression as well across control and DM1 fibroblasts. Interestingly, there was no difference in MBNL1 protein expression between NHF and DM1 cells, indicating a differential between mRNA and protein results. This discrepancy could be due to a couple of reasons. First, it could be that the expression of MBNL1 mRNA in these cells does not correlate to the amount of protein expression, possibly due to endogenous translational inhibitory mechanisms in place to limit MBNL1 protein expression in the face of induced MBNL1 mRNA expression. The other possibility, which we decided to investigate further, would be that we are simply not extracting the total amount of MBNL1 protein from our cultured cells, and that the relative expression of MBNL1 protein analyzed thus far does not represent the entire picture.

Protein extraction in the DM1 literature has been done using a standard RIPA lysis buffer

protocol (Oddly, the expression of MBNL1 mRNA and protein in DM1 cells has not extensively explored in previous reports). To investigate total MBNL1 extraction, we adopted a protein extraction protocol similar to as outlined by Winton et al. 2008, whereby through a series of consecutive extraction phases, we attempted to extract potentially insoluble MBNL1 proteins. In their experiments, Winton and colleagues were able to identify an insoluble fraction of TDP-43, which is a disease protein for FTLD-U and ALS (Winton et al., 2008). Similarly, in our experiments, healthy and DM1 fibroblasts were subjected to three rounds of RIPA lysis, with each round initiating at the leftover cell pellet from the previous extraction phase. This was followed up further by a final extraction phase with 7M urea buffer, which has shown to be effective in extracting insoluble as well as soluble protein fractions from samples. In the end we had 4 extraction fractions, each with considerably less protein quantity than its previous phase. By employing a more quantitative stain-free Western blot technique, we were able to quantitatively measure the amount of MBNL1 protein expressed, and thus extracted, in each fraction, to then be compared across our cell line samples (Figures 15-17). By comparing results across multiple cell lines (3 NHF and 7 DM1), we were better able to compare MBNL1 protein extraction in NHF vs. DM1 cells.

Serial-extraction of MBNL1 in fibroblasts revealed that most of the MBNL1 protein is extracted in the initial phase of the extraction. However, a certain portion of MBNL1 proteins eluded in either the second and third extraction phase in both control and DM1 fibroblasts, suggesting that a proportion of MBNL1 in these cells are less RIPA-soluble than the majority. In addition, we detected MBNL1 protein in DM1 cells that is only extracted via 7M urea buffer and not RIPA lysis buffer. Intriguingly, this RIPA-resistant fraction of MBNL1 was detectable in 6/7 DM1 cells (the exception was DM66, which had no nuclear foci detected), whereas it was absent

in NHFs. GAPDH was used as a positive soluble extraction control in these experiments, most of GAPDH proteins was extracted in the initial RIPA-extraction phase (Figure 16). Oversaturation of the MBNL1 signal in these blots revealed the RIPA-insoluble MBNL1 fraction in greater detail (Figure 17). The expression of RIPA-insoluble MBNL1 in DM1 cells did not correlate with the number of CTG repeats in these samples, however was at a significantly higher concentration compared to NHFs. (Figure 18A). We also compared our results with another finding in our lab, done by Nafisa Tasnim, which quantified the number of foci per nucleus in control and DM1 fibroblasts via FISH analysis. The presence of foci directly correlates with the presence of detectible insoluble MBNL1 fractions in DM1. This is further supported by the observation that DM66 fibroblasts not only lack detectable foci in their nucleus and not elicit aberrant splicing defects, but also lack detectable insoluble MBNL1 fractions (Figure 18B). To further explore the source of insoluble MBNL1 fractions in DM1, we decided to fractionate cell lysates to cytoplasmic and nuclear portions, followed by the same serial extraction protocol on the nuclear fraction of these cells (Figure 19). HDAC1 and GAPDH were used as positive extraction controls in nuclear and cytoplasmic fractions respectively. Similar to previous experiments, we observed an insoluble fraction of MBNL1 proteins in DM1 cells, specifically within the nuclear fraction, whereas it was absent in NHFs (Figure 19B). This result suggests that insoluble MBNL1 fractions are present within the nucleoplasm of DM1 cells. We hypothesize foci in DM cells may be a source of increased insolubility of a small fraction of MBNL1 in these cells.

Our extraction protocol is limited in it's scope of quantitatively measuring the concentration of insoluble MBNL1 in DM1 fibroblasts. Development of a more quantitative and comprehensive biochemical assay would shed light on insoluble MBNL1 fractions, as well as the

ratio of soluble to insoluble MBNL1 protein present *in vitro*. Moreover, the significance of these results and whether or how they contribute to disease phenotype remains to be seen. One untested hypothetical model of how insoluble MBNL1 might be interacting in DM1 could be by tightly binding and interacting with DMPK mutant RNA as a first line of interaction, possibly misfolding – changing conformation – and insolubilizing in the process, which would then promote the transient binding of additional MBNL1 proteins in a dynamic structure. A recent report has suggested that MBNL1 binding to foci is dynamic, actively separating and binding to this structure (Jahromi et al., 2013). In their study, introduction of a known potent chemical inhibitor of the CUG-MBNL1 complex was able to bind this complex without dislodging MBNL1, but rather increasing the dissociation rate of MBNL1 from MBNL1-CUG-Ligand complex compared to the initial MBNL1-CUG complex. Although the thermodynamics of this complex interaction have not been studied in detail, it is interesting to note that a change in dissociation rate of MBNL1 to CUG might signify a conformation change in structure of MBNL1 or foci or both as a result of binding one another. Additionally, other reports have shown that MBNL1 knockdown reduces foci formation significantly (Kino et al., 2015 and Dansithong et al., 2005); CUG-repeats alone are not able to form foci aggregates and require co-expression of MBNL1 (Kino et al., 2015). When taken together, these results suggest significant structural role of MBNL1 in foci formation, rather than a passive sequestration. Ultimately, we are unsure of the proportion of total cellular MBNL1 protein this insoluble fraction represents.

Given that the central dogma of DM1 pathology centers on the idea of a lack of bioavailable MBNL1 present in the nucleus of DM cells, it begs the question as to how much MBNL1 would need to be present in order to "saturate" the foci and gain therapeutic benefit by freeing up a sufficient amount of MBNL1 to correct for missplicing. In addition, reduction in the

area and count of foci present in these cells would theoretically free up MBNL1 and make it available. With our recent findings of the presence of an insoluble fraction of MBNL1 in DM1 fibroblasts, MBNL1-CUG interactions could be studied in more detail, potentially providing novel therapeutic approaches in understanding and treating DM1 and related families of pathogenic satellite RNA repeat-expansion diseases.

CONCLUSIONS

PACT presents itself as a potential therapeutic target for the treatment of DM-related phenotypes. Further work, as highlighted in previous sections will need to be done in order to establish confidence on PACT affect in DM1. Furthermore, downstream pathways can be studied in detail to reveal other targets involved in this process. More importantly, other discovered downstream targets might be easier to target with drugs that will make them better therapeutic candidates. DICER on the other hand did not yield highly positive results and might not be a suitable target for drug therapy. Investigation into the miRNAome however remains an interesting field of study that would open more paths towards finding suitable targets. In addition, the discovery of insoluble MBNL1 fractions present in DM1 is novel and has not been discussed previously. Contribution to disease phenotype or rational therapies based on solubilization of MBNL1 present themselves as further research routes in this field.

The premise behind this research is to further our understanding of the mechanisms involved in DM1 pathology, in order to establish better-targeted treatments for this disease. The aim is to provide a novel therapeutic avenue for this disease, which will be mainly focused in translational aspects in order to find key targets that could be regulated with drugs/compounds readily available on the market. This will allow for a reduction in time and cost for developing therapies that are specifically targeted to specific molecular pathways. Further research will provide knowledge in this regard not only for DM1, but also for other rare neuromuscular diseases.

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