MEF2 Isotypes During Skeletal Myogenesis

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ABSTRACT

The MEF2 family of transcription factors (MEF2A, MEF2C, and MEF2D) are crucial during skeletal muscle differentiation. Although the roles of MEF2D isoforms are well established, the roles of MEF2A and MEF2C are not as well understood. This thesis, we investigated the expression, localization, and function of MEF2A and MEF2C, using specific antibodies. While MEF2A is expressed in both proliferating and differentiated myoblasts, protein levels of MEF2C were only detected during differentiation. During early stages of differentiation MEF2A is expressed in both the cytoplasm and the nucleus. However during later stages of differentiation, it is localized predominately in the nucleus. MEF2C appears to be localized differently depending on which isoform is being investigated. Using an affinity purification and mass spectrometry based approach we identified PRMT1 as a unique interacting protein with MEF2A during skeletal muscle differentiation. PRMT1 is a protein arginine methyltransferase which mediates the addition of methyl groups onto various proteins including histone H4 arginine 3 (H3R4) which is associated with gene activation. Both MEF2A and PRMT1 occupy genomic targets of MEF2A. Inhibition of PRMT1 with a specific inhibitor delays C2C12 myoblast differentiation in the early stages of differentiation but no effect was observed during late stage differentiation. The MEF2 family of transcription factors show distinct but overlapping function during skeletal muscle differentiation.

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LIST OF ABBREVIATIONS

Base pairs: bp
Histone methyltransferases: HMTs
Histone acetyltransferases: HATs
Hostone deacetylase: HDACs
MRF: Myogenic Regulatory Factors
Basic helix-loop-helix: bHLH
Myogenic factor 5: MYF5
MEF2: Myocyte enhancer factor 2
Histone Deacetylase: HDAC
Actin: Acta1
Troponin 1 type 2: tnni2
Leiomodin: lmod2
Rhabdomyosarcoma: RMS
Forkhead box protein O1: FOXO1
Mitochontrial DNA: mtDNA
NAHD dehydrogenase 6: ND6
Adenosine Triphosphate: ATP
Ataxia telangiectasis mutated gene: ATM
Protein arginine methyltransferase: PRMT
S- adenosylmethionine: AdoMet
coactivator-associated arginine methyltransferase 1: CARM1
Histone 2A Arginine 3: H2AR3
Histone 2A Lysine 119: H2A K119
Histone 2B Lysine 120/123: H2B K120/123
Histone H3 Arginine 2: H3 R2
Histone H3 Lysine 4: H3 K4
Histone H3: Lysine 9: H3 K9
Histone H3 Arginine 8: H3R8
Histone H3 Arginine 17: H3R17
Histone H3 Lysine 27: H3 K27
Histone H3 Arginine 26: H3R26
Histone H3 Lysine 36: H3 K36
Histone H3 Lysine 56: H3 K56
Histone H3 Lysine 76: H3 K79
Histone H Serine 10: H3 S10
Histone H4 Arginine 3: H4R3
Histone H4 Lysine 16: H4 K16
Histone H4 Lysine 20: H4 K20
Deoxyribonucleic Acid: DNA
Ribonucleic acid: RNA
Ribonucleic acid sequencing: RNA-seq
paired related homeobox : PRRX1
Myosin heavy chain: MYH3
Packed cell volume: PCV
Bovine serum albumin: BSA

Phosphate buffered saline: PBS
Sodium didecyl sulfate polyacrylamide gel electrophoresis: SDS-PAGE
Immunoglobulin heavy chain: IgH
RNA Polymerase II: Pol II
Absent, Small, or Homeotic 2 Like: ASH2L
Mitogen-activated protein kinase: MAPK
Lysine-specific histone demethylase 1A: LSD1
Nonidet P-40: NP-40
Rotations per minute: RPM
Phenylmethanesulfonylfluoride: PMSF
Dimethyl sulfoxide: DMSO
Micrococcal nuclease: Mnase
Chromatin immmunoprecitation: ChIP
Glyceraldehyde 3-phosphate dehydrogenase: GAPDH
Co-Immunoprecipitation: Co-IP
Chromatin Immunoprecipitation Sequencing: ChIP-seq

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CHAPTER 1

INTRODUCTION

Epigenetics and transcription

Almost every cell in a multicellular organism contains the same DNA. However not all cells perform the same function. The differences between cells can be partially explained through differences in the epigenome. Epigenetics adds another level of gene regulation that can allow for cell commitment and differentiation while not changing the DNA (Waddington 1957, Goldberg et al. 2007). Epigenetic changes do not change the sequence of DNA, but they can modify the activity of genes. Epigenetic changes can be classified into DNA methylation, histone methylation, acetylation, ubiquitination, phosphorylation, and sumoylation (Fischle et al. 2003).

In eukaryotic cells, DNA is packaged into nucleosomes, which contain approximately 146 bp of DNA wrapped around a histone octomer which contains 2 copies of histones H2A, H2B, H3 and H4. The nucleosomes are then folded into more compact secondary structures, which are then compacted into the tertiary structures, called chromosomes. The DNA is extremely compacted, in order to fit within the nucleus of a cell. This compaction could create a problem for transcription however this compact organization allows for the establishment and maintenance of specific gene expression through the addition of different marks to histone tails and DNA methylation (Conaway 2012).

Histone proteins are composed of 2 major domains, the globular region and the N-terminus tails that are less structured. Both regions can be highly post-translationally

modified by acetylation of lysine residues, methylation of lysine or arginine residues, phosphorylation of serines or threonines, ubiquitylation of lysine residues, sumoylation of lysine residues. These marks have specialized functions to regulate transcription and in some cases DNA repair, replication, or condensation (Kouzarides 2007). Histone-modifying enzymes and chromatin remodelers modify specific residues on the histones to allow for the specific activation or repression of gene transcription (Conaway 2012). Methylation marks are catalyzed by histone methyltransferases (HMTs) and can be removed by histone demethylase enzymes. The addition of acetyl groups to the histones are carried out by histone acetyltransferases (HATs) and the acetyl groups are removed by histone deacetylases (HDACs) (Allis et al. 2006, Brown et al. 2000). A summary of the specific histone marks, their location, enzyme responsible, and function during transcription can be seen in Table 1.1

Table 1.1. A summary of the histone marks, the location, enzyme which catalyzes the reaction, and the marks' function during transcription (Table modified from Li et al. 2007).

Modification	Position	Enzyme in a mammal	Function during transcription
Lysine Methylation	H3 K4	Mll, All-1, Set9/7, ALR-1/2, ALR, Set1	Activation
	H3 K9	Suv39h, G9a, Eu-HMTaseI, ESET1, SETBD1	Repression, activation
	H3 K27	Ezh2/Ezh1	Repression
	H3 K36	HYPB, Smyd2, NSD1	Repress internal initiation
	H3 K79	Dot1L	Activation
	H4 K20	PR-Set7, SET8	Silencing
Arginine Methylation	H3 R2	CARM1	Activation
	H3 R17	CARM1	Activation
	H3 R26	CARM1	Activation
	H4 R3	PRMT1	Activation
Phosphorylation	H3S10		Activation
Ubiquitination	H2B K120/123	UbcH6, RNF20/40	Activation
	H2A K119	RING1/RNF2	Repression
Acetylation	H3 K56		Activation
	H4 K16	hMOF	Activation

Muscle Differentiation

Skeletal muscle regeneration begins with activation of muscle stem cells, called satellite cells. In healthy muscle, satellite cells reside between the plasma membrane and the basal lamina in a quiescent state and become activated upon muscle injury. The quiescent state of satellite cells is characterized by the expression of the transcription factor PAX7. Skeletal myogenesis is driven by is the expression of the Myogenic Regulatory Factors (MRFs): Myf5, MyoD, Myogenin, and Mrf4 which are all characterized by a basic helix-loop-helix (bHLH) domain. These Pax7+ cells remain in a quiescent state until injury, when they exit the quiescent state. Satellite cells become

activated upon injury, and they commit to the myogenic lineage. Myoblasts are characterized by the expression of Myf5 and MyoD. Myoblasts undergo multiple rounds of cell divisions. On the onset of differentiation the master regulator MyoD activates muscle differentiation specific genes, like myogenin. Ultimately, these myocytes fuse to the damaged muscle fibers and the muscle repair process is complete. Myogenin and Mrf4 are important during the final stages of differentiation (Hinits and Hughes 2007; Rudnicki and Jaenisch 1995, Zhang et al. 2015, Bentzinger et al. 2012, Schienda et al. 2006, Seale et al. 2000, Relaix et al. 2005). When the four MRFs are expressed in non-muscle cells, there is the ability to initiate myogenic gene expression though MyoD and Myf5 appear to be most efficient at this process (Ishibashi et al. 2005).

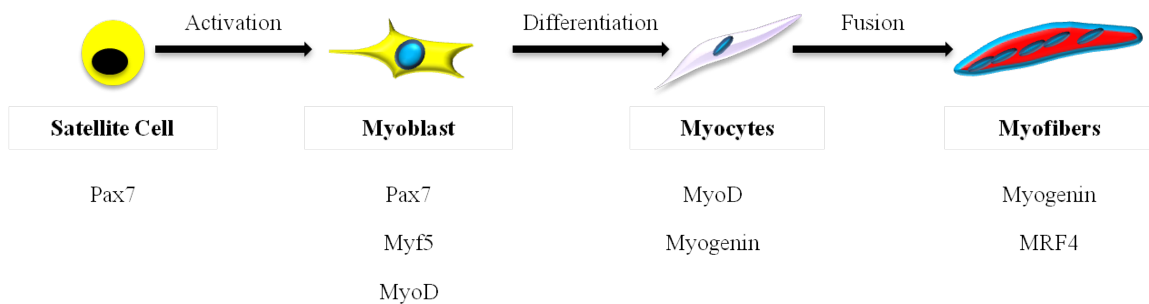


Figure 1.1 The process of adult muscle regeneration in normal healthy muscle. Before injury, the Pax7+ satellite cells remain in a quiescent state. Upon injury they are activated where they proliferate, differentiate, and fuse to give functional muscle. Pax7 identifies the satellite cells; MyoD, myogenin, and MRF4 are important for expressing muscle specific genes (Adapted Bentzinger et al. 2012, Zhang et al. 2015).

The MEF2 Family of Transcription Factors

Another important family of proteins during skeletal muscle myogenesis is the myocyte enhancer factor 2 (MEF2) family of transcription factors. MEF2 was originally identified to have a muscle-specific DNA-binding role during myotube differentiation

(Molkentin et al. 1995). Expanded research has identified their function in differentiation, proliferation, morphogenesis, survival, and apoptosis in several cell types. MEF2 transcription factors play a central role in the transmission of extracellular signals to the genome to ensure the correct genetic program is activated and the transcription factors act with epigenetic machinery, as well as microRNA mediators (Potthoff and Olson 2007).

The MEF2 transcription factors belong to the MADS family of transcription factors. Lower organisms, like *Saccharomyces cerevisiae, Drosophila,* and*, Caenorhabditis elegans* possess a single *MEF2* gene, whereas vertebrates have four genes – *MEF2 a, b, c,* and *d.* The N-terminus of all MEF2 proteins contain the highly conserved MADS-box. Immediately adjacent is the MEF2 domain which mediates dimerization, DNA binding, as well as cofactor interactions. The C-terminus is the transcriptional activation domain, which is divergent among the vertebrate *MEF2* genes. This domain contains sites for post-translational modifications as well as can display variable primary amino acid sequence based on alternative splicing sites selection (Potthoff and Olson 2007) (Figure 1.2).

	MADS	MEF2	
Yeast MEF2	61%	11%	7%
***D. melanogaster* MEF2**	90%	68%	14%
***C. elegans* MEF2**	95%	84%	7%
hMEF2A	100%	100%	100%
hMEF2B	91%	68%	6%
hMEF2C	98%	87%	11%
hMEF2D	95%	82%	16%

DNA Binding, Dimerization, Co-Factor Recruitment

Transcriptional Activation, Post Translational Modifications, Alternative Splicing

Figure 1.2. Schematic representation of the domain organization in the MEF2 family of transcription factors. The MADS and MEF2 domains are relatively conserved throughout evolution. These 2 domains facilitate DNA binding, dimerization with other members of the MEF2 family and co-factor recruitment. The remaining region is relatively less conserved. This is the location are responsible for transcriptional activation. This region is a hot spot for post-translational modifications and also undergoing alternative splicing to generate multiple isoforms. The percentages represents sequence homology relavent to hMEF2A (Figure modified from Potthoff and Olson 2007).

MEF2 and Muscle Regeneration

The MEF2 binding consensus sequence is CAT(A/T)$_4$TAG which is found in the control regions of many muscle specific genes (Sebastian et al. 2013). MEF2 proteins cannot induce the expression of muscle specific genes when transfected in non-muscle cells. However when they are co-expressed with MRFs, there is a dramatic increase in the expression of myosin heavy chain, which is used as an indicator of myogenic conversion. Expression of MRFs increases the expression of MEF2, which then plays a role in amplifying and maintaining the signal in a feed forward mechanism. MRFs

heterodimerize with other bHLH family members and binds to E-box consensus sequences present in the promoter of genes. MEF2 cooperates with this heterodimer to activate transcription of muscle specific genes. (Molkentin et al. 1995).

During muscle regeneration there are slight differences in the timing of the MEF2 family member expression. The expression of *MEF2A* is observed first during differentiation around day 1, *MEF2D* expression begins around day 2, and *MEF2C* expression begins around day 3 as seen in C2C12 and primary myoblasts (Snyder et al. 2013). It appears that during muscle regeneration the different MEF2 isotypes compensate for the loss of each other. When the individual MEF2 isotypes are knocked out in Pax7 expressing cells, there is no difference between the individual knockouts and the control muscle following cardiotoxin injury, indicating possible redundant functions of MEF2A, C, and D during adult skeletal muscle regeneration. However, when all the MEF2 isotypes are knocked out, there is a severe muscle regeneration defect following cardiotoxin injury. This indicates that whatever role the MEF2 isotypes play during skeletal muscle regeneration, it is important for this process (Liu et al. 2014).

A summary of the effects of knockout and knockdown of MEF2 isotypes during development and adult muscle regeneration is mentioned in Table 1.2. The MEF2 family of transcription factors do play a role during skeletal muscle regeneration shown by the extremely poor regeneration following cardiotoxin injury when all of the MEF2 isotypes are knockout (Liu et al. 2014). During muscle development it appears that the MEF2 family of transcription factors play a role in organizing different aspects of the muscle, and some isotypes are important for muscle fiber type decisions (Table 1.2). However in order to fully understand this role, the molecular mechanisms by which these proteins

function must be investigated, and by using a molecular approach the distinct differences will become more apparent.

Table 1.2 A summary of the different phenotypes associated with MEF2 knockdown or knockout mice

Knockdown		
Isotype	Phenotype	References
MEF2A	*In Vitro:* Delay in differentiation, increase in apoptosis Downregulation of muscle structure development genes, genes associated with muscle contraction, and muscle cell differentiation	Snyder et al. 2013 Wales et al. 2014
	In Vivo: Delayed regeneration after cardiotoxin induced muscle damage	
Knockout		
Total MEF2	In Skeletal muscle driven knockout: Poor muscle regeneration following cardiotoxin injury	Liu et al. 2014
MEF2A	Homozygous knockout: No observable phenotype in muscle but MEF2A is required for mitochondrial content and architecture integrity in post-natal cardiac myocytes. Individual knockout in skeletal muscle: no observable phenotype following cardio toxin injury	Potthoff et al. 2007 (A and B) Naya et al. 2002 Ewen et al. 2011 Liu et al. 2014
MEF2D	Homozygous knockout: No observable phenotype in muscle Skeletal Muscle Knockout: reduction in slow-twitch fibers in the soleus Individual knockout in skeletal muscle: no observable phenotype following cardio toxin injury	Potthoff et al. 2007 (A and B) Liu et al. 2014
MEF2C	Homozygous knockout: exhibit early lethality at E9.5 due to cardiovascular defects MEF2C knockout driven by myogenin: Lethality at P1 with disorganized muscle MEF2C knockout driven by muscle creatine kinase: myofibers rapidly deteriorate after birth due to a disorganized sarcomeres, there is also a decrease in the slow fiber types in the soleus muscle Individual MEF2C knockout in skeletal muscle: no observable phenotype following cardio toxin injury	Potthoff et al. 2007 (A and B) Liu et al. 2014

Regulation of MEF2 Activity

There are many different levels of regulation of *MEF2* expression. An interesting feedback loop is the negative feedback loop created with MEF2 and histone deacetylase 9 (HDAC9). HDAC9 is a direct transcriptional target of MEF2 in vitro and in vivo. HDAC9 acts on MEF2 to inhibit its transcriptional activity. This creates a negative feedback loop between MEF2 and HDAC9 (Haberland et al. 2007). Class II HDACs include HDAC4 and 5, and have been shown to inhibit myogenesis and block MyoD function. Class II HDACs do not perform this activity by acting upon MyoD, but rather act upon MEF2 to suppress myogenic activity. This suppression can be overcome by CaM kinase, which induces the shuttling of HDAC5 from the nucleus to the cytoplasm and prevents the formation of the MEF2-HDAC5 complex, insulin-like growth factor (IGF) signaling, as well as an increase in MyoD (McKinsey et al. 2000, Lu at el. 2000).

There are also many posttranslational modifications that can modulate the activity of MEF2 proteins. The posttranslational modifications usually occur in the C-terminus transcriptional activation domain of MEF2 (Potthoff and Olson 2007). MyoD generates a positive feedback loop with MEF2 activity. This pathway allows for MyoD and MEF2 binding on myogenic promoters and aids in MEF2D role in recruiting Pol II (Penn et al. 2004). p38 MAPK signalling is important during muscle differentiation to help fine tune muscle specific gene expression. The Ash2L containing methyltransferase complex mediates the addition of three methyl groups to histone H3 at lysine 4 (H3K4me3) to allow for the expression of the muscle specific gene expression program. The recruitment of Ash2L to muscle specific promoters is regulated through the phosphorylation of MEF2D by p38 MAPK (Rampalli et al 2007, and Aziz et al. 2010). MEF2A, MEF2C,

and MEF2D all have p38 phosphorylation sites. In MEF2A Thr-312, Thr-319, and Ser-453 are phosphorylated by p38. p38 phosphorylates Thr-293, Thr-300, and Ser-382 on MEF2C. In the ubiquitously expressed isoform of MEF2D, MEF2Dα1, p38 phosphorylates Thr-308 and Thr-315. In the muscle specific isoform of MEF2D, MEF2Dα2, p38 phosphorylates Thr-307 and Thr-314 (Zhao et al. 1999). Lysine methylation of K-267 by G9a represses the transcriptional activity of MEF2D specifically and LSD1 is able to counteract this repression. As C2C12 differentiate the methylation by G9a decreases allowing an increase in the MEF2D target myogenic genes (Choi et al. 2013). In MEF2D phosphorylation by PKA on S119 and S190, enables the class II HDAC binding, inhibiting the transcriptional activity of MEF2D (Du et al. 2008).

Alternative Splicing of MEF2D and MEF2C

Transcripts of MEF2 family members also undergo splicing in order to generate more diversity in regards to function. The schematic in Figure 1.3 demonstrates the different isoforms of MEF2C and MEF2D that will be further discussed. MEF2D has a muscle specific isoform, MEF2Dα2, and a ubiquitously expressed isoform, MEF2Dα1 (Martin et al. 1994). There is also alternative splicing during skeletal muscle myogenesis, where the β exon is included during differentiation of each MEF2 gene isoform. It appears that when this alternative splicing event occurs, there is an increase in the expression of MEF2 downstream targets. The MEF2 β+ isoforms induce a stronger expression of downstream targets (Zhu et al. 2005). Sebastian et al. 2013 did an in-depth examination of the muscle specific isoform of MEF2D during skeletal muscle differentiation. It was found that the exon switching allowed the muscle specific isoform

of MEF2D, MEF2Dα2, to escape inhibitory phosphorylation by PKA but this isoforms still retains its p38 MAPK phosphorylation site which allows for the recruitment of Ash2L to activate muscle genes. When MEF2D α2 induced to be expressed in an inducible C2 line, there is an increase in differentiation and myotube formation compared to both control and MEF2Dα1. The cell line expressing MEF2Dα1 did not induce differentiation and fusion. When there was forced expression of MEF2Dα2 after cardiotoxin muscle injury in mice, there was improved muscle regeneration, indicated by an increase in muscle fiber caliber. Also, there was a fiber type switch from primarily fast twitch muscle to a slower muscle isotype with both MHC-IIb and MHC-IIa expressing myofibers. RNA-seq analysis showed that MEF2Dα2 drives the expression of late muscle genes during myogenesis. This difference in phosphorylation events allows for the correct temporal expression of muscle genes, as well as, the optimal level of gene transcription (Sebastian et al 2013).

MEF2C has the γ domain which is excluded from the predominant isoform of MEF2C. When the γ domain is included in MEF2C its transactivation activity is downregulated which allows for the correct level of MEF2C activity (Zhu and Gulick 2004). There also appears to be a muscle specific isoforms of MEF2C, the MEF2Cα2, and a ubiquitously expressed MEF2Cα1 isoform. It appears that the MEF2Cα1 isoform interacts more strongly with HDAC5 compared to MEF2Cα2 in co-immunoprecipitation analysis which could repress muscle specific genes during myogenesis. When the different isoforms of MEF2C were individually transfected into C2C12, there was also an increase in the relative mRNA levels from actin (acta1), troponin 1 type 2 (tnni2), and leiomodin 2 (lmod2). The cells with an overexpression of MEF2Cα2 differentiated better

than both the vector control and the cells transfected with MEF2Cα1, whereas the C2C12 expressing MEF2Cα1 did not differentiate at all (Zhang et al 2015).

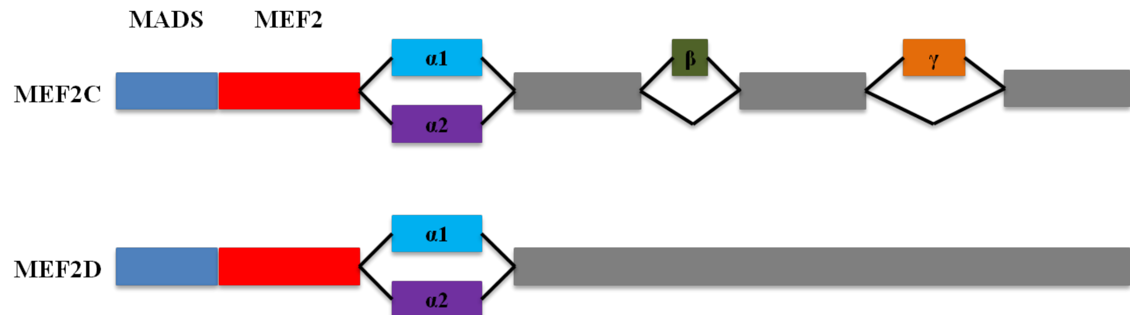


Figure 1.3 A simplified diagram MEF2C and MEF2D splicing (Modified from Sebastian et al. 2013 and Zhang et al. 2015).

Clinical Importance of the MEF2 family of Transcription Factors

Understanding the function of the MEF2 isoforms is not only interesting given the complexity of their regulation but also has clinical applications to allow better understanding of different diseases. MEF2D and MEF2C show downregulated expression in rhabdomyosarcoma (RMS) (Zhang et al 2013; Zhang et al 2015). RMS is a highly malignant tumour and it is believed to be caused by the inability of myogenic precursors to differentiate into normal muscle. MEF2D does not appear to be expressed in RMS cell lines nor does it appear to bind to the TNNI2 promoter. When MEF2D was transfected into RMS cell lines there was an increase in the expression of muscle specific genes, decrease in the proliferation, migration, and the anchorage ability of the RMS cells. The expression of MEF2D inhibited tumour formation in vivo (Zhang et al. 2013). Overexpression of MEF2Cα2, the muscle specific isoforms, appears to increase the myogenic activity to drive differentiation as seen through the expression of myosin heavy

chain (MYH3) in RMS cells while inhibiting proliferation and decreasing the number of anchorage independent colonies (Zhang et al 2015).

Outside of the muscle system, it appears that MEF2D plays an important role in various neuronal processes. She et al. (2014) found that MEF2D can also regulate genes found in the mitochondrial DNA (mtDNA), specifically the NADH dehydrogenase 6 (ND6) which is an important component of complex I of the oxidative phosphorylation system. When MEF2D function was blocked, there was a decrease in complex I activity, increases H_2O_2 levels and reduced ATP production. In the mouse model of Parkinson disease there was a disruption of MEF2D's ability to function and in post-mortem brain samples of patients with Parkinson disease there was decreased MEF2D in the mitochondria and decreased levels of ND6 (She et al. 2014). It also appears the MEF2D plays a critical role in the survival of cerebellar granule cells. The ataxia telangiectasis mutated (ATM) gene is a kinase which phosphorylates and activates MEF2D which contributes to neuronal survival after DNA damage and when MEF2D is knocked out in cerebella, the mice had an increased susceptibility to DNA damage (Chan et al. 2014).

PRMT Family of Arginine Methyltransferases

PRMT1 is a member of the protein arginine methyltransferases (PRMT) family that mediates the methylation of arginine. There are currently eleven characterized members of the PRMT family of methyltransferases that have been identified. This family of methyltransferases mediates the transfer of a methyl group from S-adenosylmethionine (AdoMet) to arginine creating a methylarginine. PRMT1, along with CARM1/PRMT4, PRMT3, and PRMT6 are type I arginine methyltransferases which

generate monomethyl-arginine and asymmetric dimethyl-arginine derivatives. Type II arginine methyltransferase, PRMT5 and PRMT7, generates monomethyl-arginine and symmetric dimethyl-arginine derivatives. PRMT1 methylates arginine3 of histone 4 (H4R3) and to a lesser extent arginine 3 of histone H2A, and this mark is associated with gene activation. CARM1/PRMT4 forms a complex with SWI/SNF ATP remodelling factors, when it is recruited to transcriptional promoters there is an increase in histone H3 arginine 17 (H3R17) and H3 arginine 26 (H3R26) methylation. These marks are associated with transcriptional activation. PRMT5 methylates arginine 8 of histone H3 (H3R8) and arginine 3 of histone H4 (H4R3), this methylation is associated with gene repression (Wysocka et al. 2006, Strahl et al. 2001, Bedford and Richard 2005, Yang and Bedford, Nicholson et al 2009, Bedford and Clarke, 2009). In addition to adding methyl-groups to specific arginines on different histones, PRMTs can also mediate the addition of methyl groups onto arginines of different proteins. PRMTs have roles in a wide variety of cellular functions that include: RNA processing, transcriptional regulation, signal transduction, and DNA repair. PRMTs have a function in RNA processing by mediating the methylation of RNA binding proteins, which acts as a maturation signal as well as regulating RNA-protein interactions by modifying the charge with the addition of the methyl group. Transcription factors can recruit different PRMTs to the promoters of various genes to facilitate histone methylation which could allow for the activation or repression of genes depending on which PRMT was active to mediate transcriptional regulation. Signal transduction can be governed through PRMTs through the addition of the methyl groups to downstream target proteins which could alter protein function and protein-protein interactions allowing the cells to respond to the signal. The role of protein

methylation in DNA damage responses is less studied but it has been shown that a component of the double-strand break-repair complex, MRE-11 is methylated by PRMT1 (Bedford and Richard 2005). PRMT1 appears to be responsible for over 85% of the methylated arginines in mammalian cells (Tang et al. 2000). It also appears that the PRMTs family has a role in cancer, Table 1.3 summarizes the roles of PRMTs in various cancers.

Table 1.3. Table summarizing current knowledge of the associated between PRMT overexpression in different cancer types (modified from Yang and Bedford, 2013).

PRMT	Possible role in Cancer
PRMT1	Overexpressed in: breast, prostate, lung, colon, bladder, leukemia
PRMT2	Overexpressed in: ERα positive breast cancer
PRMT3	High PRMT3 activity in breast cancer with DAL1 loss
PRMT4/CARM1	Overexpressed in: breast, prostate, and colorectal
PRMT5	Overexpressed in: gastric, colorectal, lung, lymphoma, leukemia
PRMT6	Overexpressed in: bladder and lung
PRMT7	Mutations in PRMT8 found in ovarian, skin, and large intestine

PRMTs and Muscle

CARM1/PRMT4 has been shown to be required during muscle differentiation. CARM1, and its cofactor GRIP-1, interact with MEF2C directly to activate MEF2C target genes. When CARM1 activity is inhibited, there is misexpression of important muscle specific genes, like MEF2C and myogenin, which blocks terminal differentiation. CARM1/PRMT4 is also required for chromatin remodelling to allow for gene expression during myogenesis (Chen et al. 2002). PRMT5 appears to be an important regulator of muscle stem cells in adult mice. PRMT5 is not required during fetal myogenesis, but it is required during adult muscle regeneration. PRMT5 directly silences cell cycle inhibitor p21, which allows for the proliferation of muscle stem cells during the early phase of

muscle regeneration (Zhang et al 2015). Dacwag et al (2009) found that both PRMT5 and PRMT4/CARM1 are required for muscle differentiation, however their timing was different. PRMT5 is required for early gene expression, and is not required for late gene expression. PRMT4/CARM1 is required for late gene expression. PRMT5 mediated the dimethylation of H3R8 during early stage differentiation, whereas PRMT4/CARM1 mediates the dimethylation of H3R17 during late stage differentiation. The addition of these methyl marks at the specific times during differentiation facilitates the Brg1-based SWI/SNF chromatin remodelling complex interaction with the correct genes at the correct time, to allow to proper gene expression (Dacwag et al. 2009). Mallappa et al (2011) found that both PRMT5 and CARM1/PRMT4 are required for the expression of myogenic microRNAs. PRMT5 is indirectly required for myogenic microRNA, by acting through myogenin and not directly methylating the histones of the regulatory regions of different microRNAs. PRMT4/CARM1 was found to directly facilitate the dimethylation of H3R17 of the regulatory regions of different myogenic microRNAs. During rat myoblast fusion, it was found that PRMT1 mediated the methylation of lamin A/C (Kim et al. 2011). Iwasaki et al (2007) found that exposing L6 myotubes to insulin caused an increase in PRMT1 catalytic activity, as well as an increase in arginine-methylated proteins, indicating a role of PRMT1 during insulin action in skeletal muscle. PRMT1 also mediates the addition of methyl groups to Arg248 and Arg250 in FOXO1. When these 2 arginines are methylated, there is a block in Akt-mediated phosphorylation at Ser253. When Ser253 is phosphorylated, FOXO1 is exported from the nucleus, and Akt-mediated phosphorylation can lead to FOXO1 proteasomal degradation through polyubiquinitation. By blocking this phosphorylation, PRMT1 prevents the export and

proteasomal degradation of FOXO1, and also increases the transactivation function of FOXO1 (Yamagata et al. 2008).

RESEARCH RATIONAL

Regulation of the skeletal muscle specific gene program is crucial for the development of functional muscle. This is a tightly regulated process where different elements of transcriptional and epigenetic machinery assemble on the regulatory regions of muscle specific genes that allows for the expression of muscle genes at correct times and levels. The MEF2 family of transcription factors play an important role in the fine tuning of the muscle specific gene expression program. This study looks into the role of MEF2A and MEF2C during muscle differentiation to find differences in, not only expression, but also function. We used antibodies specific for the different MEF2 isotypes to investigate specific expression and localization. Protein-protein interactions were performed to identify unique protein interactions between MEF2A and other proteins during skeletal muscle differentiation. This knowledge will add to the growing body of literature of the MEF2 family of transcription factors which will allow for the differential roles of the MEF2 isotypes to become clearer.

HYPOTHESIS

The members of the MEF2 family of transcription factors have overlapping yet distinct roles required for fine-tuning the muscle gene expression program during myogenesis.

OBJECTIVES

1) To characterize expression profile of MEF2 family members during muscle differentiation.

2) To identify a functional difference between members of the MEF2 family.

CHAPTER 2

Materials and Methods

Antibodies

The MEF2A, MEF2Dα1, and MEF2Dα2 rabbit polyclonal antibodies were generated by Genscript against peptides specific for each isotype – SPLSEDRFSKLNEDC for MEF2A, DSVGHSPESEDKYRC for MEF2C, ELDGLFRRYGST for MEF2Dα1, CPQTEEKYKKIDEEF for MEF2Dα2. The MHC antibody was obtained from Developmental Studies Mybridoma Bank. Pan-MEF2 (Santa Cruz Biotechnology, catalog no. sc-13917), Pan-MEF2 – for antibody specification only (Santa Cruz, catalog no. sc-13917), myogenin (Santa Cruz Biotechnology, catalog no. sc12732), HDAC5 (Active Motif, catalog no. 40970), PRMT1 (Abcam, Catalog no. ab7027), tubulin (Developmental Studies Hybridoma Bank, catalog no. 6G7), Histone H3 (Upstate, catalog no. 06-755, 1:1000, PBS).

Antibody specification

Flag tagged MEF2A, MEF2C, and MEF2D proteins were purified from SF-9 cells overexpressing the proteins. The concentrations of each protein was estimated and approximately equal volumes were separated by polyacrylamide gel electrophoresis (PAGE) at a constant 80V for approximately 1.5 hours. Samples were transferred to nitrocellulose membranes (Bio-Rad catalog no. 1620112) after overnight at 20V. Membranes were blocked with 5% milk in PBS for one hour. The membranes were incubated with primary antibodies for 4 hours at room temperature. The membranes were

washed for 30 min with PBST, and incubated with secondary antibody (1:5000) for 1 hour at room temperature. The membranes were washed again for 30 mins. The membranes were developed and protein levels were analyzed.

Cell Culture

C2 myoblasts (Yaffe and Saxel 1977) were grown in Dulbecco's modified Eagle medium with 10% fetal bovine growth serum (FBS), and 1% penicillin/streptomycin. The cells were not allowed to become more than 80% confluent. To differentiate the C2 myoblasts, the cells were grown to 90% confluency, the medium was then changed to Dulbecco's modified Eagle medium supplemented with 2% horse serum and 1% penicillin/streptomycin. The cells were then differentiated for the specified number of days, with a media change every 48 hours.

Whole cell lysate

C2 myoblasts were differentiated for the specified number of days, washed twice with PBS and collected by scraping. Cells were lysed with a NP-40 lysis buffer (50mM Tris HCl pH7.5, 150mM NaCl, 0.5mM $MgCl_2$, 10% glycerol, 0.5% NP-40) on ice for 15 mins, centrifuged at 14800 rpm for 10 min. The supernatant containing the lysate was transferred to a new tube and was quantitated and equal amounts of proteins was applied into individual wells along with 5µL of molecular weight marker with a concentration of 1.56 µg/µl (Bio-Rad catalog no. 1610373). Proteins were separated by SDS-PAGE, the voltage was applied at a constant 80V for approximately 1.5 hours. Samples were transferred to nitrocellulose membranes (Bio-Rad catalog no. 1620112) overnight at 20V. Membranes were blocked with 5% milk in PBS for one hour. Primary antibodies were

incubated for 4 hours. The blots were washed for 30 min with PBST, and incubated with secondary antibody (1:5000) for 1 hour at room temperature. The membranes were washed again for 30 min. The membranes were visualized and protein levels were analyzed.

Immunofluorescence

C2C12 myoblasts were grown on coverslips for up to 5 days of differentiation. Cells were crosslinked using 1% formaldehyde, permeabilized with 0.2% Triton X-100/PBS, and blocked with 5% BSA in PBS. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C. Cells were washed with PBS, after cells were incubated with secondary antibody conjugated with Alexa Fluor546 (Invitrogen, A11010). The nuclei of the cells were stained with 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc., H-1500), mounted and imaged using a Zeiss LSM 510 Meta confocal microscope or Zeiss Observer Z.1.

Subcellular Fractionisation

Cells were resuspended in buffer A in 5xPCV (10mM Hepes K^+ pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 5mM β-glycerol phosphate, 1mM PMSF), cell were centrifuged and resuspended in 2xPCV of Buffer A and homogenized 10 times with a B-pestle homogenizer. The nuclei were centrifuged. The supernatant was mixed with 0.11 volumes of buffer B (0.3M Hepes K^+ pH7.9, 1.4M KCl, and 0.03M $MgCl_2$) and labeled 'cytoplasmic fraction'. The pellet was resuspended in 1.5xPNV of Buffer C (20mM Hepes K^+ pH 7.9, 2mM $MgCl_2$, 420mM KCl, 0.2mM EDTA, 25% glycerol, 5mM β-

glycerol phosphate, 1mM PMSF), homogenized 15 times with a B-pestle homogenizer. Fifty units of benzonase (Merck Millipore, catalog no. 70664) was added to the lysate, and incubated for 1 hour at 4°C. The lysate was centrifuged and loaded into pre-soaked Slide-A-Lyzer dialysis cassettes (Thermo Scientific, Catalog no. 66330). Lysate was dialyzed in buffer D (20mM Hepes K^+ pH 7.9, 1.5mM $MgCl_2$, 100mM KCl, 0.2mM EDTA, 20% glycerol, 5mM β-glycerol phosphate, 1mM PMSF) overnight at 4°C. Nuclear extracts were recovered by centrifugation, and labeled 'nuclear fraction'. Both fractions were quantitated and equal amounts were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad catalog no. 1620112) overnight at 20V. Membranes were blocked with 5% milk in PBS for one hour. Primary antibodies were incubated for 4 hours. The membranes were washed for 30 min with PBST, and incubated with secondary antibody (1:5000) for 1 hour at room temperature. The blots were washed again for 30 min. The membranes were developed and protein levels were analyzed.

<u>Co-Immunoprecipitation</u>

Nuclear extracts were prepared from C2 myoblasts that were induced to differentiate. After 5 days of differentiation, the myotubes were incubated with MG-132 to inhibit protease activity (Calbiochem catalog no. 474790) for 3 hours and then collected by scraping. Cells were resuspended in buffer A in 5xPCV (10mM Hepes K^+ pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 5mM β-glycerol phosphate, 1mM PMSF), cell were centrifuged and resuspended in 2xPCV of Buffer A and homogenized 40 times with a B-pestle homogenizer. The nuclei were centrifuged and resuspended in 1.5xPNV of Buffer C (20mM Hepes K^+ pH 7.9, 2mM $MgCl_2$, 420mM KCl, 0.2mM EDTA, 25% glycerol,

5mM β-glycerol phosphate, 1mM PMSF), homogenized 15 times with a B-pestle homogenizer. 50U of benzonase (Merck Millipore, catalog no. 70664) was added to the lysate, and incubated for 1 hour at $4^{o}C$. The lysate was centrifuged and loaded into pre-soaked Slide-A-Lyzer dialysis cassettes (Thermo Scientific, Catalog no. 66330). Lysate was dialyzed in buffer D (20mM Hepes K^{+} pH 7.9, 1.5mM $MgCl_2$, 100mM KCl, 0.2mM EDTA, 20% glycerol, 5mM β-glycerol phosphate, 1mM PMSF) overnight at $4^{o}C$. Nuclear extracts were recovered by centrifugation, quantitated before being used for interaction studies.

To immunoprecipitate endogenous proteins, 10μg of antibody or control IgG were prebound to protein A Dynabeads (Life Technologies 10002D) and crosslinked using dimethyl pimelimidate (DMP). Nuclear extract was pre-cleared with control IgG and protein A beads for 1 hour at $4^{o}C$ and then added to beads for an overnight incubation at $4^{o}C$. The beads were washed with IP300 (25mM Tris HCl pH7.9, 5mM $MgCl_2$, 300mM KCl, 0.25% NP40, 10% glycerol). The proteins were eluted from the antibodies using the peptide that was used to create the antibody. 200nM of peptide was resuspended in IP100 (25mM Tris HCl pH7.9, 5MM $MgCl_2$, 100mM KCl, 0.25% NP40, 10% glycerol) and added to the beads. The samples were incubated on ice for 10 minutes, and then the supernatant was collected. Two elutions were completed and pooled. If peptide was not available for the antibody, the proteins were eluted using a urea elution buffer (6M urea, 50mM Tris HCl pH 8.3, 5mM EDTA, 0.05% SDS). Samples are separated by SDS-PAGE and visualized by western blot or silverstain.

Mass Spectrometry Analysis

Protein identification was performed at the Ottawa Hospital Research Institute Proteomics Core Facility (Ottawa, Canada) on bands that were excised from a SDS-PAGE gel. A full procedure explanation provided by the proteomics core facility can be found in the appendix. The MS/MS spectra were matched against *mouse* sequences from SwissProt version 2013_5 as well as a database of common contaminants.

Chromatin Immunoprecipitation

Myoblasts were differentiated for 5 days. Proteins were cross-linked to DNA using 1% formaldehyde in Dulbecco's modified Eagle medium for 30 minutes at room temperature The cross-linking reaction was quenched by adding 125mM glycine for 10 minutes, and cells were collected by scraping. Cells were resuspended in 5xPCV of hypotonic lysis buffer (25mM Hepes pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.1% NP-40, 0.3mM PMSF) and incubated on ice for 30 minutes, myotubes were lysed using a 2-mL dounce homogenizer (B-pestle). The lysate was centrifuged and resuspended in MNase Buffer (50mM Tris HCl pH 7.5, 140mM NaCl, 5mM $MgCl_2$ 1mM $CaCl_2$), MNase (Sigma) was added to the chromatin at a concentration of 0.12U per 100µg of DNA and incubated for 10 minutes at 37°C. The MNase reaction was stopped by adding one-tenth volume of MNase stop buffer (11mM EDTA, 11mM EGTA). The chromatin was centrifuged and resuspended in lysis buffer (50mM Tris HCl pH7.5, 150 mM NaCl. 1mM EDTA, 0.5% SDS, 1% Triton X-100) and sonicated using a bioruptor for 80 cycles of 30 seconds on 60 seconds off. Debris was pelleted by centrifugation and the chromatin was precleared with beads, sheared salmon sperm DNA for 1 hour at 4°C. After the chromatin was

precleared it was incubated with specific antibodies. Beads were washed and DNA was eluted and precipitated following a phenol:chloroform precipitation. DNA was analyzed by ChIP-qPCR to examine enrichment of proteins on various promoters and enhancers using SYBR green and specific primers (Qiagen, Catalog no. 204076) following manufactures' specifications in the Rotor-Gene Q real-time PCR cycler. To determine relative enrichment, qPCR amplification of immunoprecipitated samples were normalized to 'Input DNA', which was the DNA added to the beads for the immunoprecipitation. The values are represented as means ± standard deviation. Statistical significance of the difference in enrichment between either MEF2A and PRMT1 and their respective mock immunoprecipitations were estimated by unpaired student t-test.

The following are the sequences of the primers to analyze relative enrichment:

IgH Enhancer

Forward Primer: 5' GCC TGG GCT TGC TTT GTC T 3'

Reverse Primer: 5' TGA CCC CTT TCG CTC ATT G 3'

Myogenin Promoter

Forward Primer: 5' TCA CAT GTA ATC CAC TGG AAA CG 3'

Reverse Primer: 5' CCT GAG CCC CCC TCT AAG C 3'

Prrx1 Promoter

Forward Primer: 5' GAT GAG CAG CAA CTC AGA CC 3'

Reverse Primer: 5' GGG ACG TTT GAG GTG GCA TAA 3'

PRMT1 inhibitor study

C2C12 myoblasts were grown to 90% confluence in growth media, when the media was switched to differentiation media. The media was supplemented with either Dimethyl sulfoxide (DMSO) as the vehicle control or different concentrations of TC-E-5003 ranging from 0.001μ to 10μM. The cells were differentiated for 48 hours, with a media change supplemented with TC-E-5003 or DMSO after 24 hours. At both 24 hours and 48 hours of differentiation cells were fixed for immunofluorescence analysis or used for RNA extraction and qPCR analysis.

Immunofluorescence Image Anaylsis

Immunofluorescence images were taken following the protocol listed above. To quantitate the images the number of nuclei that were positive for myogenin or MYH3 were divided by the total number of nuclei in the field. The values are represented as means ± SEM. Statistical significance of the difference in gene expression between the cells incubated with TC-E-5003 and the vehicle control were estimated by unpaired student t-test.

qPCR Analysis

Total RNA was extracted using RNA Stat-60 (TEL-TEST inc. catalog no. CS-111) following the manufacture's protocol, which is a single-step protocol. A cDNA library was made using M-MuLV reverse transcriptase (New England Biolabs, Catalog no. M0253L), random primers, and 2μg of RNA following the manufacture's protocol. The cDNA was then subjected to qPCR using SYBR green mix and primer sets (Qiagen,

Catalog no. 204076) following manufactures'specifications performed by Rotor-Gene Q Real-time PCR cycler. Forty cycles of qPCR were completed to quantify mRNA transcript levels. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as an internal control and experiments were normalized to it. The values are represented as means ± SEM. Statistical significance of the difference in gene expression between the cells incubated with TC-E-5003 and the vehicle control were determined by unpaired student t-test.

The following are the sequences of the primers used to gene expression analysis:

GAPDH

Forward Primer: 5' ACT CCA CTC ACG GCA AAT TCA ACG 3'

Reverse Primer: 5'GAG CCC TTC CAC AAT GCC AAA GTT 3'

Myogenin:

Forward Primer: 5' CAT CCA GTA CAT TGA GCG CCT ACA 3'

Reverse Primer: 5' AGC AAA TGA TCT CCT GGG TTG GGA 3'

MYH3

Forward Primer: 5' ACC TTG CCA AGA AGA AGG ACT CCA 3'

Reverse Primer: 5' TGG ATG CGG ATG AAC TTG CCA AAG 3'

CHAPTER 3

RESULTS

Expression of MEF2A and MEF2C is Regulated in a Spatiotemporal Manner

The MEF2 Family members are expressed during C2C12 differentiation, we first examined how the expression of MEF2A and MEF2C is altered temporally during myotube formation. In order to investigate the specific MEF2 isotypes, MEF2 antibodies were produced by Genscript against peptides specific for the different MEF2 family members. To test the specificity of the antibodies recombinant MEF2A, MEF2C, and MEF2D proteins were expressed and purified from SF-9 cells. Western blot showed that our MEF2A and MEF2C antibodies were specific for the desired isotype. The commercially available pan-MEF2 antibody from Santa Cruz (sc-17785) did not recognize all MEF2 isotypes, whereas the Santa Cruz pan-MEF2 antibody (sc-13917) did (Figure 3.1). The Dilworth lab MEF2 antibodies are specific for each respective MEF2 isotype.

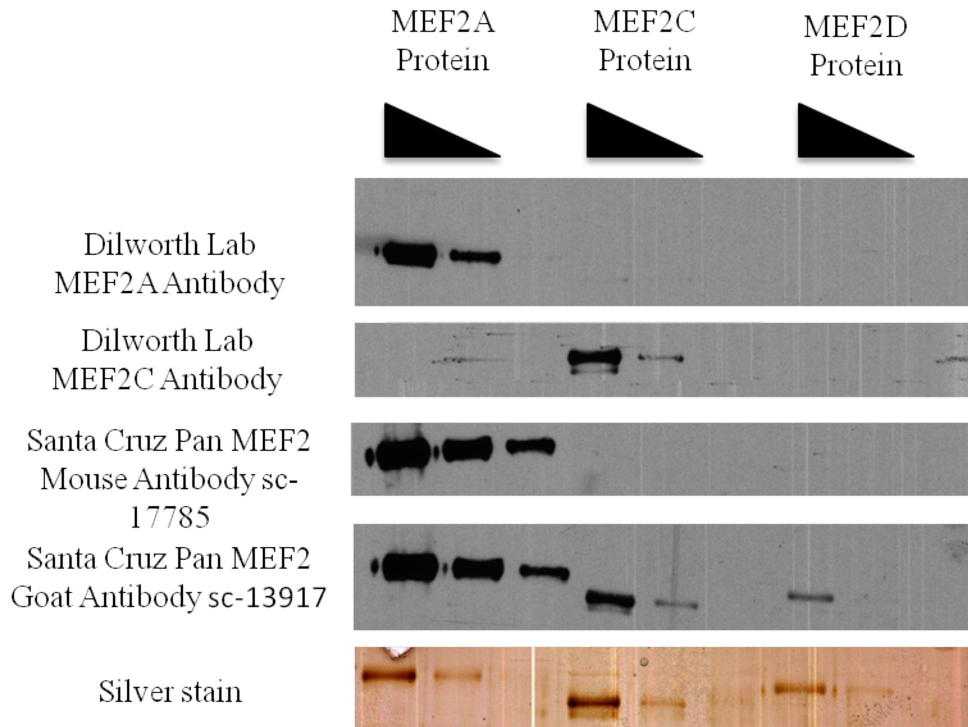


Figure 3.1 Verification of the MEF2 antibody specification. MEF2A, MEF2C, and MEF2D proteins purified from SF-9 cells were separated on SDS-PAGE gels and different MEF2 antibodies were tested to determine specificity. A gel was silver stained as a loading control.

To examine protein expression patterns for MEF2A and MEF2C, C2C12 myoblasts were differentiated for 5 days, and a sample was collected during each day of differentiation, starting at day 0 (D0) through to day 5 (D5). MEF2A expression begins at day 0 and continues through to day 5, whereas MEF2C protein only starts to express around day 2 of differentiation and remains constant between day 3 and day 5 of differentiation. Myogenin expression was used as a positive control to indicate that the C2C12 myoblasts underwent differentiation, and its protein levels increased as the C2C12 cells differentiated from day 0 to day 5, with no proteins detected at day 0 or day1, with the highest protein levels at day 5. The western blot analysis of the temporal changes in protein accumulation during C2C12 differentiation can be seen in Figure 3.2.

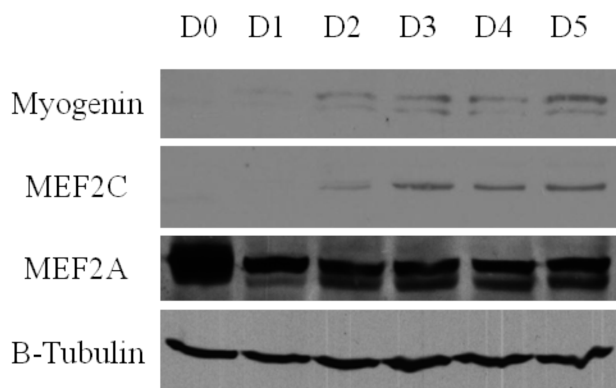


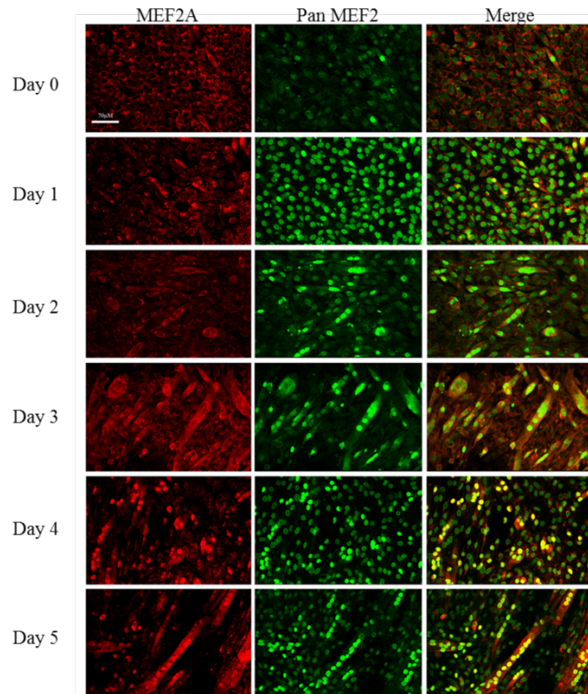
Figure 3.2: Expression patterns of MEF2A and MEF2C. Western blot preformed on whole cell extract from C2C12 cells that were differentiated over a time period of day 0 (D0) to day 5 (D5). The blots were probed for MEF2A, MEF2C, Myogenin, and β-tubulin.

In addition to the protein levels during myoblast differentiation, the protein localization during differentiation was examined for both MEF2A and MEF2C. Immunofluorescence analysis with the MEF2A or MEF2C antibodies along with a Pan-MEF2 was used to determine protein localization. The immunofluorescence images of MEF2A and Pan-MEF2 during C2C12 differentiation can be seen in Figure 3.3A. Similar to the western blot analysis MEF2A proteins were detected starting at day 0, what was striking was the change in location as differentiation continued. Pan-MEF2 remained within the nucleus during the entire C2C12 differentiation time course; whereas MEF2A was found both in the cytoplasm and nucleus during days 0, 1, and 2 of differentiation. After 3 days of differentiation MEF2A localized more strongly in the nucleus. To confirm this observation, another immunofluorescence experiment was completed that looked at 2 days and 5 days of differentiation, and the same pattern was observed at 60x magnifications, as seen in Figure 3.3B. In addition to the immunofluorescence confirmation, C2C12 cells that were differentiated for 2 days and 5 days were collected

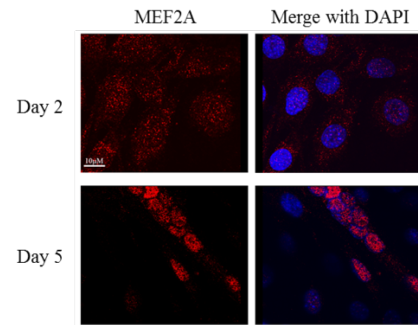
and had cytoplasmic proteins and nuclear proteins isolated through subcellular fractionation. At day 2 of differentiation, MEF2A is found in both the cytoplasm and nucleus fractions, and at day 5 there is an increase in the protein levels in the nuclear fraction. β-tubulin was a loading control for the cytoplasm fraction and histone H3 was a loading control for the nuclear fraction. MEF2C immunofluorescence images, which can be seen in figure 3.4A, show similar results to its western blot results but it was never seen within the nucleus, along with the pan-MEF2. There is limited MEF2C protein during day 0 and day 1 of differentiation. There is some protein at day 2 of differentiation, but the protein can be seen during day 3, 4, and 5 of differentiation. When cells were probed with pan-MEF2C, there was MEF2C found in both the cytoplasm and nucleus. MEF2C showed a similar expression pattern to the Genscript MEF2C, with limited protein expression at day 0 and day 1 of differentiation, some expression at day 2 of differentiation and much stronger expression during day 3, 4, and 5 of differentiation (Figure 3.4B). Both MEF2A and MEF2C have specific spatiotemporal expressions, MEF2A is expressed during the full time course of differentiation and becomes more strongly associated within the nucleus toward the end of C2C12 myoblast differentiation. MEF2C expression begins later during differentiation and its localization might be specific depending on which isoforms is being examined.

The change in MEF2 localization, as well as a lack of understanding of the different isoforms of MEF2C, made MEF2A a more interesting target for further investigation.

A



B



C

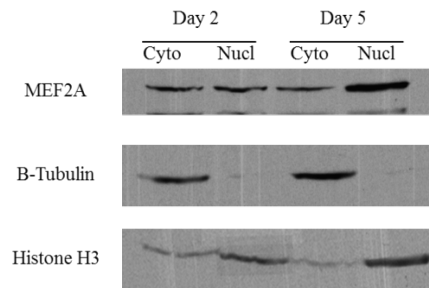


Figure 3.3. Localization of MEF2A during C2C12 myoblast differentiation. Immunofluorescence and confirmation of MEF2A location during differentiation. A) Immunofluorescence analysis preformed on C2C12 cells were differentiated over a time period of day 0 to day 5. Slides were stained using antibodies that recognize MEF2A and Pan MEF2 and imaged with a 20x lens. B) Confirmation of the difference in localization of MEF2A at day 2 and day 5 of differentiation, imaged with a 60x lens. C) subcellular fractionation separating the cytoplasm proteins (cyto) from the nuclear proteins (Nucl) at day 2 and day 5 of differentiation. The blots were probed for MEF2A, β-tubulin, and histone H3.

A B

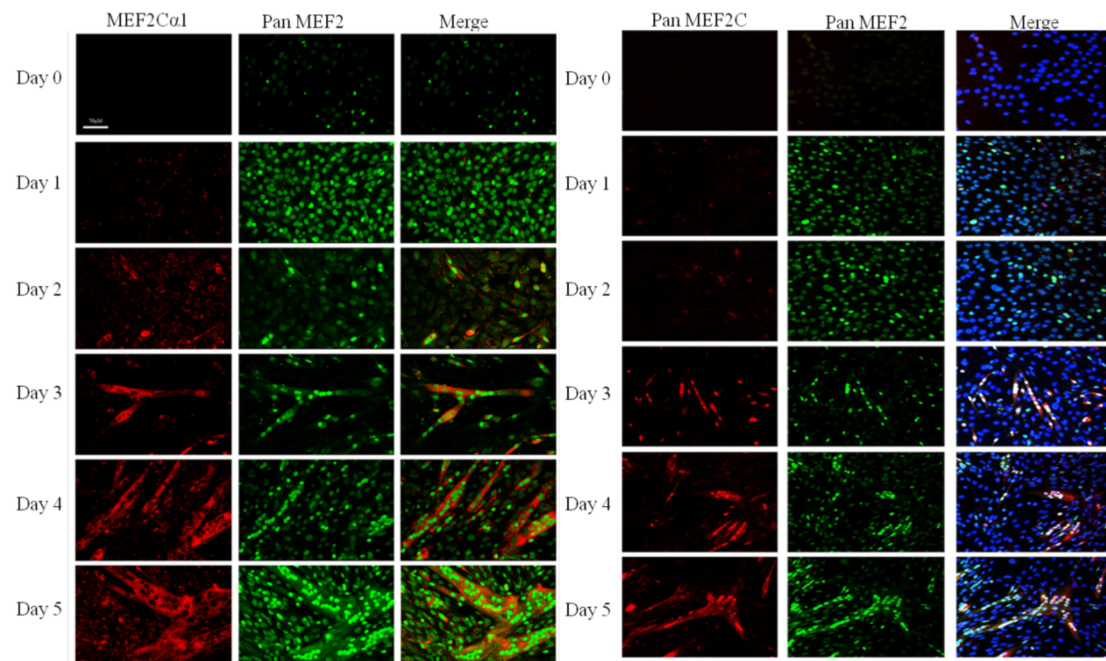


Figure 3.4. Immunofluorescence and confirmation of MEF2C location during differentiation. A) Immunofluorescence analysis performed on C2C12 cells that were differentiated over a time period of day 0 to day 5 of MEF2Cα1 and Pan-MEF2. B) Immunofluorescence analysis performed on C2C12 cells that were differentiated over a time period of day 0 to day 5 of Pan-MEF2C and Pan-MEF2.

Identification of MEF2 Interacting Proteins by Affinity Purification and Mass Spectrometry

Binding profiles between members of the MEF2 family of transcription factors are very similar. MEF2Dα1 and MEF2Dα2 had 5204 ChIP-seq peaks that overlapped, whereas MEF2Dα1 had 2706 individual peaks and MEF2Dα2 had 3899 (Sebastian et al. 2013). Further the DNA binding domain in the MADS and MEF2 domain are conserved between the MEF2 isotypes whereas the transactivation domain is not conserved (Potthoff and Olson 2007). It would be necessary to look at protein-protein interactions to elucidate individual functions between the MEF2 family of transcription factors.

Nuclear extracts were prepared from day 5 differentiated C2C12 cells, and incubated with MEF2A antibody bound to magnetic beads, the beads were washed in a high salt condition with 300mM KCl and the proteins were eluted off the beads by competition using an molar excel of the peptide that served as an epitope when the antibody was generated. MEF2 was successfully immunoprecipitated, as seen in the western blot in Figure 3.5A. The remainder of the eluate was separated by SDS-PAGE and the gel was then silver stained to examine if other proteins were immunoprecipitated along with MEF2A. There were bands in the MEF2A IP lane that were not found in the mock IP lane, as seen by the image of the silver stained gel in Figure 3.5B. The IgG and IP:MEF2A lanes with elute 1 and 2 were cut from the band and sent for proteomics analysis. Table 3.1 contains the list of hits from the mass spectrometry analysis.

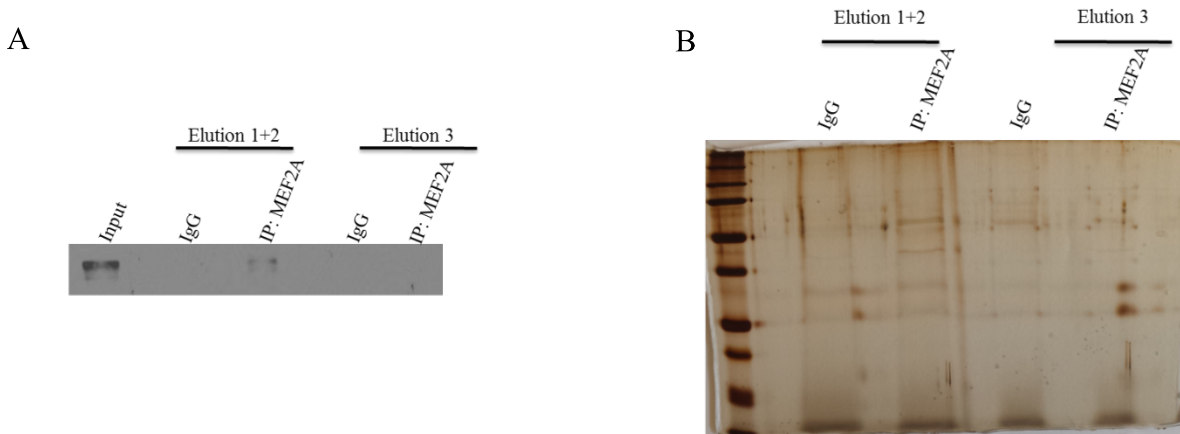


Figure 3.5. Identification of MEF2A interacting proteins using affinity purification and mass spectrometry. Co-immunoprecipitation used MEF2A as the target to find protein – protein interactions. A western blot to confirm that MEF2 proteins were immunoprecipitated by the MEF2A antibody (A). In order to complete the mass spectrometry analysis to determine other proteins that might interact with MEF2A, the eluted proteins from the MEF2A antibody were separated by SDS-PAGE and stained using silver stain (B). The MEF2A and IgG lanes from elution 1+2 were cut and sent to the OHRI proteomics core facility for protein identification.

Table 3.1. Proteins that were identified by Mass-Spectrometry analysis following MEF2A co-IP.

Protein Identified	Protein ID
MEF2A	Q60929
Protein Arginine methyl transferase 1 (PRMT1)	Q9JIF0
Wiz	O88286
Nestin	Q6P5H2
Lamin A/C	P48678
Enhancer of Rudimentry homolog	P84089
Enhancer of mRNA-decapping 4	Q3UJB9
Sodium channel, non voltage gated 1 gamma subunit	Q9WU39
Heat shock cognate 71 kDa protein	P63017
Heterogeneous nuclear ribonucleoprotein M	P49312
Ankyrin repeat domain 40	Q99NH0

The interaction between MEF2A and PRMT1 was investigated further, with a co-immunoprecipitation/western completed using MEF2A and both isoforms of MEF2D as the bait. MEF2A successfully immunoprecipitated PRMT1, whereas PRMT1 was not immunoprecipitated by either MEF2D isoforms. The MEF2D isoforms were successful at pulling down ASH2L whereas MEF2A was not. The co-immunoprecipitation/western blot results for the MEF2A and MEF2D co-immunoprecipitation can be seen in Figure 3.6A. Reciprocal co-immunoprecipitation was completed by using PRMT1 as the bait from Hela nuclear extracted, provided by a member of the Dilworth Lab. PRMT1was successful at pulling down itself and MEF2A. It did not however pull down MEF2D or MEF2C. The co-immunoprecipitation/western blots can be seen in Figure 3.6B. The interaction between MEF2A and PRMT1 is specific for MEF2A and not MEF2D or MEF2C.

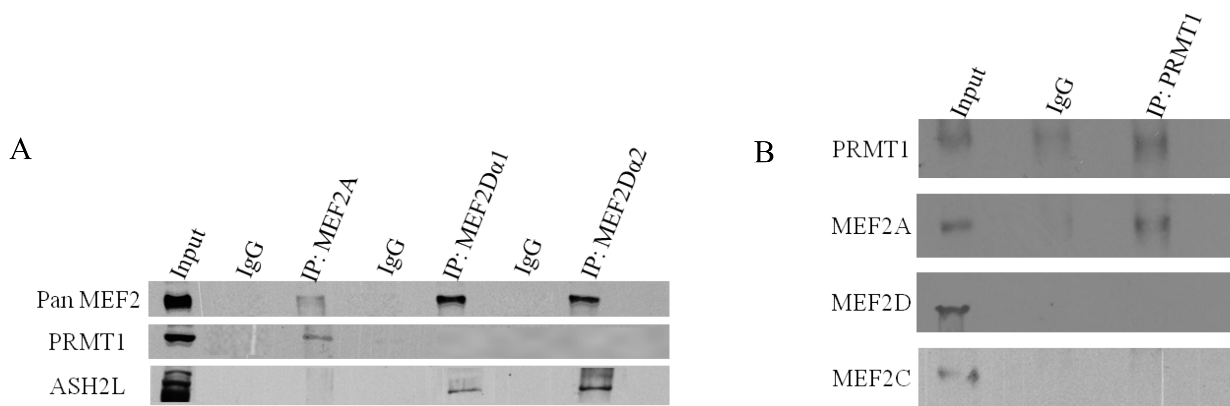


Figure 3.6. Validation of interaction between MEF2A and PRMT1 using co-immunoprecipitation after 5 days of differentiation. A) PRMT1 is only immunoprecipitated by MEF2A, not MEF2Dα1 or MEF2Dα2. ASH2L has been previously shown to interact with MEF2D and was used as a positive control for the MEF2D IPs. B) Reciprocal co-IP which used PRMT1 as the target to pull down other interacting proteins from Hela cells. MEF2A was successfully immunoprecipitated by PRMT1 whereas neither MEF2D nor MEF2C were.

To further investigate the interaction between PRMT1 and MEF2A at MEF2A genomic targets, cross-liked chromatin immunoprecipitations (X-ChIP) and qPCR analysis was completed pulling down DNA bound with either PRMT1 or MEF2A. Both MEF2A and PRMT1 are found of the promoters of myogenin and paired related homeobox 1 (Prrx1), with limited binding on the negative control region, IgH enhancer. There was also limited binding by the mock X-ChIPs. The qPCR graphs can be seen in Figure 3.7. There was a 2.5 fold increase of MEF2A compared to the mock IP, and a 6.7 fold increase in the binding of PRMT1 over the mock IP both on promoter of myogenin. On the promoter of Prrx1, there was a 3 fold increase of MEF2A binding over the mock IP and a 4 fold increase in the binding of PRMT1 over the mock IP. MEF2A and PRMT1 co-occupy the regulatory regions of MEF2A target genes.

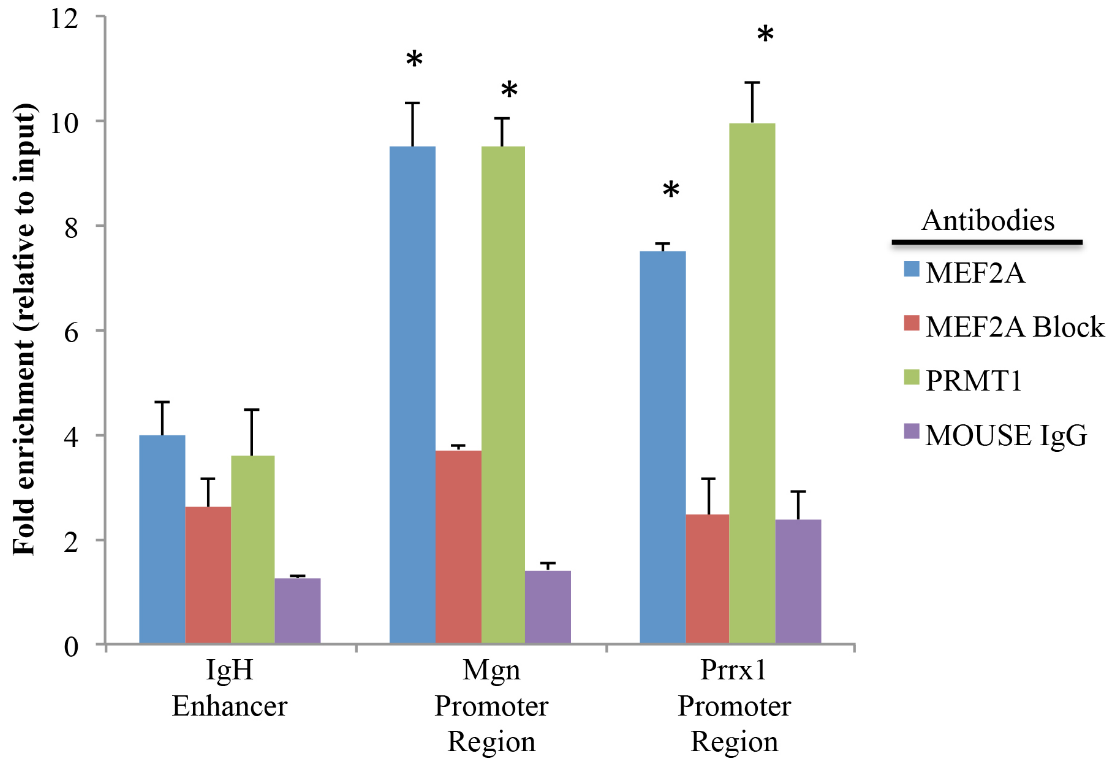


Figure 3.7. PRMT1 and MEF2A co-occupy regulatory regions of MEF2A genomic targets. MEF2A and PRMT1 both appear to bind to the myogenin promoter (Mgn Pro) and paired related homeobox 1 (Prrx1) promoter. Enrichment that significant ($p<0.05$) compared to the mock immunoprecipitation is denoted with an asterisk.

Understanding the Functional Relationship between MEF2A and PRMT1

To begin to understand the functional role of the PRMT1 and MEF2A interaction, PRMT1 inhibitor TC-E-5003 was added to C2C12 during differentiation to examine if there was an effect on myogenesis. When the differentiation media was added to the C2C12 cells, it was supplemented with either DMSO, as the vehicle control, or varying concentrations of TC-E-5003 which ranged from 0.001µM to 10µM. The cells were incubated up to 48 hours, with cells being collected at 24 hours as well as the media and TC-E-5003 being changed after 24 hours. At each time point, 24 and 48 hours of differentiation samples for immunofluorescence analysis and RNA for qPCR analysis

were taken. After 24 hours of TC-E-5003 incubation, there was a decrease in the differentiation index calculated by the number of myogenin positive nuclei and the number of myosin heavy chain 3 (MYH3) positive nuclei as the concentration of TC-E-5003 increased as seen in Figure 3.8A and 3.8B. There was a 1.2 fold decrease in the differentiation index calculated by myogenin positive nuclei in the sample treated with 0.1μM of TC-E-5003 compared to the vehicle control. When the concentration of TC-E-5003 was increased to 10μM there was a 1.35 fold decrease in the differentiation index calculated by myogenin positive nuclei compared to vehicle control. This decrease was also seen in the differentiation index calculated by the number of MYH3 positive nuclei. After 24 hours of differentiation in the presence of 0.1μM of TC-E-5003, there was a 1.75 fold decrease in the differentiation index calculated by the number of MYH3 positive nuclei divided by the total number of nuclei compared to the vehicle control. After 24 hours of differentiation in the presence of 10μM of TC-E-5003 there was a 2 fold decrease in this calculation of the differentiation index. There were also decreases in the mRNA levels, normalized to GAPDH, of both myogenin and MYH3 after incubation with TC-E-5003 for 24 hours, as seen in figure 3.8C. After 24 hours of incubation with 0.1μM of TC-5-5003, there was a 1.8 fold decrease in the levels of myogenin mRNA, when the concentration of TC-E-5003 is increased to 10μM there is a 3 fold decrease in myogenin mRNA compared to vehicle control. An even more substantial decrease in MYH3 mRNA was seen, when the C2C12 cells were incubated with 0.1μM of TC-E-5003 there was a 2 fold decrease compared to the vehicle control, and a 3.7 fold decrease after 10μM incubation compared to the vehicle control. After 48 hours of incubation with varying concentrations of PRMT1 inhibitor, there was no longer any difference between

any of the TC-E-5003 and the vehicle control when comparing the differentiation index, fusion index, or myogenin and MYH3 relative mRNA levels as seen in Figure 3.8. There was no difference between the differentiation index, calculated by the number of myogenin positive nuclei, of the vehicle control or any of the TC-E-5003 incubated samples or the fusion index, calculated by the number of nuclei per tube as seen in Figure 3.9A and 3.9B. There was also no difference between the relative mRNA levels of myogenin or MYH3 between the vehicle control and any of the TC-5-5003 treated samples. The interaction between MEF2A and PRMT1 does appear to have a role during C2C12 muscle differentiation, when PRMT1 is inhibited there appears to be a delay in C2C12 differentiation that is compensated for when other MEF2 proteins are expressed.

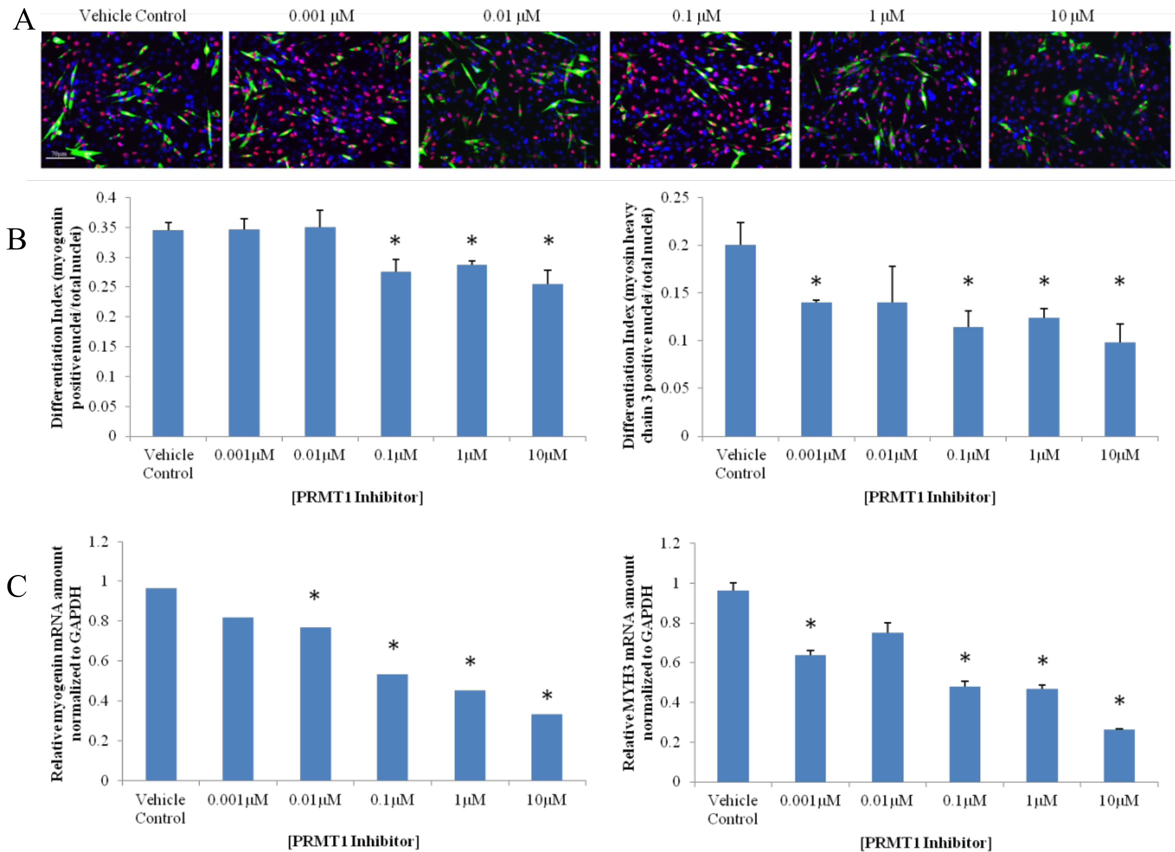


Figure 3.8. The effect of PRMT1 inhibition during skeletal myogenesis causes a decrease in differentiation after 24 hours in differentiation media. TC-E-5003, which is a PRMT1 specific inhibitor, was incubated on C2C12 myoblasts at varying concentration during 24 h of differentiation. A) Immunofluorescence images were taken and quantitated. B) There was a decrease in the differentiation index calculated by both the number of myogenin positive nuclei or MYH3 positive nuclei compared to the total number of nuclei. C) There was a decrease in both the mRNA levels of myogenin and myosin heavy chain 3. Changes in myogenin or MYH3 mRNA or differentiation indices that are significant ($p<0.05$) between a concentration of TC-E-5003 and the vehicle are denoted by an asterisk.

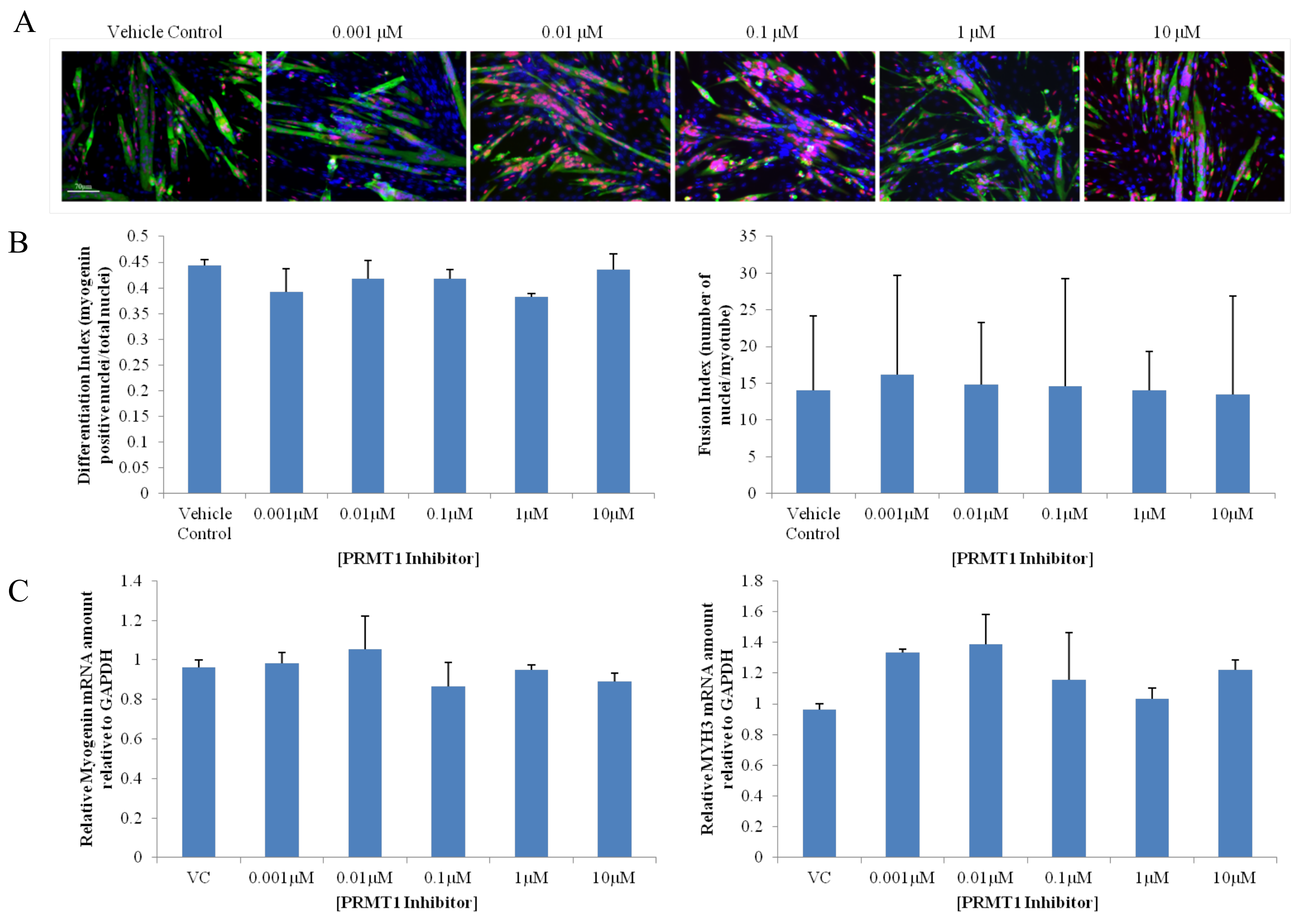


Figure 3.9. After 48 hours of differentiation, the differences between the vehicle control treated cells and the cells treated with TC-E-5003 are no longer visible. A) Immunofluorescence images after 48 h of either TC-E-5003 treatment or vehicle alone. B) The differentiation index, the total number of myogenin positive nuclei divided by the total number nuclei in each field, and the fusion index, the number of nuclei per tube, were calculated. C). The mRNA levels of myogenin and MYH 3 normalized to GAPDH.

CHAPTER 4

Discussion

It was hypothesized that the MEF2 family of transcription factors have overlapping, yet distinct roles required for fine tuning the muscle gene expression program during myogenesis. To begin to understand the role of the MEF2 family of transcription factors, their individual expression profiles were examined. Similar to other published reports (Snyder et al 2013), MEF2A was the first of the MEF2 family members to be expressed throughout differentiation. Sebastian et al (2013) showed that MEF2Dα1 begins to be expressed around day 1. MEF2C begins to be expressed around day 2, and the muscle specific isoform of MEF2D, MEF2Dα2 is expressed at day 3 of differentiation. MEF2Dα1 and MEF2Dα2 remain within the nucleus throughout their individual expression (Sebastian et al 2013). MEF2A is present in both the cytoplasm and nucleus during early differentiation, and it becomes more strongly localized in the nucleus during late differentiation, as confirmed by high resolution immunofluorescence imaging and western blotting. When MEF2A is within the cytoplasm, it has limited access to the transcription machinery. According to the immunofluorescence images of MEF2Cα1 during C2C12 differentiation its expression matches the western blot data however it is never located within the nucleus. When a Pan-MEF2C antibody was used there appeared to be staining in both the cytoplasm and nucleus.

This indicates that there might be multiple isoforms of MEF2C present within the C2C12. Zhang et al (2014) found that MEF2Cα2 is a muscle specific isoform and exhibits the ability to increase the myogenic potential of cells whereas MEF2Cα1 is

ubiquitously expressed and does not appear to have this ability. C2C12 cells might keep MEF2Cα1 out of the nucleus, possibly in favour of MEF2Cα2, during myogenesis, however further study is required to confirm this difference in localization profiles of both MEF2Cα1 and MEF2Cα2 and to better understand the possible mechanism and role MEF2Cα1 and MEF2Cα2 play during myogenesis. Each MEF2 isotype has its own particular expression profile, and while there are some overlapping features, such as each isotype is expressed toward the end of differentiation, there are some very clear differences in when each isotype is expressed or where it is primarily localized.

In order to begin to investigate a possible function of MEF2A, it was important to examine protein-protein interactions. Given the homology between the MEF2 isotypes in the DNA binding domains, looking at genomic patterns of binding might not lead to an understanding of the differential roles (Potthoff and Olson 2007). Sebastian et al. (2013) found substantial overlap in MEF2Dα1 and ME2Dα2 binding patterns throughout the genome. In order to get a clear understanding of the biology of the MEF2 isotypes, having specific antibodies is important. The differences between the interacting proteins might be very minute, and if a pan antibody is used the differences between interacting proteins might not be identified, which is why interacting proteins with MEF2C was not explored at this time. Mass spectrometry analysis of proteins that were immunoprecipitated by MEF2A showed a variety of proteins. On the list PRMT1 appears to be an interesting target given its known role of gene activation by mediating the addition of methyl groups to H4R3 (Wysocka et al. 2006, Strahl et al. 2001). PRMT1 was found to interact only with MEF2A. To further confirm this interaction, X-ChIP was performed and both MEF2A and PRMT1 bind to MEF2 target genes. There was

substantial binding of MEF2A and PRMT1 on the promoter of Prrx1, which was found to be a MEF2A target by MEF2A ChIP-seq (Wales et al. 2014) as well as myogenin. Together this indicates that MEF2A is the only MEF2 isotype that interacts with PRMT1, and this interaction has a duel functional role given that both MEF2A and PRMT1 can bind to promoter of genes involved in the muscle specific gene expression program.

PRMT1 is the methyltransferase that mediates the methylation of arginine 3 of histone 4 (H4R3), which is an activation mark (Strahl et al. 2001). MEF2A could recruit PRMT1 to the necessary promoters and/or enhancers, PRMT1 would then methylate H4R3, much like p53 or YY1 (An et al. 2004, Rezai-Zadeh et al. 2003). This would allow for the correct timing and levels of those genes during myogenesis. To further investigate this possible mechanism, it would be interesting to complete a PRMT1 ChIP-seq, and an H4R3me ChIP-Seq and compare the two with a MEF2A-ChIP seq to investigate the amount of overlap between the 3 different binding profiles. In addition a knockdown of MEF2A cell line can be created and then the recruitment of PRMT1 to regulatory regions can be examined in both the knockdown and wild-type cells. Another possibility to investigate further understand this interaction is to determine if MEF2A is a target for PRMT1 arginine methylation. The MEF2 family members of transcription factors can be differentially modified by the addition of different post-translational modifications. The different phosphorylation sites between MEF2D isoforms allows for the different functions each play during myogenesis (Sebastian et al. 2013), so MEF2A could be methylated by PRMT1 to allow for proper timing and expression of the muscle specific gene expression program. Investigation into MEF2A as a target of PRMT1 methylation would be required to further examine this possibility. To further understand

the function of the individual MEF2 isotypes, knockdown all the endogenous MEF2 isotypes then express the individual isotypes could possibly determine on the individual functions of the MEF2 family of transcription factors.

PRMT1 specific inhibitor TC-E-5003 was used to begin to understand the functional importance of the MEF2A and PRMT1 interaction. During the early portion of C2C12 differentiation, PRMT1 inhibition resulted in an inhibition of differentiation, as seen by the decrease in myogenin and MYH3 at the mRNA and protein levels. During late stage differentiation, there was no longer an effect of the PRMT1 inhibition on C2C12 differentiation. A result that is consistent with a role for MEF2A in fine-tuning the timing of gene expression during muscle differentiation. The myogenin and MYH3 mRNA and protein levels were all similar, and there was no difference when examining the fusion index, which is the number of nuclei in each tube. This result could be explained by the compensatory effect the other MEF2 isotypes can play by acting through alternative pathways. Liu et al (2014) showed that only a complete MEF2 knockout was able to give a muscle regeneration defect. When individual MEF2 family members were knocked out in adult skeletal muscle forced to regenerate following cardiotoxin injury there were no visible differences between the control regenerating muscle and the individual MEF2 knockouts. However when MEF2A, C, and D, were all knocked out there was a severe regenerating defect. The authors, Liu et al, explained this by indicating that the other MEF2 family members are able to compensate the loss of one of the MEF2 genes. During the early portion of C2C12 differentiation MEF2A is expressed, while the other MEF2 isotypes are either not expressed, or are expressed at lower levels so a defect in differentiation can be observed after PRMT1 inhibition.

However during later differentiation, MEF2D isoforms and MEF2C are expressed which could rescue the defect. The order of PRMT1 first, then CARM1/PRMT4 for optimal functionality has been previously seen p53 dependent transcription, where the optimal level of gene p53 dependent activity was seen when PRMT1 was added before PRMT4/CARM1, indicating a preferred order of activation (An et al. 2004). To investigate this possible rescue, it would be interesting to examine what would happen in cells that had the other MEF2 isotypes depleted, except for MEF2A and see if the rescue still occurs.

The interaction between PRMT1 and MEF2A has yet to be described in any cell type. However, it is not the first time a member of the PRMT family of methyltransferases and a MEF2 isotype have been found to interact. Liang et al (2002) found that CARM1/PRMT4 interacts with MEF2C to activate MEF2C dependent transcription during myogenic differentiation. Like PRMT1 inhibition, the inhibition of CARM1 also inhibited muscle differentiation as seen by decreased myogenin and MYH3 protein levels after treatment. Liang et al (2002) reported differences after 72 hours of differentiation, which would be consistent with the expression of MEF2C only beginning around day 2 of differentiation and becoming very strong around day 3. This could be the mechanism that explains why after 48 hours of differentiation we no longer see a defect with PRMT1 inhibition. PRMT1 and PRMT4/CARM1 have been shown to cooperate in gene regulation. When PRMT4/CARM1 and PRMT1 are individually knocked down in HeLa cells there was no significant changes in gene expression, however when they were both knockdown there were more misregulated (Kleinschmidt et al 2008) indicating that there is cooperation between CARM1 and PRMT1 in gene regulation. PRMT1 and

PRMT4 have been shown to interact work together in other systems, Huang et al (2013) showed that PRMT1 and PRMT4 are both involved in the regulation of the antioxidant responsive element (ARE), allowing the transcription factor NRF2 to bind to the ARE and activate transcription of its downstream genes.

A possible mechanism for the interaction between MEF2A and PRMT1 can be seen in Figure 4.1. MEF2A could recruit PRMT1 to muscle specific gene promoters, PRMT1 would methylate H4R3 at the promoters which would activate the expression of the genes.

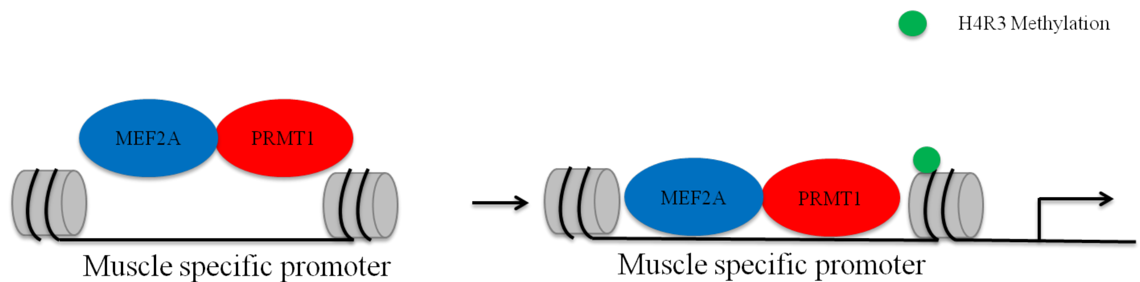


Figure 4.1 A possible mechanism for MEF2A and PRMT1 interaction. MEF2A and PRMT1 bind to regulatory elements of genes. PRMT1 mediates the methylation of H4R3 which activates gene expression.

Understanding the biological function can lead to better understanding of the biology of different diseases, like rhabdomyosarcoma. When either MEF2D or MEF2C are expressed in alveolar rhabdomyosarcomas (aRMS) cell lines, the cells differentiate (Zhang et al 2013. Zhang et al. 2015), indicating a possible treatment option for patients with aRMS. The majority of aRMS patients have either PAX3-FOXO1 or PAX7-FOXO1 chimeric transcription factors, which were created through chromosomal translocations (Renshaw et al. 2013). FOXO1 can be phosphorylated by Akt, which is part of the PI3K/Akt/mTOR pathway. This pathway is active in aRMS clinical samples and this phorphorylation event can be inhibited by methylation of FOXO1 that is mediated by

PRMT1 (Renshaw et al. 2013 and Yamagata et al. 2008). The part of the FOXO1 affected by either Akt or PRMT1 is retained in the chimeric transcription factors (Greer and Brunet 2005). It has been shown that blocking of the PI3K/AKT/mTOR pathway can inhibit RMS cell growth, understanding that in normal cell biology, cells can use PRMT1 methylation to inhibit AKT-mediated phosphorylation. This could lead to better treatment options for patients with overactive PI3K/AKT/mTOR pathway, or at least provide a better understanding of the disease model.

The MEF2 family of transcription factors have a distinct yet overlapping role in fine-tuning the muscle gene expression program during myogenesis. Each family member has a unique expression and localization pattern, with some overlap between family members. MEF2A and MEF2Dα1 are the first family to be expressed during the beginning stages of skeletal muscle differentiation, whereas MEF2C and MEF2Dα2 are expressed during the later stages of differentiation. MEF2A becomes more strongly associated within the nucleus during the later stages of differentiation, whereas the location of MEF2C might depend on which isoform is being examined. PRMT1, the arginine methyltransferase, interacts specifically with MEF2A. The interaction might allow proper timing of an early set of muscle genes.

Future Directions

The MEF2 isotype story is far from complete; there are still large gaps in the understanding of these complex proteins. The mechanism of PRMT1 activity in conjunction with MEF2A needs to be fully understood. As previously mentioned I would like to examine the genomic binding of MEF2A and PRMT1 by ChIP-seq, to examine

which genes have both PRMT1 and MEF2A binding on their regulatory regions which would identify the genes which might be under the control of MEF2A/PRMT1. It would also be interesting to examine if there are differences in H4R3 methylation on the regions that have MEF2A/PRMT1 binding. It would also be interesting to examine the mechanism of this interaction in further detail. To examine if MEF2A is required for PRMT1 binding onto MEF2A/PRMT1 target regulatory regions I would create a MEF2A knockdown cell line and examine PRMT1 binding when MEF2A is knockdown by PRMT1 X-ChIP. To further examine the possible compensation by the other MEF2 family members, I would create a cell line with MEF2C and MEF2D knocked down, and examine the effect PRMT1 inhibition has on differentiation at both the RNA and protein levels.

To better understand the story of MEF2C the differences between the isoforms must be examined. As Zhang et al. 2015 showed, there are differences between MEF2Cα1 and MEF2Cα2 and these differences should be examined in much more details. Similar to the MEF2D isoforms, MEF2C isoforms specific antibodies could be created to examine differences between the isoforms. If isoforms specific antibodies for MEF2C are created, then it might be possible to examine protein-protein interactions, localization and genomic binding patterns examine that could lead to a better understanding of MEF2C biology.

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APPENDIX

Proteomics Core Facility - Service Report

Date:	*May 4, 2015*
Client:	Katherine Reilly / J DILWORTH
Internal Reference Number:	DP559

Sample #	Customer Sample Name
DP559-1	Rabbit IgG
DP559-2	Rabbit IgG
DP559-3	Rabbit IgG
DP559-4	Rabbit IgG
DP559-5	Rabbit IgG
DP559-6	Mef2a 1
DP559-7	Mef2a 2
DP559-8	Mef2a 3
DP559-9	Mef2a 4
DP559-10	Mef2a 5

⮤ *This number uniquely identifies each sample processed at the Core Facility. Please quote service number DP559 in any follow-up correspondence.*

Notes
Since "IgG" vials were not numbered, numbering was randomly assigned to these vials.

Scaffold Formatted Results
The results of the analysis have been exported using a program called *Scaffold* and a "*.sf3*" file will be provided to you. To view the *.sf3* file you will need *Scaffold Viewer* which is a free download for Mac/PC/Linux from
http://www.proteomesoftware.com/products/free-viewer/

Scaffold quick-start tutorial is available at
http://dropbox.ogic.ca/proteomics/Scaffold_Primer.ppsx

Methods

Proteomics
Proteomics analysis was performed at the OHRI Proteomics Core Facility (Ottawa, Canada).

Protein Digest
Proteins were digested in-gel using trypsin (Promega) according to the method of Shevchenko (Nat Protocols 2006; 1(6):2856-60). The resulting peptide extracts

were concentrated by vacufuge (Eppendorf) and resuspended in 1% formic acid (Fisher).

LC-MS/MS

Peptides were analyzed by LC-MS/MS (liquid chromatography – tandem mass spectrometry) on a system comprised of an UltiMate 3000 RSLC nano HPLC, LTQ Orbitrap XL hybrid mass spectrometer and nanospray ionization source (Thermo Scientific). The system was controlled by Xcalibur software version 2.0.7 (Thermo Scientific).

Peptides were loaded by autosampler onto a trap column (Acclaim PepMap C18, Thermo, USA) in 3% acetonitrile, 0.1% formic acid at a flow rate of 15 microlitres per minute for 5 minutes. Peptides were eluted over a 60 minute gradient of 3% - 45% acetonitrile at a flow rate of 300 nanolitres per minute through a 10-cm long column with integrated emitter tip (Picofrit PF360-75-15-N-5 from New Objective packed with Zorbax SB-C18, 5 micron from Agilent), and nanosprayed into the mass spectrometer. Nanoflow HPLC solvents contained 0.1% formic acid and 5% DMSO (Nature Methods 2013; 10(10):989). MS scans were acquired in FTMS mode at a resolution setting of 60,000. MS^2 scans were acquired in ion trap CID mode using data-dependent acquisition of the top 5 ions from each MS scan.

Protein Identification Using Mascot

MASCOT software version 2.5.1 (Matrix Science) was used to infer peptide and protein identities from the mass spectra. The observed MS/MS spectra were matched against *mouse* sequences from SwissProt version 2013_05 and also against a database of common contaminants. Mass tolerance parameters were MS ±10 ppm and MS/MS ±0.6 Da. Enzyme specificity was set to 'Trypsin' with <=2 miscuts. Oxidation of methionine, protein N-terminal acetylation, pyrocarbamidomethlyation of N-terminal cysteine, and conversion of glutamine to pyroglutamate were allowed as variable modifications. Carbamidomethylation of cysteine was set as a fixed modification.

Protein Identification – Peptide numbers

Protein Identified	Peptide number
MEF2A	4
Protein Arginine methyl transferase 1 (PRMT1)	1
Wiz	1
Nestin	1
Lamin A/C	1
Enhancer of Rudimentry homolog	1
Enhancer of mRNA-decapping 4	1
Sodium channel, non voltage gated 1 gamma subunit	1
Heat shock cognate 71 kDa protein	1
Heterogeneous nuclear ribonucleoprotein M	1
Ankyrin repeat domain 40	1