Regulation and Function of Myocyte Enhancer Factor 2 (MEF2) in Myogenic and Neurogenic Cells

Jahan Salma

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Abstract

In eukaryotic organisms, regulation of gene expression at the transcriptional level is a fundamental mechanism which is evolutionary conserved in all cellular systems. It tightly regulates the diversification in expression patterns of genes and proteins required for biological complexity and function. Transcriptional regulation is mediated by the physical interaction between transcription factors and specific *cis-acting* regulatory elements in gene promoter regions. Myocyte enhancer factor 2 (MEF2) is a transcription factor highly conserved in eukaryotes involved in differentiation, proliferation, and survival/apoptosis. The MEF2 gene family (MEF2 A-D) regulates development of various tissue types including muscle (skeletal, cardiac, and smooth muscle), bone, lymphocytes and neurons. The regulation of MEF2 activity is complex, and is coordinated at multiple levels including posttranslational modifications and protein-protein interaction, that together modulate MEF2's function. Conversely dysregulation of MEF2 activity underlies pathogenesis in muscle and neuronal cells. MEF2 is responsive to various signaling cascades which provide a way for distinct stimuli to differentially regulate MEF2-dependent gene expression. It is known that phosphorylation by kinases is an important process through which the activity of MEF2 is up- or down-regulated. Several kinases (p38MAPK, CDK5, PKC, and ERK5) have been linked to muscle and neuronal development, as well as survival in part due to their modulation of MEF2 function. In addition MEF2 is known to be targeted by co-repressors, such as class IIa histone deacetylases (HDAC 4, 5, 7 and 9). This interaction contributes to repression of

MEF2-dependent gene expression. Although MEF2 family members are critical regulators of skeletal muscle differentiation and cardiovascular function their individual roles within nervous system are less well characterized.

In current studies, we attempted to investigate the posttranslational regulation of MEF2 both in myogenic and neurogenic cells. The cAMP/protein kinase A (PKA) signaling pathway regulates a variety of cellular functions and numerous important biological processes. Many of the effects of cAMP/PKA are mediated via changes in gene expression. We have previously documented that cAMP/PKA signaling negatively regulates MEF2 activity and inhibits myogenesis by direct phosphorylation of MEF2 proteins. MEF2 A-D are highly expressed in multiple regions of the brain, including cortex, cerebellum, and hippocampus. Distinct patterns of expression during pre- and postnatal development suggest specific functions for MEF2 proteins at different stages of neuronal maturation and survival. However, whether the cAMP/PKA pathway inhibits MEF2 mediated gene expression in neurons was unclear. Recently, we evaluated whether cAMP/PKA signaling can inhibit MEF2-dependent gene expression directly or indirectly and survival role of MEF2D in hippocampal neurons. We performed survival assays to determine PKA effects in neuronal cells. We observed that experimental induction of cAMP/PKA signaling promotes apoptosis in primary hippocampal neurons as indicated by TUNEL and FACS analysis. Luciferase reporter gene assays revealed that PKA potently represses MEF2D *trans*-activation properties in neurons. Krüppel-like factor 6 (KLF6) was identified as a key transcriptional target of MEF2 in hippocampal neurons and siRNA mediated knockdown of KLF6 expression promotes neuronal cell

death and also antagonizes the pro-survival role of MEF2D. In this study, we found that cAMP/PKA signaling represses KLF6 transcriptional activity and induce neuronal apoptosis by phosphorylating MEF2 and preventing HDAC4 export from the nucleus. These observations characterize a potent inhibitory effect of PKA on the transactivation properties of MEF2D leading to repression of KLF6 expression and compromising neuronal survival (Chapter III).

Next, we were interested to determine how MEF2 controls diverse cellular processes in muscle development in the presence/absence of cofactors. Tandem Affinity Purification (TAP) combined with mass spectrometry analysis was employed in the current studies to identify MEF2 interacting cofactors. We identified Strawberry notch 1(Sbno1) as a novel interacting factor of MEF2D which is known to be downstream effecter of Notch signaling. Notch signaling is known to block the expression and activity of myogenic factors such as MEF2s. We therefore characterized the mechanism of myogenic inhibition by Notch-Sbno1 signaling. C2C12 myoblasts provide a useful *in vitro* model to study skeletal muscle differentiation. We determined the expression patterns, by western blot analysis, of muscle specific gene expression during myogenesis. Sbno1 represses MEF2 transactivation properties and plays a critical role in inhibition of skeletal muscle differentiation. Immunocytochemistry analysis suggests that Notch-Sbno1 might be involved in maintaining the "reserve" cell population. Our data suggested that protein-protein interactions between Sbno1 and MEF2D result in interference with the function of myogenic factors (Chapter IV).

MEF2D is a known transcriptional regulator of muscle differentiation. Current studies identified KLF6 as a novel MEF2D target gene which is involved in hippocampal neuronal survival. TGFβ has been reported as a potent inhibitor of myogenic differentiation by maintaining myoblasts in a proliferative state (undifferentiated myoblasts). Further, TGFβ and KLF6 regulate each other's expression in other cell types. We therefore sought to investigate the possible role of KLF6 in a myogenic context and assessed whether TGFβ activation regulates KLF6 protein expression and function in a MEF2 dependent manner in C2C12 myoblasts (Chapter V).

Taken together, these studies indicate that differential activation of signaling cascades and co-factors regulate the MEF2 transcriptional complex which has profound effects on gene expression in myogenic and neurogenic cells.

Dedication

I dedicate this dissertation to my parents with thanks for their love, support, encouragement and faith…

...but most especially to my father, my favourite person, Muhammad Ibrahim (Late), for his never ending love, support and inspiration
Thank you for everything…

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I would like to thank first to Almighty God, the most benevolent, merciful and Whose blessings enabled me to perceive and pursue the higher ideals of life. Special praise to his last messenger (PBUH), Who is forever a torch of knowledge and guidance for humanity as a whole, that enabled me to complete this work.

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Jahan Salma

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List of abbreviations

ACTH	Adrenocorticotropic hormone
Arc	Activity-regulated cytoskeleton
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BDNF	Brain-Derived Neurotrophic Factor
bHLH	Basic Helix-Loop-Helix
BMK	Big mitogen-activated protein kinase
BNST	Bed nucleus of the stria terminal
BPKDi	Bipyridyl PKD inhibitor
BSA	Bovine serum albumi
BTEB	Basal transcription element binding
CaMK	Ca2+/calmodulin-dependent kinase
cAMP	Cyclic adenosine 3′,5′-monophosphate
CARM1	Co-activator-associated arginine methyltransferase1
CBF1	C promoter-binding factor 1
CBP	CREB Binding Protei
CDK	Cyclin Dependent Kinase
CGN	Cerebellar granule neuron
CHO	Chinese hamster ovar
ChIP	Chromatin Immuno-Precipitatio
CKII	Casein kinase II
CMV	Cytomegalovirus
CNS	Central nervous system
CPBP	Core Promoter Binding Protein
CREB	Cyclic AMP Responsive Element Binding Protein
CtBP	C'-terminal Binding Protein
DAB	diamino benzidine
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
dbcAMP	Dibutyryl cyclic adenosine monophosphat
DG	Dentate gyrus
DM	differentiation mediu
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DSL	Delta, Serrate/ Jagged, and Lin-12 and Glp phenotype-2
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid tetrasodium salt dehydrate

EGF	Epidermal Growth Factor
EKLF1	Erythroid Kr¨uppel-like factor 1
ERK	Extracellular signal-Regulated Kinase
FITC	Fluorescein Isothiocyanate
FSK	Forskolin
β-Gal	β-galactosidase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA	Globin transcription factor1
GBF	GC-rich sites binding factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GM	Growth medium
GTP	Guanosine-5'-triphosphate
GRIP-1	Glucocorticoid receptor-interacting protein 1
GSK3β	Glycogen Synthase Kinase-3β
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
Hes1	Hairy and enhancer of split-1
HRP	Horse radish peroxidase
HS	Horse serum
IgG	Immunoglobulin G
IGF1	Insulin-like Growth Factor-1
IL	Interleukin
JNK	c-Jun N'-terminal Kinase
KLF6	Kruppel-Like Factor6
LAG 1	Longevity-assurance gene-1
LS	Lateral septum
LTD	Long-term depression
LTP	Longterm potentiatior
LTC4S	Leukotriene C4 synthase
MADS	MCM1, Agamous, Deficiens, Serum-response facto
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein Kinas
MASH1	Mammalian achaete-scute homologs
MB	Myoblast
MCK	Muscle creatine kinase
MCM1	Yeast mating type decisions
MEF2	Myocyte Enhancer Factor-2
MITR	MEF2 interacting transcriptional repressoı
MRF	Myogenic Regulatory Factor

MS	Mass spectrometry
Myf5	Myogenic Factor-5
MyoD	Myogenic Differentiation 1
MyoG	Myogenin
NES	Nuclear Export Sequence
NFAT	Nuclear factor of activated T
NGF	Nerve growth factor
NICD	Notch Intracellular Domain
NLS	Nuclear localization signal
NMDA	N-methyl-D-aspartate
Numb	Negative regulator of Notch
OPNG	O-nitrophenyl-beta-d-galactopyranoside
p38MAPK	p38 Mitogen Activated Protein Kinase
PCAF	P300/CREB-binding protein-associated factor
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PGC-1 α	PPAR-gamma coactivator 1-alpha
PI	Propidium iodide
PKA	Protein Kinase A
PKC	Protein Kinase C
PKD	Protein Kinase D
PMSF	Phenyl methyl sulfonyl fluoride
PNS	Peripheral nervous system
PP1	Protein phosphatases
PPAR γ	Peroxisome proliferators activated receptor γ
PSG5	Pregnancy-specific glycoprotein 5
RMS	Rostro-migratory stream
RNAi	RNA interferance
RT-PCR	Reverse transcriptase PCR
Sbno1	Strawberry notch 1
SCA1	Spinocerebellar ataxia type-1
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SIK	Salt-Inducible Kinase
SIRT1	Sirtuin (silent mating type information regulation-2) homolog
Smad	Mothers against decapentaplegic homolog
Sp1	Trans-acting transcription factor 1
SRC	Steroid receptor co-activator
SRF	Serum Response Factor
Su (H)	Suppressor of Hairless

SUMO	Small Ubiquitin-like Modifier
SVZ	Subventricular zone
synGAP	Synaptic Ras GTPase activating protein
TAD	Transcriptional activation domai
TAP	Tandem Affinity purification
TEMED	N,N,N',N'-tetramethylethylene diamine
TEV	Tobacco etch virus
TGFβ	Tumour Growth Factor β
TNF	Tumor necrosis factor
TRITC	Tetramethylrhodamine iso-thiocyanate
TSA	Trichostatin A
TSS	Transcriptional Start Site
TUNEL	Terminal deoxynucleotidyl transferase UTP-biotin nick end labelling
Ubc	Ubiquitin-conjugating enzyme
Upa	Urokinase plasminogen activato
VSMC	Vascular smooth muscle cells

Chapter I: Literature Review

In higher eukaryotes, each cell type has a unique molecular organization that is ultimately controlled by a complex network of transcription factors, which activate or repress transcription of a multitude of downstream genes (Brivaniou & Darnell Jr., 2002). Transcription factors are DNA binding proteins that regulate gene expression by binding to promoter regions proximal to gene transcription start sites (TSSs) or to more distal enhancer regions that regulate expression through long-range interactions (Farnham, 2009). The instructions for gene expression are started with the initiation of transcription process that involves the transcribing of genetic information from DNA to RNA. There are three major steps to the process of DNA transcription including binding of transcription machinery to DNA, elongation, and termination (Brivaniou & Darnell Jr., 2002). Transcription factor binding to DNA requires appropriate binding *cis*-elements which help to define regulatory elements initiation sites within the genome. Transcription factor binding depend upon cell types, and one major factor contributing to this cell type-specific binding is physiological function (Howard & Davidson, 2004). However, the binding of transcription factors to their cognate sites is often insufficient to account for the patterns of expression of their target genes (**Figure 1**). Many transcription factors are also relatively weak transcriptional activators and function by recruiting co-activators/co-repressors that do not bind DNA directly but regulate transcription in a DNA sequence specific manner by associating with DNA-bound factors (Spiegelman & Heinrich, 2004).

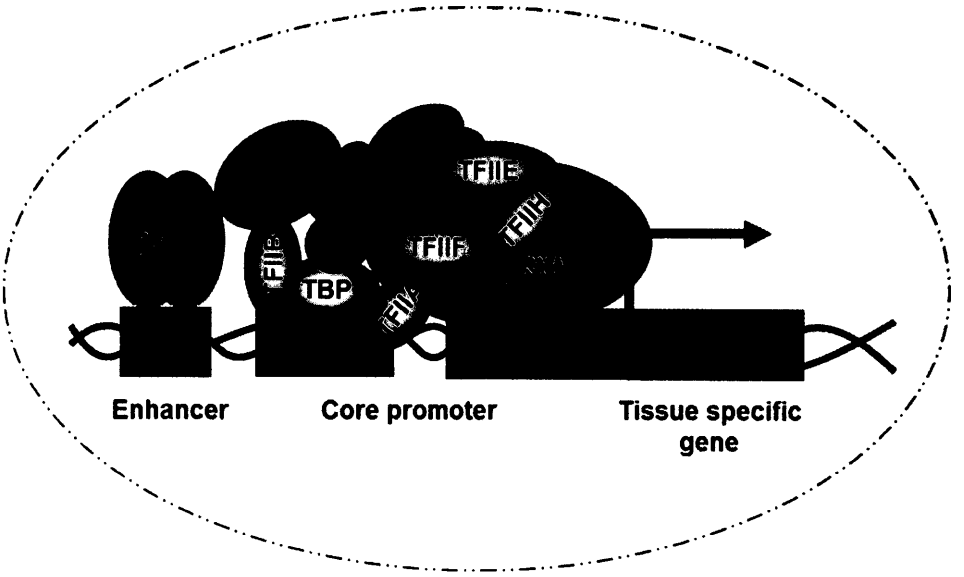

Figure 1: Schematic of regulation of transcription initiation of a typical eukaryotic gene.

The process of embryonic development, cell growth, differentiation, and survival are tightly connected with the regulation of gene expression that require extracellular and intracellular signals and subsequent activation of cascades of signalling machinery (McKinsey et al. 2002). The myocyte enhancer factor (MEF2), a family of transcription factor, have been shown to play critical role in transmitting extracellular signals to the genomic machinery and in the activation of the genetic programs that control critical cellular processes including cell proliferation, differentiation, morphogenesis, and survival of wide range of tissue types including muscle, T cells, and neurons (Matthew & Olson 2007). In adult tissues, MEF2 also serves as a key regulator of stress responses and adaptive

programs in response to environmental signals, including fiber-type switch of skeletal muscle, cardiac hypertrophy, activity-dependent remodeling of neuronal synapses, and neuronal survival (Ornatsky & McDermott, 1996; Flavell et al. 2006 & 2008; Salma & McDermott, 2012; Akhtar et al. 2012).

1. Evolution and origin of MEF2 genes

Changes in genes encoding transcriptional regulators represent the most important determinants of morphological evolution in plants and animals (Degnan et al. 2009; Wu et al. 2011). A duplication event occurred more than a billion years ago in the MADS (MCM1, agamous, deficiens, serum-response factor) box lineage before the divergence of plants from fungi and animals (Theissen et al. 1996; Alvarez-Buylla et al. 2000). The evolution of the MADS-box gene subfamilies based on the taxonomic distribution revealed that each family member comprises highly conserved putative orthologs and recent paralogs (Theissen et al. 1996; Wu et al. 2011). Phylogenetic analysis has shown that at least one ancestral MADS-box gene duplicated in the common ancestor of the major eukaryotic kingdoms to give rise to the distinct Type I (SRF-like) and Type II (MEF2-like) lineages found in plants, animals, and fungi (Alvarez-Buylla et al. 2000) (**Figure 2**). The defined species distribution, specific function, and strong evolutionary conservation of the MADS-box gene subfamilies suggest that establishment of different subfamilies occured during eukaryotic evolution by rapid fixation (Alvarez-Buylla et al. 2000). The evolution of MEF2 proteins is linked to their diverse role to the origin of

novelties in multicellular eukaryotes and function at the core of an ancient regulatory network for diverse tissue formations (Genikhovich & Technau, 2011).

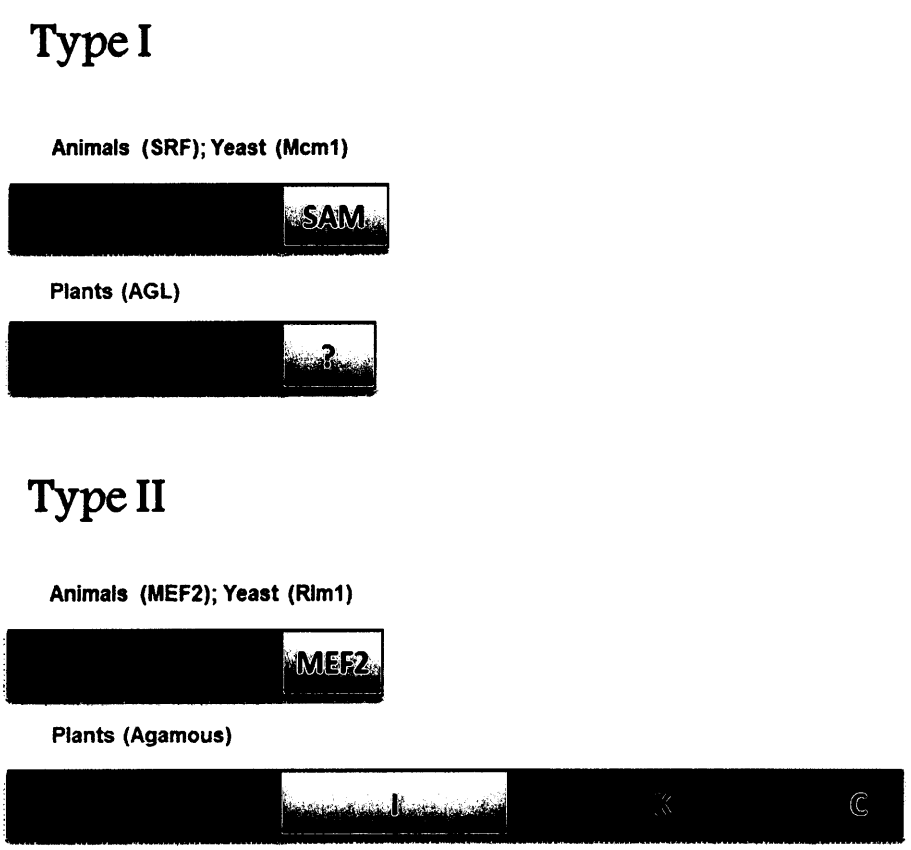

Figure 2: Schematic representation of the protein domains of plant, animal, and fungal Type I (SRF-like) and Type II (MEF2-like) MADS-domain proteins.

1.1. MADS-box proteins

The MADS-box encodes a novel DNA-binding domain found in a diverse group of transcription factors from yeast, animals, and plants. Members of the MADS-box transcription factor family play essential roles in developmental process

in plants and animals (Shore & Sharrocks, 1995). Many MADS-box genes have conserved functions across the animals, but some have acquired novel functions in specific species during evolution. The analyses of MADS-domain protein interactions and target genes have provided new insights into their molecular functions (Shore & Sharrocks, 1995). The MADS- box genes encode for a family of highly conserved transcriptional regulators. The earliest members of MADS family identified as MCM1 from yeast (*Saccharomyces cerevisiae*) (Passmore et al. 1989), AGAMOUS from plant (*Arabidopsis thaliana)* (Yanofsky et al. 1990), DEFICIENS from plant (*Antirrhinum majus)* (Sommer et al. 1990), and serum response factor (SRF) from human (*Homo sapiens)* (Norman et al. 1988). Plants contain a considerable number of MADS-box genes (e.g. 107 in *A. thaliana).* By contrast, only a few MADS-box genes are present in animals (two in *Drosophila melanogaster*, and five in human) and four in yeast, *S. cerevisiae* (Shore & Sharrocks, 1995). MADS- box proteins involves in a diverse range of important biological functions such as morphogenetic development in plants (Thakare et al. 2008; Adamczyk & Fernandez, 2009), immediate early gene expression, differentiation and maintenance in animals (Black & Olson, 1998; Potthoff & Olson, 2007; Flavell et al. 2008), cell–type-specific transcription and pheromone response in yeast (Herskowitz, 1989; Shore & Sharrocks, 1995). These gene subfamilies may have been essential prerequisites for the establishment of several complex eukaryotic body structures, such as muscles in animals and certain reproductive structures in higher plants, and of some signal transduction pathways (Molkentin & Olson, 1996; Thakare et al. 2008; Adamczyk & Fernandez, 2009).

The MADS-box proteins are characterized by the MADS domain considered to consist of 55–60 amino acids in the N terminal region responsible for DNA binding, protein dimerization, and cofactor interactions (Pellegrini et al. 1995; Shore & Sharrocks, 1995; Ng & Yanofsky, 2001; Immink et al. 2002; Messenguy & Dubois 2003). The MADS domain folds into an N-terminal extension, followed by a long amphipathic α-helix and two antiparallel β-strands. Most MADS proteins bind to A/T-rich DNA sequences in common. However, there are distinct consensus sequences: SRF binds as a homodimer specifically to a 10-bp consensus region $CC(A/T)_6GG$, called the CArG-box or SRF site (Pollock & Treisman, 1990), whereas MEF2 proteins bind to another 10-bp consensus $CTA(A/T)_4TAG$, called MEF2 site (Pollock & Treisman, 1991; Andres et al. 1995). In general, MADS-box family members tend to share highly similar sequences, expression patterns, and related functions. Previous analyses revealed that the highly conserved MADS-box domain, in SRF and MEF2 proteins, is highly critical for maintaining the configuration of the DNA binding and for protein dimerization (Shore & Sharrocks, 1995). There are few striking differences between SRF- and MEF2-type MADS domains. SRF-type MADS domains has a highly conserved basic residue Lysine (K), whereas the corresponding position of MEF2-type MADS domains has a conserved acidic amino acid, which cannot interact with the DNA phosphate, as the basic residue (K) of SRF-type does. Despite the absence of C-terminal, the MADS domains of SRF and MEF2 remain sufficient to mediate the correct DNA-binding specificity (Nurrish & Treisman, 1995; West et al. 1997; Santelli & Richmond, 2000). In addition, the analysis of MADS domains in bacteria and phage showed

that these MADS domains are more similar to MEF2-type MADS domains than to SRF-type MADS domains. In mammals, there is only one SRF and four members of the MEF2 family, in addition with their greater diversity by alternative splicing variants, belonging to the MADS-box superfamily (Theissen et al. 1996). The binding sites for these factors are similar to each other containing A/T-rich DNA sequences. However, there are many different properties between SRF- and MEF2-binding sites in line with the differences between the SRF- and MEF2-type MADS domains (Chambers et al. 1992).

1.2. MEF2 proteins

MEF2 are evolutionary conserved proteins among all family members and act as a key determinant factor in both muscle and neuronal development in vertebrates and invertebrates (Potthoff & Olson, 2007). The N-terminus of MEF2 proteins share about 50% identity overall and 95% similarity in the highly conserved MADS-box (consists of 58 amino acids) and an adjacent MEF2 domain (consists of 29 amino acids) (Shore & Sharrocks, 1995; Black & Olson, 1998). The MADS-box domain mediates dimerization of MADS-box proteins and DNA-binding to an A/T rich sequence in gene regulatory region, whereas MEF2 domain binds preferentially to the consensus sequence $CTA(A/T)_4TA(G/A)$, found in the control region of most muscle specific genes (Gossett et al. 1989; Pollock & Treisman, 1991). The MEF2 domain is specific to MEF2 family of proteins require for high affinity DNA-binding, co-factor interaction and dimerization (McDermott et al. 1993; McKinsey

et al. 2002). The MADS-box and MEF2 domain are mandatory and sufficient for DNA binding but lack transcriptional activity on their own (Ornatsky et al. 1996) **(Figure 3A).** Interestingly, previous studies revealed that MEF2 domain are not required for DNA binding but are essential for site-specific transcription, suggesting a functional relationship between MEF2 domain and transcriptional activation domain (TAD) (Molkentin et al. 1996). Mutation of MEF2 binding sites severely diminishes its expression and MEF2 mediated target genes (Molkentin et al. 1996; Potthoff & Olson, 2007). The organization of the MEF2 genes is identical within conserved regions, from *Drosophila to Homo sapiens,* indicate that they evolved from a common ancestral MEF2 gene present in invertebrates (Breitbart et al. 1993; Lilly et al. 1994).

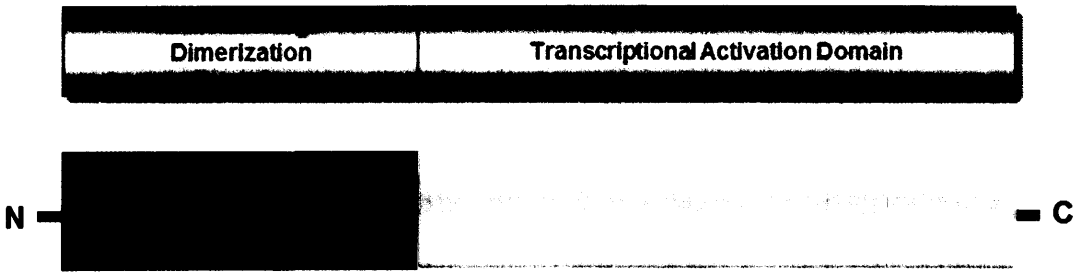

Figure 3A: Schematic of MEF2 protein. The MADS and MEF2 domains are shown at the N-terminus and transcriptional activation domain locates at C-terminus.

The C-terminus of MEF2 proteins is more divergent amongst the MEF2 members and acts as a transcriptional activation domain (TAD), as revealed by sequence analysis. This region is subject to extensive alternative splicing, multiple post-translational modifications, and interactions with a number of other proteins which regulate the ability of MEF2 to activate transcription (Black & Olson, 1998) **(Figure 3A).** Recent studies have demonstrated that structure-function relationship of the C-terminus of MEF2 is very important in several tissue types (Han & Molkentin, 2000; Potthoff & Olson, 2007). MEF2A has been shown to contain a nuclear localization sequence (NLS) at its extreme C-terminus, which is conserved in MEF2 family members. When the NLS is deleted, MEF2 fails to localize to the nucleus (Yu et al, 1992).

1.2.1. MEF2 isoforms

In vertebrate, MEF2 proteins are encoded by four genes MEF2 A, -B, -C, and –D, which are expressed in distinct but overlapping temporal and spatial expression patterns during embryogenesis and in adult tissues, with highest expression in mature skeletal, cardiac and smooth muscle cells, as well as in neurons and at lower levels in several other cell types. (Pollock & Treisman, 1991; Yu et al. 1992; Breitbart et al. 1993; McDermott et al. 1993; Leifer et al. 1993; Martin et al. 1993, Martin et al. 1994; Edmondson et al. 1994; Lyons et al. 1995; Ticho et al. 1996). MEF2 A, -C, and –D bind the MEF2 consensus sequence with high affinity as a

homo/heterodimer whereas MEF2B fails to bind as a homodimer *in vivo* or *in vitro* (Pollock & Treisman, 1991; Yu et al. 1992) **(Figure 3B).**

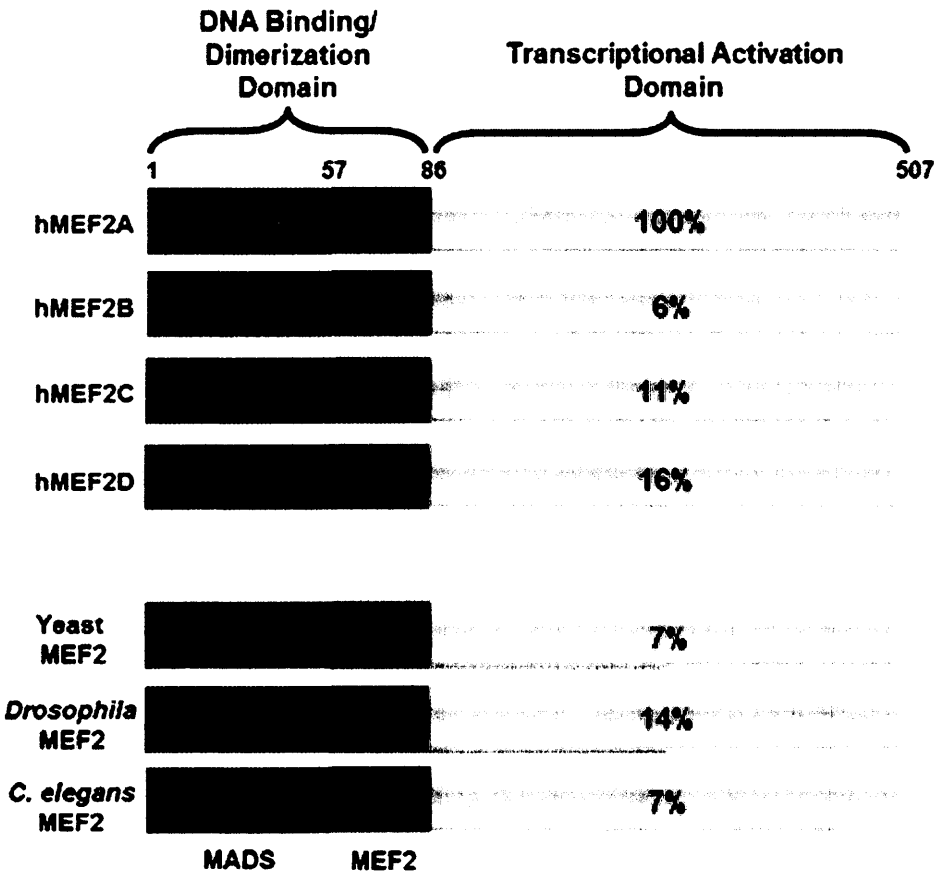

Figure 3B. Schematic of Myocyte Enhancer Factor2 in diverse species. Vertebrates have four MEF2 (MEF2 A, B, C, and D) whereas, Yeast *Drosophila* and *C. elegans* possess a single MEF2 gene. The conserved N-terminal contains the MADS box and MEF2 regions, which together mediate DNA binding, dimerization, and co-factor interactions. The C-terminal regions are divergent among family members that mediate transcriptional activities, interactions with other proteins and subject to complex patterns of alternative splicing and posttranslational modifications. This figure is adapted from the article by Potthoff & Olson 2007.

In contrast, in *Drosophila*, *Caenorhabditis elegans,* and *S. cerevisiae,* there is a single MEF2 gene (Potthoff & Olson, 2007). In yeast (*S. cerevisiae)*, the MEF2 homolog Rlm1 binds the same DNA sequence as the vertebrate MEF2 proteins, regulated specific gene expression in response to mitogen-activated protein (MAP) kinase activation (Dodou & Treisman, 1997). The number of MEF2 genes in primitive vertebrates is not yet evident, but invertebrate chordates, including a Urochordate (sea squirt, *Ciona sp*) and a Cephalochordate (amphioxus, *B floridae*), have but one. All established mammalian genomes have four MEF2 gene isotypes (Wu et al. 2011). By contrast, metazoa ranging from Porifera (sponges) and Cnidaria (coral, hydra, and jellyfish) through Arthropoda (insects and crustaceans) and Echiniodermata (sea urchin) have a single MEF2 gene (Genikhovich & Technau, 2011).

A cnidarian MEF2 homologue, *PcMef2*, was first reported in the hydrozoan *Podocoryne carnea* (Spring et al. 2002). *Nvmef2* was identified in the sea anemone, *Nematostella vectensis, and* mentioned as an ectodermally expressed gene (Martindale et al. 2004). The *Nvmef2* transcript was found in single cells throughout embryonic and larval development (Martindale et al. 2004; Govich & Technau 2011). These cells are considered to be precursors of neurons or sensory stinging cells (cnidarian-specific nematocytes) (Watanabe et al. 2009).

1.2.2. Alternative Splicing

Alternative splicing is a major mechanism of generating protein diversity in higher eukaryotes. Recent genome-wide studies suggest that more than 50% mammalian genes, including many therapeutic target genes, produce multiple protein isoforms through alternative splicing and alternative usage of transcription initiation and/or termination (Caceres & Kornblihtt, 2002; Pan et al. 2008; Luco et al. 2011). The role of alternative promoter is particularly critical in transcriptional regulation, since their precise utilization allows the balanced expression of corresponding transcript variants in different tissues and developmental contexts (Genikhovich & Technau, 2011). Altered expression of transcript variants and protein isoforms for numerous genes is linked with diseases (Singh & Cooper, 2012). MEF2 mRNAs, proteins, and sequence-specific DNA-binding activities are widely expressed, but target gene activation is highly restricted among tissues and cell types but additional condition may also be pertinent, including regulated expression of splicing isoforms with distinct functions (Zhu et al. 2005).

The C-terminal region of each vertebrate MEF2 transcripts is capable of generating multiple isoforms through a complex pattern of alternative splicing that are conserved among vertebrates. These splicing patterns use alternative exons and splice versus no-splice options that include or exclude a short domain (Zhu et al. 2005). Several splicing variants of the four MEF2 transcripts were identified in the original cDNA cloning work (Zhu et al. 2005). MEF2A was the first MEF2 to be identified (Yu et al. 1992; Pollock & Treisman, 1991), and four splicing variants are encoded by the MEF2A gene of all vertebrates (Zhu et al. 2005). Three additional

vertebrate MEF2 isoforms, MEF2B, -C, and -D, were subsequently identified (Black & Olson, 1998). There are four alternatively spliced exons/domains, termed α1, α2, β, and γ, which give rise to numerous tissue-restricted MEF2 isoforms **(Figure 4).**

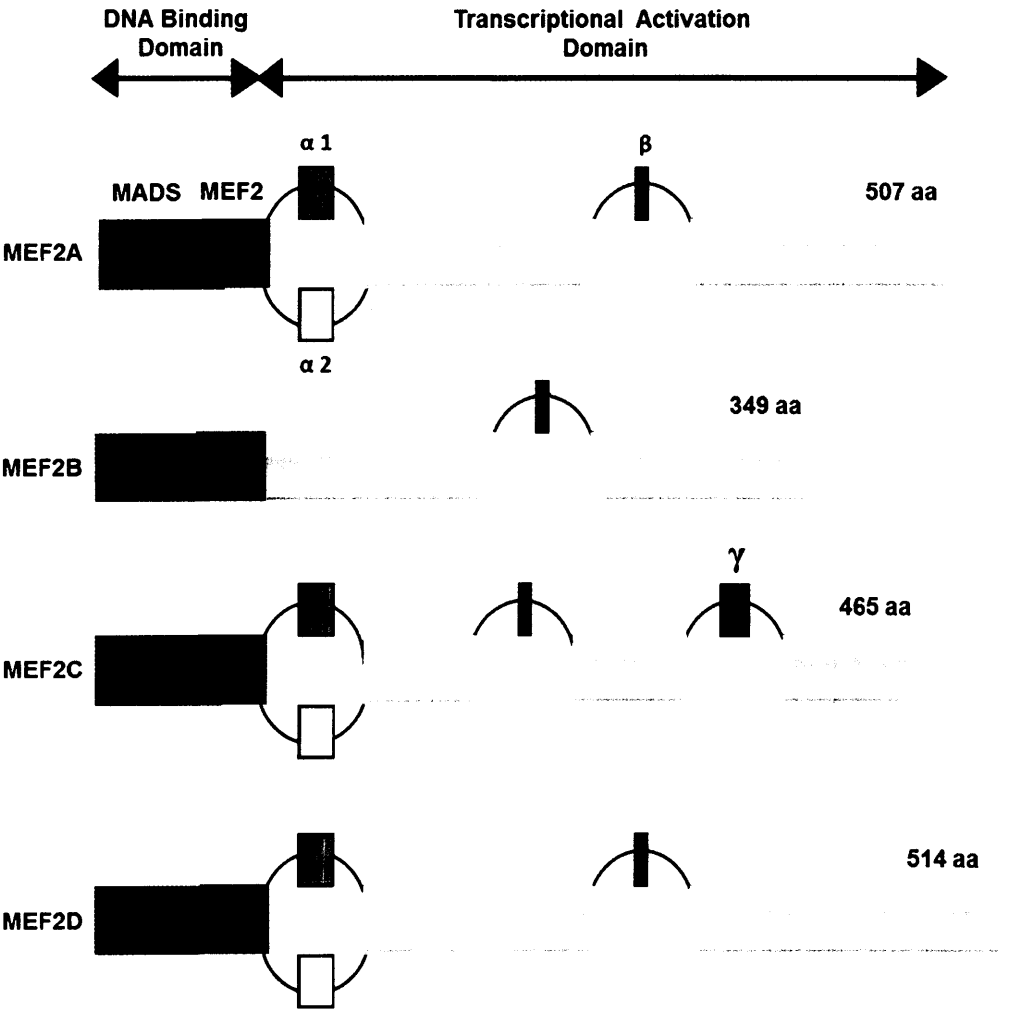

Figure 4: Major forms and splice variants of MEF2. Four vertebrate MEF2 isoforms with indicated alternative splicing exons within the C-terminal transcriptional activation domain labelled as α1/α2, β and γ. The MADS and MEF2 domains are shown at the N-terminus of each isoform. This figure is adapted from the article by Black & Olson 1998.

MEF2D contains an acidic exon (TEDHLDL), which is present only in skeletal muscle, heart and brain (Breitbart et al. 1993; Martin et al. 1994). MEF2D alternatively spliced exon α1 (isoform 1a) is expressed ubiquitously in kidney, heart, stomach, PC12 cells, and neuronal cells, whereas the other exon α2 (isoform 1b) expression is restricted in skeletal muscles, a good example of tissue specific splice (Martin et al. 1994). MEF2 β+ isoforms are more robust than β- isoforms in activating MEF2-responsive gene expression. β function is position-independent and exists in all MEF2 splicing variant contexts. MEF2A mRNAs containing an acidic β-exon (SEEEELEL) are expressed predominantly in striated muscle and brain, the corresponding region in MEF2C (SEDVDLLL) is present only in skeletal muscle and brain (McDermott et al. 1993) and MEF2D (TEDHLDL) is restricted to skeletal muscle, heart and brain (Breitbart et al. 1993, Martin et al. 1994).

MEF2A spliced transcripts that include the β exon, are induced during myocyte differentiation (Yu et al. 1992). Studies has shown that MEF2s with the β+ exon seem to be more potent in activating MEF2 responsive reporter gene expression than MEF2 without the β exon, suggesting that alternative splicing variants may possess distinct regulatory capabilities *in vivo* (Zhu et al. 2005). The third exon (γ) is unique to MEF2C genes. This γ domain is constitutive in other MEF2 isoforms, but γ- variants of MEF2C have distinctive function within this family (Zhu & Gulick, 2004). The MEF2C γ− isoforms are expressed exclusively in heart tissue with no evidence of γ+ isoforms, however γ− isoforms are expressed predominantly in other adult tissues such as skeletal muscle and brain (Zhu &

Gulick, 2004). MEF2C γ− isoforms are much more robust than γ+ forms in activating MEF2-responsive reporter gene expression (Zhu & Gulick, 2004).

Expression patterns of the various vertebrate MEF2 isoforms demonstrate a conserved pattern of alternative splicing that involve in distinct functions in diverse tissue types. However, the expression of the different splice variants is mostly overlapping, and there might be some redundancy in function. Alternative splicing emerges as critical regulators of MEF2 function that interferes with almost every biological function such as proliferation, differentiation, and survival of myogenic and neurogenic cells.

1.3. MEF2 expression in diverse tissue

The MEF2 family of proteins was originally identified as a regulator of muscle specific gene expression that recognized with an A/T rich *cis* element in the muscle creatine kinase (MCK) enhancer (Gossett et al. 1989; Black et al. 1995). MEF2 binding sites have subsequently been identified in the promoter or enhancer regions of the skeletal, cardiac and smooth muscle genes (Braun et al. 1989; Iannello et al. 1991; Nakatsuji et al. 1992; Molkentin & Markham, 1993; Wong et al. 1994; Katoh et al. 1994; Kolodziejczyk et al. 1999; Anderson et al. 2004; Creemers et al. 2006a). However, unlike skeletal muscle restricted transcription factors, MRFs, MEF2 proteins are extensively expressed in many tissues types, including lymphocytes, neural crest, endothelium, bone and neurons (Leifer et al. 1993; McDermott et al. 1993; Leifer et al. 1994; Dodou et al. 1995, Ikeshima et al. 1995; Lyons et al. 1995;

Ornatsky & McDermott, 1996; Lin et al. 1996; Arnold et al. 2007; Lam & Chawla, 2007). Several reports have also provided evidence that expression of MEF2 proteins are ubiquitous (Pollock & Treisman, 1991; Yu et al. 1992; Breitbart et al., 1993; McDermott et al. 1993; Martin et al. 1994). MEF2 expressed in different tissues appear to have distinct preferences for sequences flanking the core region, but such selectivity is depending on specific role of MEF2 in diverse tissue types (Andres et al. 1995).

1.3.1. Expression of MEF2 in muscle

During embryogenesis, the MEF2 transcripts are highly enriched both in vertebrates and invertebrates developing muscle cell lineages (Black & Olson, 1998; Naya et al. 1999). MEF2C is the first member of MEF2 family to be expressed during mouse and chick embryo development (Edmondson et al. 1994), appearing in the cells of precardiac mesoderm at 7.5 dpc that give rise to the heart (Edmondson et al. 1994) Soon thereafter, transcripts for MEF2A, MEF2C and MEF2D were detected in the developing myocardium at 8.5 dpc. In the skeletal muscle, expression of MEF2C appears in the somite myotomes few hours after Myf5 and myogenin expression at 9.0 dpc which implicate the critical role of MEF2 required for the myoblats differentiation/for the activation of myogenin gene expression during the process of myogenesis (Edmondson et al. 1994). MEF2A and MEF2D expression are found at lower level than MEF2C in the myotome at 9.5 dpc. However, MEF2A, -D are detected more in embryonic tissues than MEF2C

transcript. Throughout the embryo development of mouse, MEF2 transcripts are expressed in the smooth muscle cells where it precedes the expression of muscle structural genes (Anderson et al. 2004). After 12.5 dpc, MEF2 transcripts are detected at high levels in various regions of the brain (Lyons et al. 1995). By 14 dpc, MEF2A, -B, and –D transcripts begin to be transcribed in a wide range of tissue types of vertebrates. In addition, after birth, MEF2A, -B, and -D transcripts are also detected ubiquitously in various tissues, except in the brain, where they display highly localized expression pattern (Pollock & Treisman, 1991; Edmondson et al. 1994; Dodou et al. 1995; Lyons et al. 1995). Only MEF2C transcript is restricted to skeletal, cardiac muscle, spleen and brain tissues (Lyons et al. 1995; Black & Olson, 1998).

In cultured skeletal muscle cells, the expression pattern of MEF2 genes during differentiation follows a sequentially timing of appearance. MEF2D has been reported to be expressed in proliferating myoblasts prior to the onset of differentiation, but in fact it does not activate muscle target genes until myoblasts exit from the cell cycle (Breitbart et al. 1993; Martin et al. 1994). MEF2A proteins start to express as cells enter the differentiation pathway after serum withdrawl and MEF2C appears later in the differentiation program (McDermott et al. 1993; Martin et al. 1993). It is clear that MEF2 protein is regulated at multiple levels during development and differentiation which allow the cells to maintain tight control of MEF2 levels in a precise temporospatial pattern (Black & Olson, 1998).

MEF2 mRNA accumulation appears in a wide range of tissue types but MEF2 transcriptional activity and protein expression has been reported to be highly

restricted to muscle specific cells and neuronal cell lineages (Naya et al. 1999). The possible explanation of this disparity may be the repression of posttranscriptional and translation regulatory mechanism which limits the MEF2 transcriptional activity. Furthermore, MEF2 proteins, particularly MEF2D, demonstrate a considerable role in regulating serum induced gene expression in non-muscle cells. Previously, it has been found that HeLa cells and NIH3T3 fibroblasts contain MEF2 binding activity and considerable expression of MEF2 proteins but MEF2 lack transcriptional activity in these non-muscle cells (Pollock & Treisman, 1991; Ornatsky & McDermott, 1996). This suggests that MEF2 activity is tissue-specific and depends on posttranslationally regulated mechanisms (Yu et al. 1992; Breitbart et al. 1993). For example, while both muscle (C2C12) and non muscle cells (HeLa, Schneider, and L6E9) contain considerable MEF2 expression, only C2C12 muscle cells demonstrate a MEF2 dependent transcriptional activation of muscle specific reporter gene (Ornatsky & McDermott, 1996). These observations are also supported by *in vivo* data where MEF2 is restricted to the specific cell lineages in the MEF2 sensor mouse during embryo development (Naya et al. 1999). MEF2-dependent expression was observed in developing myogenic cell lines of the MEF2 sensor embryo but not in other cell types that also contain MEF2 (Naya et al. 1999). In certain conditions MEF2 remain silent and transcriptional activity of MEF2 proteins can be regulated independent of DNA binding in various cell types (Black & Olson, 1998).

1.3.2. Expression of MEF2 in neurons

MEF2 family members are highly expressed in postmitotic neurons throughout the central nervous system (CNS) during embryogenesis, as well as in the adult (Leifer et al. 1993; McDermott et al. 1993; Leifer et al. 1994; Lyons et al. 1995; Ikeshima et al. 1995; Black et al. 1996). In vertebrates, each MEF2 isoform shows a unique temporal expression pattern in different regions of the brain. The timing of MEF2 expression in the CNS is consistent with a role for MEF2 factors in neuronal differentiation and survival (Lam et al. 2007). In human brain, MEF2C transcripts are preferentially expressed in certain neuronal layers of the postnatal cerebral cortex in a temporal manner declining from postnatal day 2 to adult (Leifer et al. 1993; Lyons et al. 1995). During brain development the level of MEF2 expression increases significantly following withdrawal from the cell cycle in differentiating neurons, which suggests the possible contribution to development and function of the nervous system (NS) by MEF2 (Ikeshima et al. 1995). In an invertebrate NS, MEF2 expression has been found in neurons of *C. elegans* and in mushroom bodies of *D. melanogaster,* and these studies concluded that MEF2 plays a functional role in neurogenesis (Schulz et al. 1996; Dichoso et al. 2000).

In the vertebrate brain, each MEF2 genes shows a unique expression pattern in different regions of the brain. All MEF2 isoforms are highly expressed in the olfactory bulb, cortex, thalamus, cerebellum, and hippocampus (Leifer et al. 1993; Lyons et al. 1995; Lin et al. 1996). The expression pattern of MEF2 correlates directly with neuronal maturation during development of the brain (Leifer et al. 1994). MEF2C is the most extensively characterized of the four MEF2 proteins in

the CNS. Indeed, MEF2C was initially cloned on the basis of its enrichment in the brain. MEF2C regulates the laminar differentiation of central neurons but absence in dividing neurons in the subventricular zones (Leifer et al. 1993; Speliotes et al. 1996). An alternative splice variant of MEF2C is found exclusively in the brain and contains a unique SEDVDLLL peptide sequence in the transactivation domain that may serve to mediate tissue specific protein-protein interactions (Leifer et al. 1994). In the developing rat cerebral cortex, a high level of MEF2C protein is expressed at embryonic day 17 to 21 in the cortical plate where postmitotic neurons further differentiate and become mature (Mao & Wiedmann, 1999). Cortical cells expressing MEF2C also express beta-tubulin type III, but not glial fibrillary acidic protein (GFAP), indicate that MEF2C expression is restricted to neurons. More importantly, antibody against MEF2C does not stain proliferating neuronal precursors identified in primary culture by BrDU, suggesting that MEF2C positive neurons are postmitotic. Again, the pattern of expression of MEF2C protein in central cortex correlates with the pattern of expression of MEF2C mRNA. Interestingly MEF2C is expressed in the adult brain, but not in the developing neurons in the cerebral cortex (McDermott et al. 1993; Leifer et al. 1993; Allen et al. 2002; Zhu & Gulick, 2004). In contrast, the other MEF2 family members show a more restricted expression pattern. MEF2A expressed in the hippocampus, thalamus, and internal granular layer of the cerebellum (Lin et al. 1996). The pattern of MEF2B expression largely follows that of MEF2A developmentally, but it is undetectable outside the olfactory bulb, cortex, and dentate gyrus by adulthood (Lyons et al. 1995). MEF2D is highly enriched throughout the developing CNS

through adulthood. In the cerebellum and hippocampus, MEF2A and –D proteins appear to be the dominant forms, although MEF2C is also expressed (Leifer et al. 1993; Lyons et al. 1995). In postnatal brain, all four MEF2 transcripts are present in the dentate gyrus. This dynamic pattern of MEF2 expression during pre- and postnatal development suggests that different MEF2 isoforms may perform unique roles at different stages of neuronal development.

In the mouse brain, MEF2C mRNA is first detected in the telencephalon at postnatal day 11.5. This region of the brain is one of the first to begin neuronal differentiation. At postnatal day 13.5, MEF2C is expressed in a layer of cells in the intermediate zone of the frontal cortex and in the olfactory bulb (Lyons et al. 1995). At this time MEF2 transcripts are also localized in different regions of the neural tube. MEF2A is distributed in a gradient with highest levels in the dorsal portion and lowest levels in the ventral portion. MEF2C is expressed only in the dorsal region, whereas, MEF2D is distributed throughout the neural tube. At postnatal day 14.5, MEF2C and MEF2D are detected in frontal cortex, hippocampus, amygdala, midbrain, olfactory bulb, and cerebellum. MEF2B is abundant in frontal cortex, present at lower levels in the hippocampus, midbrain, and amygdala, and absent in the cerebellum (Lin et al. 1996). At postnatal day 16.5, low levels of MEF2A overlap with high levels of MEF2C in the hippocampus, midbrain, and frontal cortex and midbrain cortex. MEF2D is the most widely distributed of the four genes at this time. At birth, the expression pattern of MEF2B and MEF2C overlap in frontal cortex and olfactory bulbs (Lyons et al. 1995). MEF2B transcripts appear in the cerebellum. In the two weeks postnatal brain, all four genes transcripts are

present in dentate gyrus. MEF2A, -C, and –D appear in neurons of the horn of Ammon. MEF2C and MEF2D are expressed equally throughout the layers of the frontal cortex. Between 2-6 weeks after birth, MEF2 transcripts show a striking pattern of differential expression in the mature cerebellum. MEF2A and MEF2D are found predominantly in the granule layer of the cerebellum. MEF2C is expressed primarily in the Purkinje neurons and MEF2B is very low or absent. This dynamic pattern of MEF2A expression during pre- and post-natal development in the mouse suggests that different MEF2 isoforms may perform unique roles at different stages of neuronal maturation (Lyons et al. 1995; Lin et al. 1996).

The immunocytochemistry and in-situ hybridization studies indicate that MEF2 transcription factors are primarily expressed in differentiating neurons but not in dividing neuronal precursors. To date, the extensive immunocytochemical studies have not been performed to identifiy the detailed localization of specific MEF2 proteins in the brain partly due to the insufficiency of high affinity isoform-specific antibodies. The data so far indicate that MEF2 protein expression in the brain correlates with mRNA expression (Lin et al. 1996). Together, these studies suggest distinct MEF2 isoforms may mediate similar functions in different populations of neurons, but because of their structural variations they might be regulated in MEF2 isoform-specific and cell-type specific manners. Consider the possible role of MEF2, sub family of MADS proteins, in neuronal differentiation.

1.3.3. Role of null MEF2 isoforms in diverse tissue types

Functions of MEF2 isoforms are observed partially overlap, but distinct roles for the diverse tissue types. Therefore, assessing MEF2 function of specific isoform has been complicated and challenging due to their overlapping expression patterns during development. However, recent studies identified numerous MEF2 target genes, indicating their diverse role during the myogenesis and neuronal survival (Sandmann et al. 2006; Flavell et al. 2008). Murine gene mutation/deletion studies provide genetic evidence in support of discrete MEF2 isoforms-specific functions in vertebrates (Bult et al. 2008). For example, the phenotypes of *mef2* null mice showed distinct role of MEF2 in skeletal and cardiac muscle. Targeted inactivation of *mef2 isoforms* in mice results in embryonic lethality (Lin et al. 1998; Naya et al. 2002), and a failure of normal bone development (Arnold et al. 2007).

The essential role of MEF2 in muscle development was first observed in *Drosophila* (Bour et al. 1995). Deletion of single *D-mef2* gene in *Drosophila* leads to complete loss of MEF2 function result in block differentiation of all muscle cell types like somatic, cardiac, and visceral muscle throughout embryonic and larval development (Nguyen et al. 1994; Bour et al. 1995; Lilly et al. 1995; Ranganayakulu et al. 1995). This indicates the absolute requirement of MEF2 for myogenesis and morphogenesis (Bour et al. 1995). During embryogenesis, MEF2C is expressed at the onset of differentiation of the cardiac and skeletal muscle lineages and is followed by expression of the other MEF2 genes in vertebrates (Edmondson et al. 1994). The *Mef2c* null mice die at embryonic day 9.5 due to cardiovascular defects, and these mice also exhibit vascular defects such as heart

tube fails to undergo looping morphogenesis, the malformation of the right ventricle, and inhibition of subsets of cardiac muscle gene expression (Lin et al. 1997; Phan et al. 2005). The other *Mef2* isoforms are expressed at normal or supraphysiological levels in the *Mef2c* null mice but lack of compensation by other isoforms indicating the unique role of *Mef2c* in cardiac development *in vivo* (Lin et al. 1998). Recent report has been documented about skeletal muscle specific deletion of *Mef2c* leads to disorganized sacromeres and the loss of integrity of the Sacromere M-line (Potthoff et al. 2007).

The *Mef2a* null mice survive to the neonatal period, but subsequently develop severe myocardial mitochondrial deficiency, pronounced dilation of the right ventricle and sudden cardiac death (Naya et al. 2002). Only a few *Mef2a*-null mice survive to adulthood with marked reduction in size and number of cardiac mitochondria and disorganization of myofibrillar and susceptibility to unexpected death (Naya et al. 2002). A loss of *Mef2a* transcriptional regulation is account for the lack of compensation by the up-regulated *mef2* isoforms in these mice. This *Mef2a*-null mice phenotype, together with the fact that *mef2c* null embryos do not exhibit mitochondrial defects (Lin et al. 1997), indicates that MEF2A plays a specific role in mitochondrial biogenesis. The *Mef2b* null mice are also viable, and do not display any obvious phenotypic defects in skeletal and cardiac muscles (Black & Olson, 1998) Because expression of the other *Mef2 isoforms* is also up-regulated in these mice and *Mef2b* have possible functional redundancy with other *Mef2 isoforms*, indicating lack of compensation *in vivo* for selective *MEF2* gene loss (Black & Olson, 1998). The alternative splicing of *Mef2b* transcripts is altered

in *Mef2b null* embryos (Vong et al. 2006) and a significant upregulation of *Mef2b* expression was also observed in *Mef2b null* mice (Lin et al. 1997). Mice homozygous for deletions in *Mef2a* or *Mef2b* are viable, whereas the *mef2d null* mice die prior to gastrulation (Lin et al. 1997; Phan et al. 2005; Kim et al. 2008). Several lines of evidence have implicated MEF2 functional role in myogenesis, progenitor cell specification and mature myotubes formations (Black & Olson, 1998). MEF2s are also reported to be involved in cardiac hypertrophy (Kolodziejczyk et al. 1999). *Mef2d* null mice shows no abnormalities in skeletal muscle development however, these mice are resistant to cardiac hypertrophy invoked by cardiac stress or chronic β-adrenergic stimulation (Kim et al. 2008). *Mef2d* isoform is thus thought to play a crucial role in mediating stress-dependent gene expression in the adult heart (Kim et al. 2008).

Since the phenotype of *mef2a*, *mef2b*, *mef2c* and *mef2d* null mice are distinct from each other, considering that the four members share similar DNA binding activities and overlapping expression pattern, suggests that they have distinct as well as partially redundant functions (Black et al. 1996; Lin et al 1996; Han et al. 1997; Blaeser et al. 2000; Gaudilliere et al. 2002; Butts et al. 2003; Heidenreich & Linseman, 2004). Despite all MEF2 isoforms demonstrated the importance of MEF2s in muscle development, but to-date it is not clear whether *Mef2* isoforms selective functions relate solely to distinctions in temporospatial expression, or to unique features of the MEF2 forms encoded by the different genes. None of the aforementioned genetic deletions of MEF2s shows a specific effect in vertebrate skeletal muscle development *in vivo*. In this regard future studies will likely focus

on tissue-specific gene deletion of MEF2s in skeletal muscle and in other tissue abnormalities specifically cardiac related phenotypes. Generation of double and triple knockout mutant mice may be a valuable methodology to further ascertain the role of MEF2 proteins. This also lessens concern about MEF2s redundant functions among family members while analyzing MEF2 null mice. Hence conditional mutagenesis approaches may be very practical and applicable in future. Taken together, these observations suggest that differential expression of MEF2 isoforms is a mechanism of transcriptional specificity for MEF2-dependent gene expression.

1.4. Regulation of MEF2 activity:

Regulation of gene expression is crucial to cell proliferation, differentiation and survival. Context specific and signal dependent regulation of gene expression is achieved to a large part by transcription factor regulation. The MEF2, family of transcription factors are crucial regulators controlling muscle-specific gene expression during myogenesis. MEF2 also plays pivotal roles in neuronal survival and synaptic formation, and in lymphocyte selection and activation (Potthoff & Olson, 2007).

The structural organization of MEF2 proteins allows it to receive and respond to multiple inputs from various intracellular signaling pathways and thus MEF2 function is profoundly influenced by developmental cues and signals from the extracellular environment (McKinsey et al. 2002). The post-translational modifications are integral components of signalling cascades that enable cells to

efficiently, rapidly and reversibly respond to extracellular stimuli (Black & Olson, 1998). As a critical regulator in many cell types, MEF2 activity is tightly regulated by a multitude of posttranslational modifications that control and modulate signalling responses and gene expression such as phosphorylation, acetylation and sumoylation (Yang et al. 1998; Ornatsky et al. 1999; Han & Molkentin, 2000; Sterner & Berger, 2000; Cox et al. 2003; Gregoire & Yang 2005; Ma et al. 2005; Shalizi et al. 2006). As such well-defined posttranslational modification patterns also dictate the functions and protein-protein interactions of MEF2 (Black & Olson 1998).

1.4.1. Phosphorylation of MEF2

Protein Phosphorylation is one of the important mechanisms for regulation of proteins in a mammalian cell (Graves & Krebs, 1999). The phosphorylation mechanism was initially identified by Fisher & Krebs in 1955, as a regulatory mechanism from muscle tissues. In humans, more than 500 different protein kinases are documented so far and identification of their biological targets is still a very challenging and active research field (Pearce et al. 2010).

The mechanism of phosphorylation is regulated by two types of enzymes, protein kinases and protein phosphatases (Graves & Krebs, 1999). Kinases transfer a phosphate group from the nucleotide donor ATP and more seldom GTP to specific amino acid residues where as phosphatases catalyze the removal of the phosphate group to control activity of protein (Krebs & Beavo, 1979). In

eukaryotes, serine/threonine residues are more common target by one of specific protein kinases, and tyrosine residue by another kinase **(Figure 5).** The overall phosphoserine/phosphothreonine events are ~90% and ~10% respectively in contrast to phosphotyrosine which is only ~0.05% in proteins (Pearce et al. 2010). Each protein may contain multiple phospho-sites, which might be targeted by different kinases/phosphatases at different time and upon different extracellular or intracellular stimuli, and thus subsequent biological functions such as, cell cycle, proliferation, differentiation, metabolism, neurotransmission and neuronal survival (Kim et al. 2002; Bossis & Stratakis, 2004; Insel et al. 2012). Protein kinases/ phosphatases also play a major role in human diseases such as inflammation and cancer; therefore they become a prime target for therapeutic intervention (Pearce et al. 2010; Insel et al. 2012).

In general, transcription factors are substrates of a number of protein kinases that phosphorylate serine and theronine as well as tyrosine residues. Protein phosphorylation and dephosphorylation of transcription factors is the most widely described regulation process affecting their structure, subcellular localization, transcriptional activity and DNA binding, leading to regulation of specific target genes (Pearce et al. 2010). Phosphorylation plays an imperative role in the regulation of MEF2 functions in myogenic and neurogenic cells (Zhao et al. 1999; Mckinsey et al. 2002; Cox et al. 2005).

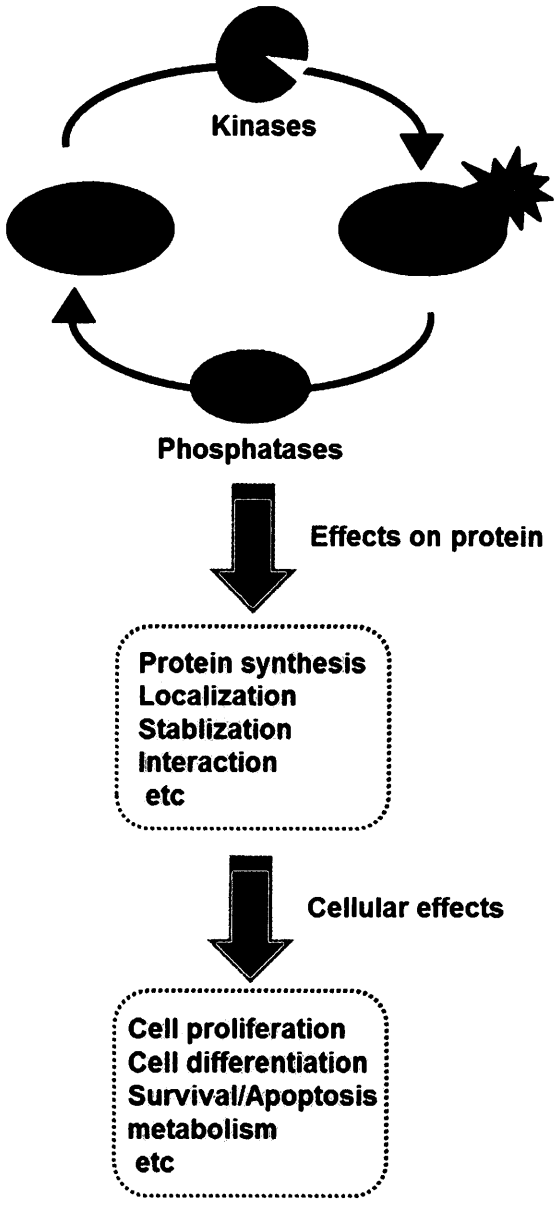

Figure 5: Schematic of Phosphorylation process. Protein phosphorylation is a reversible process which catalyzed by protein kinases and phosphatases. Phosphorylation affects the substrates by modification of their activity, localization and stabilization resulting in regulation of different biological processes such as cell proliferation, differentiation, survival, and apoptosis.

To date, many phosphorylation sites have been identified in the transactivation domain of MEF2A and numerous studies reported that the activity of MEF2 factors is tightly modulated by phosphorylation (Ornatsky et al. 1999; Yang et al. 1998; Cox et al. 2003; Gong et al. 2003). It is known that phosphorylation by kinases is an important regulatory mechanism through which the MEF2 activity is up/down-regulated leading to modulate MEF2 functions. Multiple kinases can target MEF2 proteins including p38MAPK, PKC (Han et al. 1997; Ornatsky et al. 1999), ERK5/BMK (Kato et al. 1997; Yang et al. 1998), CKII (Molkentin et al. 1996), and CDK5 (Gong et al. 2003) depending on the external and internal stimuli (Kato et al. 2000; Mckinsey et al. 2002) in a positive or negative manner.

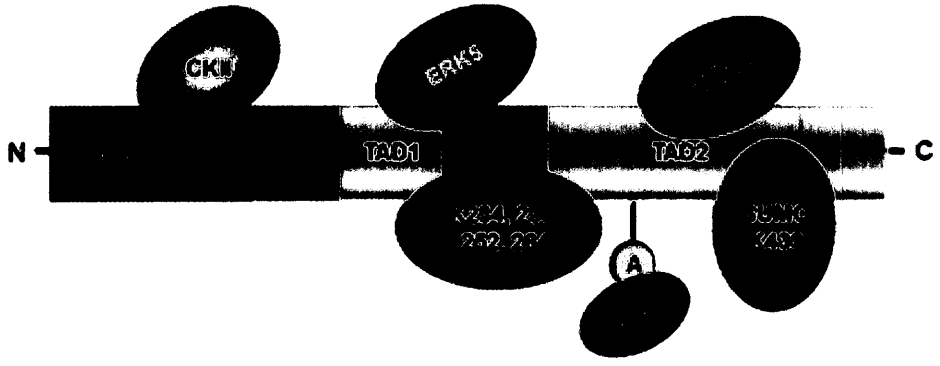

Figure 6: Schematic of posttranslational modifications of MEF2

The majority of phosphorylation sites consist of proline directed serine/theronine residues, which are more often targeted by MAPK and CDK families of protein kinases. The post-translational modification of MEF2 by various signaling enzymes may provide a way for distinct stimuli to differentially regulate MEF2-dependent transcriptional programs in various tissue types **(Figure 6).**

1.4.1.1. cAMP-dependent Protein Kinase (PKA) signaling pathway

Cyclic adenosine 3′,5′-monophosphate (cAMP) is produced as an intracellular second messenger in response to a variety of extracellular signals, including hormones, growth factors, and neurotransmitters (Tasken et al. 1997). The effect of cAMP on certain cellular functions has been shown to be dependent on cell-type and biological responses (Skalhegg & Tasken, 2000; Bossis & Stratakis, 2004; Pearce et al. 2010). cAMP regulates the effects of differentiation and gene expression, and stimulates cell metabolism through cell division, growth and promotes the G_1 to S phase transition in the cell cycle (Kim et al. 2002; Insel et al. 2012). Major intracellular effects of cAMP in mammalian cells are believed to be mediated by cAMP-dependent protein kinase (PKA), discovered as an active kinase in early 1950s (Taylor et al. 1990). In addition, cAMP has a PKA independent effect including interaction with cyclic nucleotide-gated ion channels, cAMP-guanine nucleotide exchange factors and cyclic nucleotide phosphodiesterases (Kim et al. 2007).

1.4.1.1.1. Protein Kinase A (PKA)

PKA is a well-defined kinase which plays fundamental roles in a variety of biological processes (Taylor et al. 1990). PKA, a serine/theronine kinase, is the main mediator of cAMP signaling, a ubiquitous signaling pathway that is conserved in all eukaryotes (Tasken et al. 1997; Skalhegg & Tasken, 2000; Pearce et al. 2010). The most common consensus sequences of PKA, RRX(S/T), (R/K)X(S/T), and (R/K)XX(S/T), were determined in various substrates (Shabb, 2001). PKA mediates acute as well as long-term responses to environmental changes through phosphorylation of wide range of substrates in almost all mammalian tissues (Bossis & Stratakis, 2004). PKA substrates include enzymes, ion channels, structural proteins and transcription factors. PKA must have appropriate substrate specificity to ensure correct transmission of appropriate signals (Shabb, 2001).

Structure of PKA:

The PKA holoenzyme is a hetero-tetramer composed of two catalytic subunits (C) associated with two regulatory subunits homodimer (RI and RII) (Skalhegg & Tasken, 2000). Multiple regulatory (RIα, RIβ, RIIα, and RIIβ) and catalytic (Cα, Cβ, and Cγ) subunit isoforms have been identified in mammalian tissues. The type I PKA contains either regulatory subunit RIα or RIβ in its structure and type II PKA contains either regulatory subunit RIIα or RIIβ (Uhler & McKnight, 1987; Tasken et al. 1993; Kim et al. 2007) (**Figure 7A**).

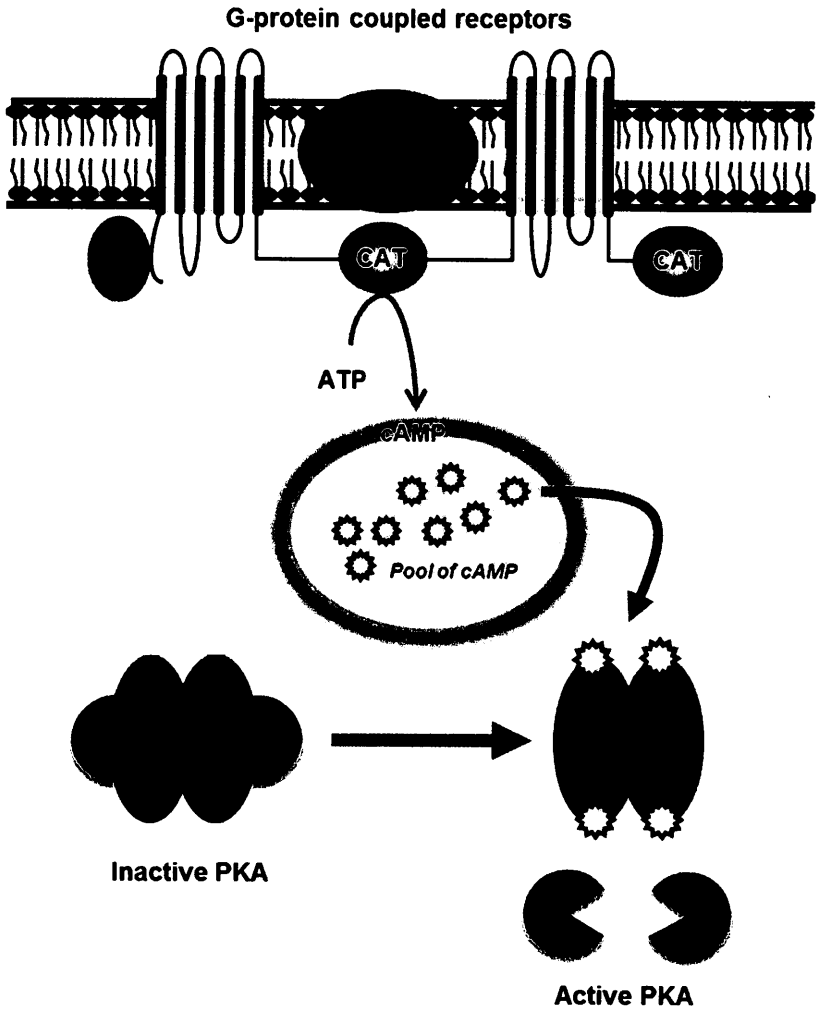

Figure 7A: Structure and Mechanism of Adenylyl Cyclase and cAMP-dependant Protein Kinase A (PKA). Adenylyl cyclase is a membrane glycoprotein possessing a total of 12 transmembrane domains (depicted as lines passing through the membrane) and two catalytic domains (CAT) within the cytoplasm. This enzyme converts ATP to cAMP upon stimulation by subunits of G_s generating pools of cAMP. In the inactive state, PKA is a complex of two catalytic subunits (C) bound to two regulatory subunits (R) that inhibit catalytic activity. When two molecules of cAMP binds to each of the regulatory subunits of PKA resulting in conformational changes and dissociation of the holoenzyme into a regulatory R-subunit dimer and two active catalytic C-subunits.

The type I PKA is generally associated with proliferation and is often overexpressed in human cancer cell lines and in primary tumors. On the other hand, preferential expression of type II PKA is found in normal nonproliferating tissues and in growth-arrested cells (Uhler & McKnight, 1987). The amino acid sequences of Cα and Cβ1 within a given species are 91% identical. However, the amino acid identity of Cα proteins from different species is significantly greater, 98–100% (Uhler & McKnight, 1987), suggesting that each kinase plays a distinct role(s) in cellular functions (Skalhegg & Tasken, 2000). Furthermore, Cα is ubiquitously expressed in mammalian tissues, whereas Cβ1 is highly expressed in brain tissues (Cadd & McKnight, 1989). The diversity of cellular responses to cAMP is the presence of multiple isoforms of PKA in different tissues (Skalhegg & Tasken, 2000; Kim et al. 2002). The regulation of PKA is achieved via unique signaling events, phosphorylation of Thr197 in the activation loop of the C- subunit is necessary for the maturation and optimal catalytic activity of PKA. Once C-subunits are phosphorylated, PKA become fully active in its catalytic potential and the Thr197 phosphate does not turn over (Uhler & McKnight, 1987).

The four regulatory subunits isoforms of PKA are similar in molecular mass and domain organization. The regulatory subunits contain N-terminal docking domain that mediates both dimerization and localization, a hinge region that interacts with the catalytic subunit and two cAMP binding domains in the C-terminus (Scott, 1991; Tasken et al. 1993) (**Figure 7A**). The regulatory subunits have multiple functions. First, they interact with the catalytic subunits and inhibit their catalytic activity. Second, the regulatory subunits serve to target AKAP (A-

Kinase Anchoring Proteins) scaffolding proteins (Wong & Scott, 2004). The regulatory subunits serve as a primary receptor of cAMP. Two stable regulatory subunits were found *in vivo*, the dissociated cAMP-bound form and the cAMP-free holoenzyme (Skalhegg & Tasken, 2000).

Regulation of PKA:

The activation of PKA is achieved by binding of the second messenger cAMP (Sands & Palmer, 2008). cAMP action starts as a response to a complex array of extracellular stimuli after agonist binding (hormones, neurotransmitters, and growth factors) to their specific G protein coupled receptors (β2-adrenergic receptor) in the plasma membrane results in activation of heterotrimeric G proteins (Woo & Xiao, 2012), which in turn stimulate adenylate cyclase to increase the intracellular level of cAMP (Skalhegg & Tasken, 2000). cAMP binding to the RIα subunits of PKA which consequently induces a conformational change in the R- subunit and leads to the dissociation of the holoenzyme into regulatory R-subunit dimer and two active catalytic C-subunits. The free active C-subunit can then affect a range of diverse cellular events by phosphorylating an array of cytoplasmic and nuclear protein substrates, including enzymes and transcription factors (Francis & Corbin, 1999; Shabb, 2001; Sands & Palmer, 2008) (**Figure 7B**). The PKA subunits perform a substantial capacity of self-regulation. In cell culture model, over-expression of Cα or Cβ results in significant compensation by an increase in RIα protein (Uhler & McKnight, 1987).

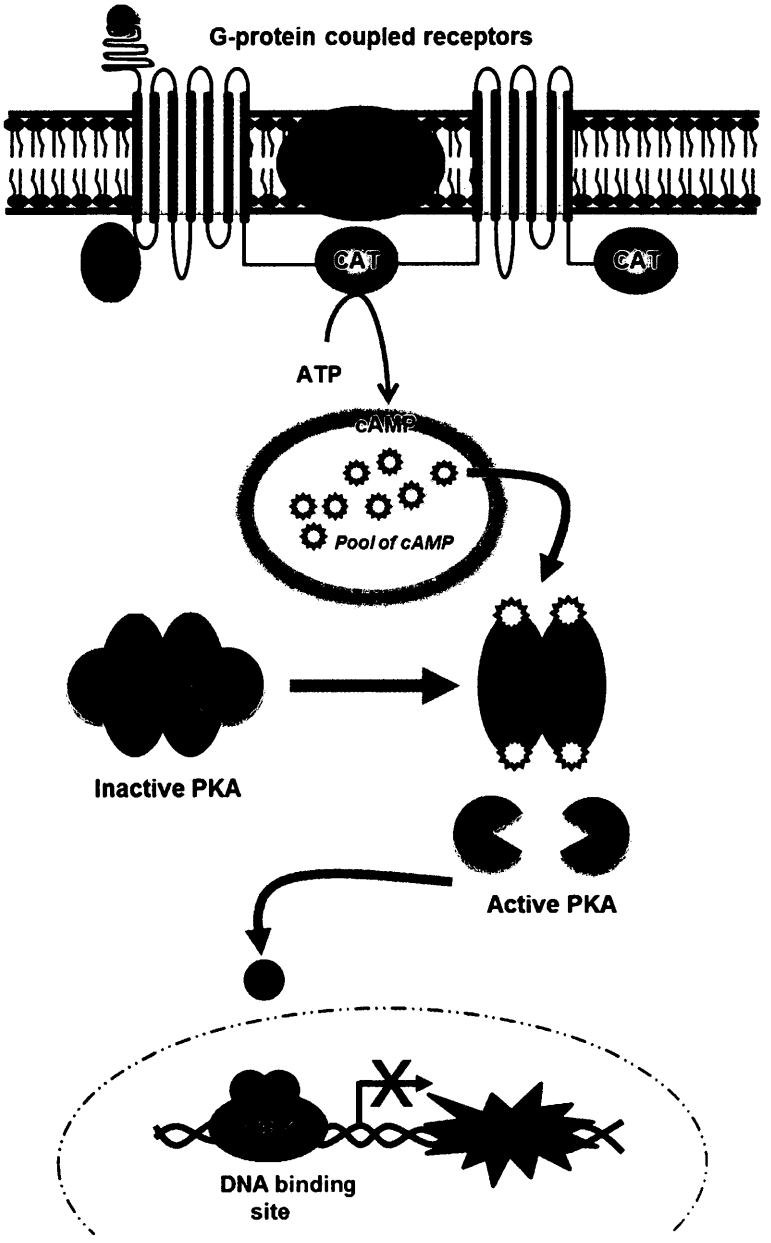

Figure 7B: Regulation of gene expression by cAMP-PKA signaling pathway. Ligand binding to a G-protein coupled receptor activates adenylyl cyclase generating pools of cAMP. PKA is activated as described in figure 6A. After dissociating from the regulatory subunits (R), the catalytic subunits (C) are translocated into the cell nucleus, where they phosphorylate transcription factor MEF2 and inhibit transcription of target gene expression.

Role of PKA:

PKA has been described to be involved in regulation of diverse biological processes including proliferation (Graves et al. 1993; Sirotkin et al. 2004; Sirotkin & Grossmann 2006), muscle differentiation (Li et al. 1992; Winter et al. 1993; Chen et al. 2005), chromatin condensation (Ueda et al. 1995), DNA replication (Costanzo et al. 1999), the immune system, and neuronal survival/apoptosis (Rydel & Greene, 1988; Hanson et al. 1998; Li et al. 2000; Jiang et al. 2009). The cellular localization and differential expression of PKA has a pivotal role in regulation of gene expression through regulation of a wide variety of transcription factors such as CREB (cAMP response element binding protein) (Chen et al. 2005; Sands & Palmer, 2008). Activation of CREB by PKA is one of the best studied transcription factor in diverse tissue types (Chen et al. 2005; Belfield et al. 2006; Pearce et al. 2010). Previous studies has shown that cAMP-dependent PKA signaling potently inhibit skeletal muscle differentiation but the precise molecular mechanism of inhibition was unknown (Li et al. 1992; Winter et al. 1993). Previously, it is revealed that MRF family member, myogenin, contain two conservered PKA phosphorylation sites in the basic region and repression of its transcriptional activation by PKA is an indirect mechanism. It is possible that repression of other myogenic regulators by PKA could involve inhibition of differentiation of skeletal muscle cells (Li et al 1992). Interestingly, *in silico* analysis reveals multiple consensus phosphoacceptor sites of PKA on MEF2 proteins. MEF2 is known as an obligatory partner for the MRFs in the myogenic program and our group was able to identify the missing link between MRFs and MEF2 (Du et al. 2008). In addition,

it has been documented that PKA is involved in development of somites in the early phase of myogenic induction by targeting CREB (Chen et al 2005). Stimulation of intracellular cAMP levels can induce to CREB phosphorylation via PKA. Phosphorylation of Ser133 is a critical event in CREB activation and induces an increase in CREB transactivation potential by allowing the recruitment and binding to co-activators such as CREB-binding proteins (CBP) and MEF2 (Belfield et al. 2006). As such PKA acts as a major physiological kinase responsible for Ser133 phosphorylation.

In the CNS, CREB family members have been shown to be essential for neuronal survival and are thought to modulate both synaptic and intrinsic plasticity in response to neuronal activity (Benito & Barco, 2010; Lonze & Ginty, 2002). Differentiation of neural progenitor cells is regulated by a coordinated change in expression of specific target genes involved in different intracellular signaling pathways. cAMP-dependent PKA signaling has also shown to be involved in neuronal differentiation during brain development (Martinez et al. 1999). PKA phosphorylate CREB leading to regulation of neuronal differentiation, neurite out-growth and neuronal plasticity (Belfield et al. 2006). All stimuli that activate neuronal CREB-dependent transcription (e.g., receptor tyrosine kinases, calcium signaling pathways, cAMP) do so by inducing phosphorylation of CREB at serine 133 (Shaywitz & Greenberg, 1999). In addition to phosphorylation of Ser133, activation of neuronal calcium signaling pathways induces phosphorylation of CREB at Ser142 and Ser143 (Kornhauser et al. 2002). These phosphorylation events occur concurrently with activation of CREB-dependent transcription, and

mutation of these serines to non-phosphorylatable alanine residues selectively inhibits calcium-regulated CREB activity in a reporter gene assay, while leaving cAMP-dependent transcription unaffected (Kornhauser et al. 2002). cAMP-dependent PKA promotes mitochondrial-dependent apoptosis in lymphoma cells (Zhang et al. 2008). Neuronal apoptosis is mediated by caspase-dependent pathways via cAMP-dependent PKA activation in hippocampal neurons (Zhao et al. 2008).

1.4.1.1.2. MEF2 and PKA

PKA functions as a negative regulator that phosphorylates a specific region of MEF2 factor, thereby inducing proteolytic modification to generate the repressor forms and inhibit muscle differentiation. The functional role of the identified PKA phosphorylation sites in the MEF2 factors has been characterized previously by our group (Du et al. 2008). Theoretical mapping shows multiple possible PKA sites residing in C-terminus region of all three MEF2 isoforms. PKA is known to phosphorylate two consensus sites: RRX(S/T) or RXS/T. (**Figure 8**). Sequence analysis of MEF2 isoforms identified four PKA sites in MEF2A at S235, S494, S302, and T415, two PKA sites in MEF2C at S181 and S228. Three possible PKA sites were detected in MEF2D at S121, S190, and S231. Interestingly, the S121 site of MEF2D is conserved and ubiquitously expressed isoform located in the alternatively spliced exon α-1 which is highly expressed in C2C12 myobalsts, PC12 cells, kidney, stomach, heart, and brain, whereas exon α-2 is restricted as striated muscle- isoforms (Martin et al. 1994). MEF2D is potently phosphorylated by PKA

whereas, MEF2A and C are moderately phosphorylated and it was concluded that they are not directly targeted by PKA (Du et al. 2008). Moreover our group did not find any PKA sites at the N-terminus of all MEF2 isoforms and these observations contradict the previous study which showed PKA phosphorylates the conserved T20 residue present in the N-terminus region (Wang et al. 2005).

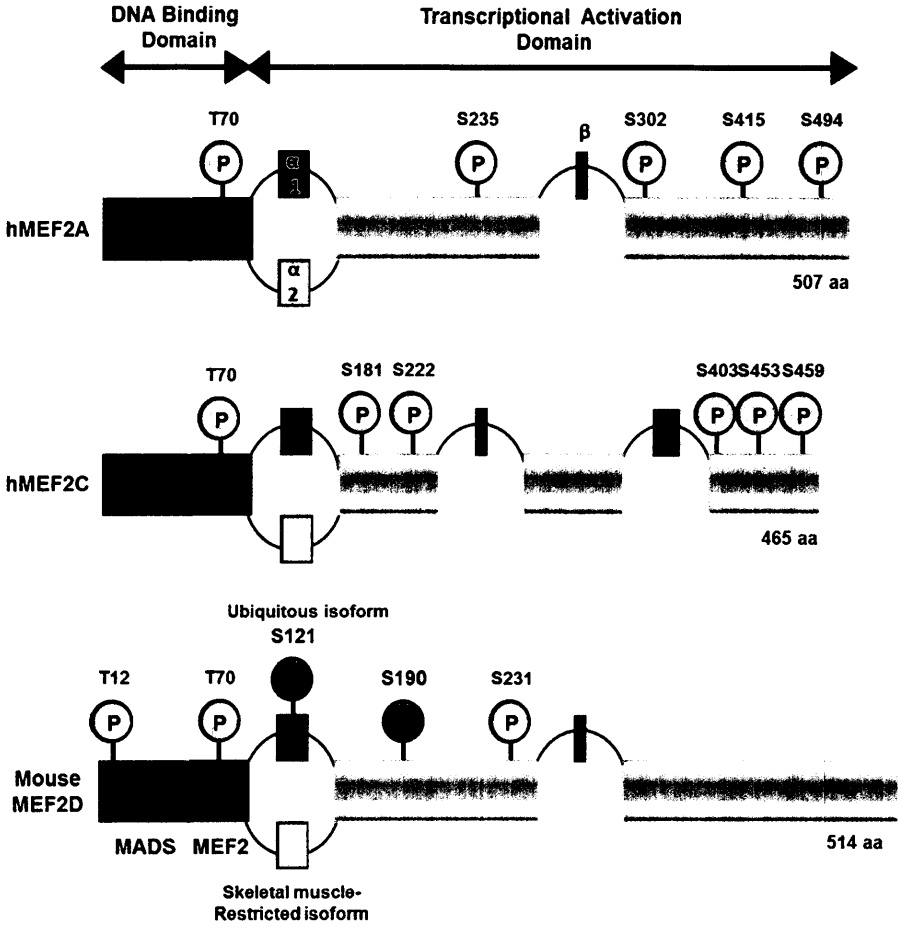

Figure 8: Theoretical mapping of PKA consensus sites (RRXS/T or RXS/T) are found on MEF2A, -C, and –D.

Mutation analysis of PKA phospho-residues in MEF2D, S121A and S190A, attenuate the PKA effect in skeletal muscle (Du et al. 2008). This demonstrates that PKA directly phosphorylates MEF2D at S121 and S190 to inhibit skeletal muscle differentiation, and mutation of these residues to neutral alanine residues rescues this inhibition (Du et al. 2008). In addition, previous studies have identified other mechanism that is involved in MEF2 inhibition by PKA phosphorylation of MEF2. PKA activation also results in an enhanced nuclear accumulation of HDAC4 and a subsequent increase in a MEF2-HDAC4 repressor complex (Backs et al. 2011) (**Figure 9**). Neutralizing mutations of S121 and S190 confers PKA-resistance to MEF2D and efficiently rescues myogenesis from PKA-mediated repression (Du et al. 2008; Gordon et al. 2009). Whereas, calcium and cAMP cooperate to activate the transcription factor CREB, cAMP can either prevents/inhibits MEF2 activation by blocking HDAC export from the nucleus and by inhibiting import of the MEF2 co-activator, NFATc3/c4 (Nuclear factor of activated T cells) (Belfield et al. 2006).

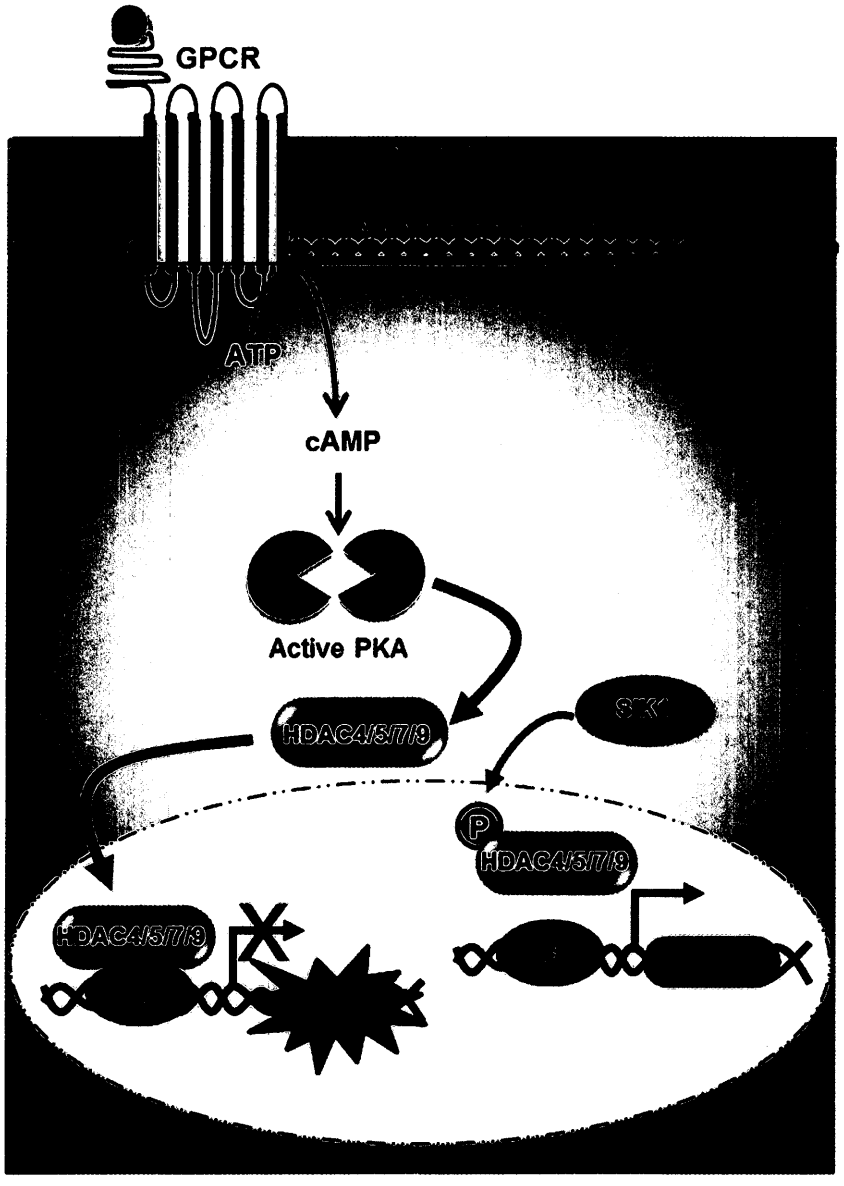

Figure 9: Regulation of gene expression by cAMP-PKA signaling pathway. Ligand binding to a G-protein coupled receptor activates adenylyl cyclase generating pools of cAMP. PKA activation results in an enhanced nuclear accumulation of HDAC4 and prevents its export from the nucleus following increase in a MEF2-HDACs repressor complex and inhibit transcription of target gene expression.

1.4.1.2. Mitogen activated protein kinase (MAPK) signaling

The mitogen-activated protein kinases (MAPKs) play an imperative role in diverse cellular programs by regulating transcription factors leading to gene expression upon receiving extracellular and intracellular signals (Cuenda & Rousseau, 2007). In eukaryotic cells, MAPKs are among the most evolutionary conserved signaling pathways ranging from yeast to human that coordinately regulate many physiological processes (Cuenda & Rousseau, 2007). MAPKs are members of a ubiquitous proline-directed proteins serine/threonine kinase family responsible for signal transduction cascades.

The major kinase cascades of the MAPK family include extracellular signal-regulated protein kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAPKs pathways. In general, ERKs are preferentially activated in response to growth factors and phorbol esters, while the JNK and p38 kinases are more responsive to stress stimuli ranging from osmotic shock and ionizing radiation to cytokine stimulations (Pearson et al. 2001). Each family of MAPKs is composed of a set of three evolutionary conserved and sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). The MAPKKKs are often activated through phosphorylation in response to extracellular mitogen stimuli or a wide range of cellular stress signals (Cuadrado & Nebreda, 2010). MAPKKK activation leads to the phosphorylation and activation of a MAPKK, which then stimulates MAPK activity through dual phosphorylation on threonine and tyrosine residues (Chang & Karin, 2001) **(Figure 10).** Once activated, MAPKs phosphorylate downstream target substrates (Cuadrado & Nebreda, 2010) and able

to modify the functions of specific transcription factors in response to specific stimuli (Chang & Karin, 2001). Previous studies documented that ERK and p38 MAPK are the key regulators of MEF2 function in wide range of tissue types including muscle and brain. These signaling cascades commonly affect MEF2 activation involved in cell proliferation, differentiation, and survival (Han et al. 1997; Yang et al. 1998; Zhao et al. 1999; Thomas & Huganir 2004).

1.4.1.2.1. The p38 MAPK

The p38 MAPKs are strongly activated by a wide range of cellular stresses but also involve in a variety of biological processes including inflammation, immune response as well as in the regulation of cell differentiation and survival/apoptosis (Han et al. 1994; Raingeaud et al. 1995; Han et al. 1997; New et al. 1998; Pearson et al. 2001; Cuadrado & Nebreda, 2010). The p38MAPK family consists of four different but functionally overlapping isoforms: p38α (MAPK14), p38β (MAPK11), p38γ [SAPK (stress-activated protein kinase) 3, ERK (extracellular-signal-regulated kinase) 6 or MAPK12], and p38δ (SAPK4 or MAPK13) (Lechner et al. 1996; Cuenda & Rousseau, 2007; Cuadrado & Nebreda, 2010). These isomers are approximately 60% identical in their amino acid sequence but differ in their expression patterns, substrate specificities and sensitivities to chemical inhibitors (Bain et al. 2007; Bae et al. 2009; Cuadrado & Nebreda, 2010). The first p38MAPK family member was identified during endotoxin-induced cell activation, which showed that p38 was rapidly phosphorylated on tyrosine residue in response to extracellular changes (Han et al. 1994; Enslen et al. 1998).

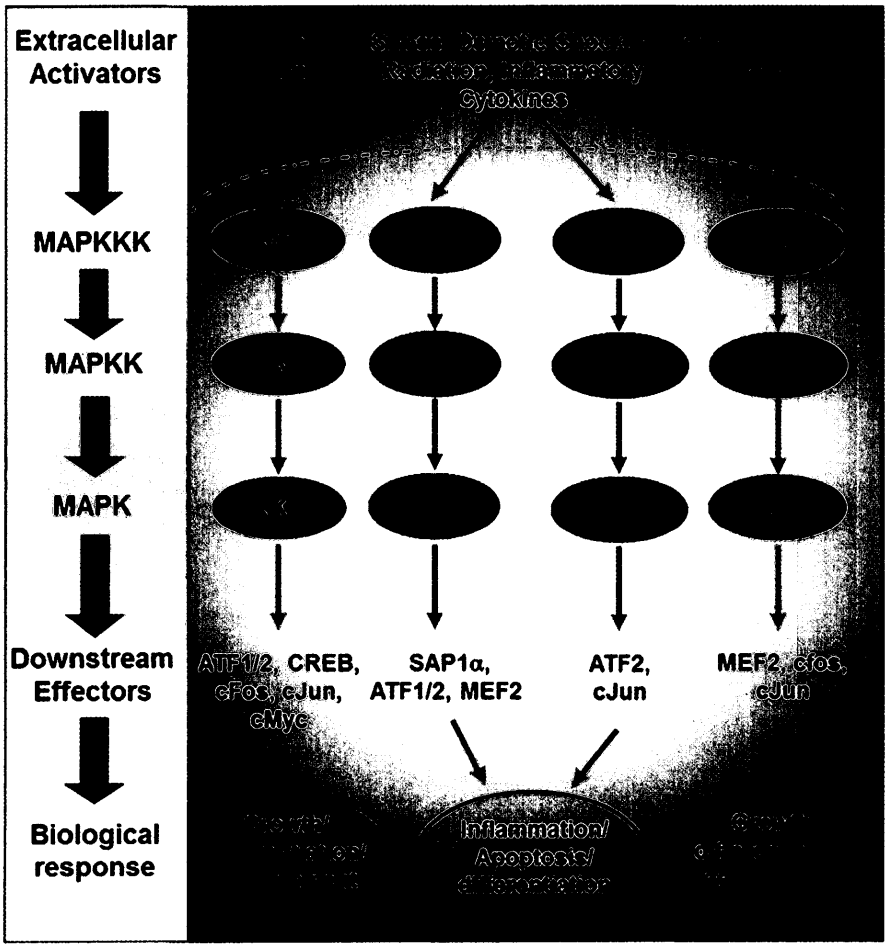

Figure 10: Schematic of MAPK signaling pathways

Based on their expression pattern, substrate specificity and sensitivity to pharmacological inhibitors, p38 isoforms can be divided into two groups: p38α and p38β (group1) while p38γ and p38δ (group2) (Jiang et al. 1996; Bae et al. 2009). Group 1 (p38α and p38β) is ubiquitously expressed in many tissues. Whereas group 2 (p38γ and p38δ) appear to have a more tissue specific expression pattern. p38γ is most abundant in skeletal muscle and p38β in brain (Lee et al. 2000; Bae et al,

2009), while p38δ is highly expressed in testes, pancreas, kidney, small intestine, and in endocrine glands (Cuenda & Rousseau, 2007; Bae et al. 2009). In the adult mouse brain, all four isoforms of p38 (α, β, γ, δ) are expressed in tissues such as the whole brain, cerebellum, and cortex, however p38α and p38β isoforms are reported to be highly localized in specific regions of the adult mouse brain including cerebral cortex and hippocampus (Lee et al. 2000). In general, throughout the brain, p38α is predominately expressed in neurons whereas p38β is highly expressed in both neuronal and glial cells (Lee et al. 2000). All isoforms phosphorylate the Serine-Proline or Thronine-Proline MAPK consensus motifs, but some substrate selectivity has been reported (Han et al. 1994; Jiang et al. 1996; Cuenda & Rousseau, 2007). The commonly used p38MAPK inhibitor SB203580 specifically inhibits p38α and p38β, but does not inhibit p38γ and p38δ activities (Bain et al. 2007; Cuenda & Rousseau, 2007). p38MAPK is present in both the nucleus and cytoplasm of quiescent cells, but upon cell stimulation p38 translocates from the cytoplasm to the nucleus (Raingeaud et al. 1995) but another research group shown that activated p38MAPK is also present in the cytoplasm of stimulated cells (Ben-Levy et al. 1998). The four p38 isoforms are strongly activated by various environmental stresses and inflammatory cytokines, including oxidative stress, UV irradiation, hypoxia, ischemia, interleukin-1 (IL-1), and tumor necrosis factor alpha (TNF-α) (Cuenda & Rousseau, 2007). A major function of p38 isoforms is the production of proinflammatory cytokines. p38MAPK can regulate cytokine expression by modulating transcription factors, or at the mRNA level by modulating their stability and translation (Jiang et al. 1996; Han et al. 1997).

In mammalian cells the functional role of the p38MAPK signaling cascade were identified in 1994. This provided the first step towards identification of several other substrates of p38MAPK and provides the first insight into the molecular mechanism involved in the activation of p38MAPK cascades (Freshney et al. 1994; Rouse et al. 1994). Several substrates of p38MAPK have been identified and characterized, for example transcription factors that are involved in cell development, myocyte differentiation and regulation of neuronal processes such as synaptic plasticity and neurodegenerative diseases (Perdiguero & Muñoz-Canoves, 2008; Corrêa, 2012). The role of p38 MAPK has been defined as a tumor suppressor, as p38α negatively regulates cell cycle progression at both the G_1/S and G_2/M transitions by number of mechanisms, including downregulation of cyclins and upregulation of CDK (Cyclin Dependent Kinase) inhibitors (Jiang et al. 1996; Thornton & Rincon, 2009). Some studies have reported prosurvival functions of p38α, however under cellular stresses cause the induction of apoptosis. These effects can be mediated by transcriptional and posttranscriptional mechanisms, which affect death receptors, survival pathways or pro- and antiapoptotic proteins (Thornton & Rincon, 2009). Classical example of transcription factors are ATF (activating transcription factor), SRF (serum response factor), p53 (tumor suppressor protein) and MEF2s. These transcription factors are directly phosphorylated and activated by p38 MAPKs in response to wide range of stimuli in mammalian cells (Perdiguero & Muñoz-Canoves, 2008).

1.4.1.2.1.1. p38 MAPK and MEF2

The p38MAPK is a major regulator of MEF2 phosphorylation and MEF2-dependent gene expression in a variety of cell types (Yang et al. 1998; Zetser et al. 1999; Cox et al. 2003). It promotes skeletal muscle differentiation, neuronal survival and also mediates the pathological effects of MEF2 in cardiac hypertrophy (Kolodziejczyk et al. 1999; Zhao et al. 1999; Okamoto et al. 2000). A two-hybrid screening with p38 kinase as bait was first revealed by researchers that p38MAPK directly phosphorylates MEF2C (Han et al. 1997; Han & Molkentin, 2000). Phosphorylation of the transactivation domain of MEF2 transcription factors has been shown to increase MEF2 activity. Previous studies have documented that p38 MAPK phosphorylate several residues in the transcriptional activation domain of MEF2A and MEF2C (but not MEF2B and MEF2D) and considerably increase MEF2 activity without affecting DNA binding (Han et al. 1997; Ornatsky et al. 1999; Cox et al. 2003). Previously it was shown that MEF2D is not phosphorylated by p38 (Zhao et al. 1999). But recently, it has been documented that MEF2D transcriptional activity is also regulated by p38 signaling–supported by other cofactors like Ash2L which is selectively recruited to muscle specific promoters through p38-dependent phosphrylation of MEF2D (Rampalli et al. 2007). *In vitro* studies in muscle cells and in fibroblasts showed that inhibition of p38 markedly reduces MEF2D activity and downstream MEF2 target genes (Penn et al. 2004; Rampalli et al. 2007). The efficient target of MEF2A and -C by p38 is mediated by conserved docking domain (D-domain) present between amino acids 266 to 282 in MEF2A and between amino acids 249 to 264 in MEF2C located in their

transactivation domain (Yang et al. 1999). The "D-domain" is sufficient to target p38MAPK to heterologous substrates (Enslen & Davis, 2001; Fantz et al. 2001). Interestingly MEF2D lacks p38 docking domain. Since MEF2D can dimerize with MEF2A, it may be possible that phosphorylation of MEF2A by p38 can potentially up-regulate MEF2D-dependent gene expression (Ornatsky et al. 1999; Yang et al. 1999; Zhao et al. 1999). Deletion of the docking site prevents MEF2A from being targeted by p38. Moreover, alteration of the docking domain from MEF2s to other proteins which are not p38 substrates confers p38-responsiveness (Yang et al. 1999) **(Figure 11).**

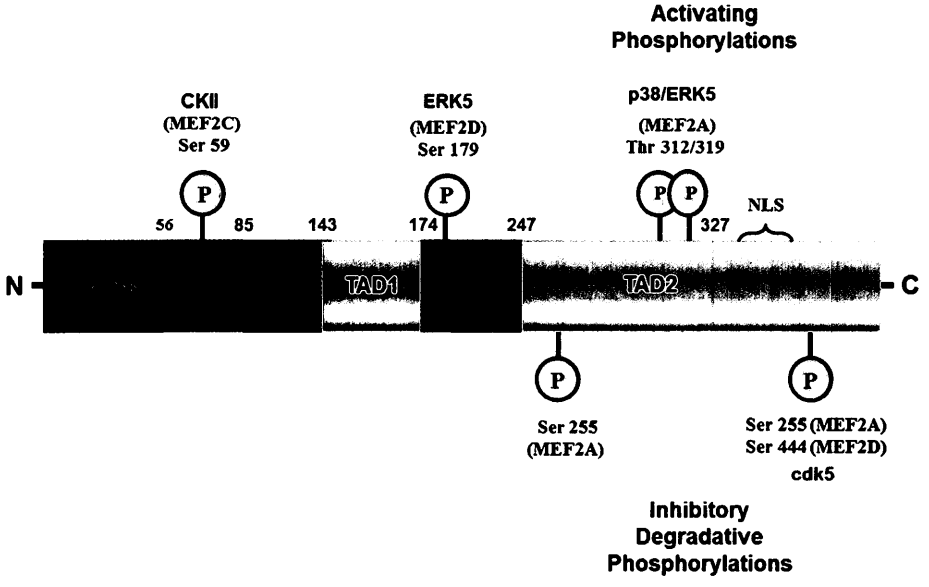

Figure 11. Schematic of multiple kinase phosphoacceptor sites of MEF2

Phosphorylation of MEF2A and MEF2C by p38MAPK enhances their transcriptional activity. Among four isoforms of p38MAPK (α, β, γ and δ), at least two of the isoforms, p38α and p38β, are well known to activate MEF2A and MEF2C by phosphorylating residues located in their transactivation domain (Yang et al. 1999; Zhao et al. 1999). Three major sites of p38-induced phosphorylation contribute to the transcriptional activity of the MEF2s (Thr312, Thr319, and Ser453). It is well documented that MEF2A can be phosphorylated at T312 and T319 residues within the transactivation domain, especially for transcriptional activation in muscle cells (Yang et al. 1999; Zhao et al. 1999; Wu et al. 2000).

Additional serine residues including S355, S453, and S479 are phosphorylated *in vitro* by p38 but their relevance to transcriptional activation is still unknown (Yang et al. 1999; Zhao et al. 1999; Ornatsky et al. 1999). Interestingly, these sites are conserved in MEF2s, however they are contained within an alternatively spliced region of MEF2C (the γ domain) that is only present in a fraction of the MEF2C protein expressed in brain (Zhu et al. 2005). Activation of MEF2C by p38 seems more complex than MEF2A and is subjected to tissue specific regulation. In response to physical-chemical stresses and proinflammatory cytokines, three prominent residues, T293, T300, and S387, in the transactivation domain of MEF2C are phosphorylated by p38 identified during *in vitro* studies (Raingeaud et al. 1995; Han et al. 1997; Zhao et al. 1999). Phosphorylation of these residues has been shown to be important for MEF2C activation by p38 in T-cells (Han et al. 1997). However, only phosphorylation of T293 is induced in differentiating myocytes and is required for MEF2C activation by p38 during muscle

differentiation, suggesting activity of p38MAPK is necessary for muscle formation (Wu et al. 2000). Mutation of these primary phospho-sites in the transactivation domain of MEF2A or MEF2C is sufficient to block myogenesis (Zetser et al. 1999; Puri et al. 2000; Penn et al. 2004). p38 also mediates pathological effects of MEF2 in cardiac hypertrophy and some forms of myotonia (Kolodziejczyk et al. 1999; Wu & Olson, 2002).

Previously it is reported that p38MAPK has also been shown to play a crucial role in somatic myogenesis. Indeed, a critical interaction between p38 and MEF2 occurs in the somites myotome during development (de Angelis et al. 2005). *In vivo* model, abrogation of p38MAPK blocks MEF2 activation in somites, and concurrently inhibits myogenic differentiation (Wu et al. 2000; Keren et al. 2006; Lluís et al. 2006; Bae et al. 2009). p38 is involved in myogenesis with expression of muscle-specific genes (Zetser et al. 1999; Puri et al. 2000; Penn et al. 2004; Lluís et al. 2006). As mentioned before among four isoforms of p38MAP kinase, p38α and p38β plays a prominent role in myogenesis. There is a persistent rise in p38α/β activity during myoblast differentiation. However, blocking of p38 inhibits induction of selective muscle-specific genes and myogenic differentiation (Wu et al. 2000; Lluís et al. 2006). p38 phosphorylates MEF2 that drive muscle-specific gene expression at several levels such as dimerization of MyoD with E-proteins, chromatin remodeling at muscle-specific genes, and stability of myogenic mRNAs (Wu et al. 2000; Simone et al. 2004; de Angelis et al. 2005). Differentiation of skeletal muscle is coordinated by MEF2 transcription factors and MyoD family, and p38 promotes myogenesis through phosphorylation of MEF2s that stimulate MyoD-

dependent muscle-specific gene expressions (Wu et al. 2000; de Angelis et al. 2005).

In past decade several studies started to focus on the role of p38MAPK in neurons and presented evidence that p38MAPK-mediated neuronal survival requires phosphorylation and activation of MEF2. Blocking the p38 signaling by p38 inhibitors promotes apoptosis of differentiating cerebellar granule neurons (Mao et al. 1999; Okamoto et al. 2000). Interestingly, MEF2-dependent transcription activity was inhibited by introducing dominant-interfering mutants of p38 in primary cerebellar granule neurons and differentiating P19 cells (Mao et al. 1999; Okamoto et al. 2000). This suggests a direct involvement of p38 in promoting neuronal survival via activation of MEF2 in neurons. Overexpression of MEF2s mutants, which cannot be phosphorylated by p38, blocked membrane depolarization and p38MAPK induced neuronal survival. The role of p38MAPK mediated neuronal survival through MEF2 was confirmed by earlier studies and later confirmed in the neurogenesis model of P19 cells (Okamoto et al. 2000). Calcium signaling, a second messenger, involve in wide range of cellular responses in neuronal cells (Ghosh & Greenberg, 1995). The importance of p38MAPK-mediated regulation of MEF2 in neurons was first observed in calcium signaling dependent neuronal survival model (Mao et al. 1999). Previously, it was shown that calcium influx triggered by extracellular stimuli induces p38MAPK activity in cerebellar granule neurons, leading to direct phosphorylation of MEF2C at Ser 387 by p38 kinase (Han et al. 1997; Mao et al. 1999). These findings indicate that calcium influx into neuronal cells results in induction of p38 cascade and phosphorylation

and activation of MEF2 by p38 which protects neurons from apoptosis in a p38 pathway-dependent manner (Mao et al. 1999). Observations from these studies contribute to our understanding regarding MEF2 regulation in neuronal cells and its requirement for survival leading to confirmation of importance of postranslational modifications of MEF2 for normal cellular functions (Okamoto et al. 2000).

1.4.1.2.2. ERK5

Another important mediator of MAPK signaling is ERK5, the extracellular signal-regulated protein kinase 5. ERK5 (also known as BMK1, for big mitogen-activated protein kinase-1) is a member of MAPK family (Lee et al. 1995; Zhou et al. 1995). ERK5 is twice the size of other MAPK family members (~100 kDa), and its N-terminal contains a kinase domain similar to that of ERK1/2. ERK5 has a relatively large C-terminal with unique structure that contains a nuclear localization signal (NLS) and a proline-rich region which makes it distinct from other family members. Three isoforms of ERK5 have been reported (ERK5a, -b, and -c) (Zhou et al. 1995). ERK5 was initially reported as a MAPK family member that is activated by stress stimuli. ERK5 activity is increased in response to oxidative stress and hyperosmolarity (Wang et al. 2006). Another study showed that ERK5 can be activated in response to serum (Kato et al. 1997), and nerve growth factor (NGF) (Encinas et al. 1999; Chang et al. 2004). ERK5 activation is correlated with the dual phosphorylation of Thr and Tyr residues within a conserved Thr-Glu-Tyr (TEY) motif in the activation loop of the kinase domain (Kasler et al. 2000). Upon stimulation, MEKK2 and MEKK3, members of the MAPKKK family, activate

MEK5, a specific MAPKK for ERK5. Subsequently, MEK5 phosphorylates and activates ERK5, and then the activated ERK5 phosphorylates substrates including MEF2 (Kato et al. 1997). Interestingly, PD98059 and U0126 were identified as MEK1/2-specific inhibitors, also known to efficiently inhibit the MEK5–ERK5 pathway (Kamakura et al. 1999; Mody et al. 2001). However, MEK5 is less sensitive to PD184352 and is also known as a MEK1/2 inhibitor (Mody et al. 2001).

ERK5 is ubiquitously expressed in several tissues, including heart and skeletal muscle and particularly expressed in high levels in the brain, thymus, and spleen (Kondoh et al. 2006). ERK5 is essential for early embryonic muscle and normal vascular development as well as neuronal survival (Dinev et al. 2001; Regan et al. 2002; Finegan et al. 2009). Targeted deletion of ERK5 in mice has revealed the critical role of ERK5 in cardiovascular development and maintenance of vascular integrity (Regan et al. 2002). Regarding localization, endogenous ERK5 found in cytoplasm and in nucleus, depending on the cell types (Buschbeck & Ullrich, 2005; Kondoh et al. 2006). In resting cells, ERK5 localizes in cytoplasm and translocates to the nucleus when co-expressed with constitutively active MEK5 or upon stimulation (Kato et al. 1997). Endogenous inactive ERK5 localizes either in the cytoplasm or diffusively throughout the whole cell, and translocates to the nucleus on stimulation in myoblast C2C12 cells (Kondoh et al. 2006). It has been shown that when ERK5 translocate to the nucleus, where it directly interacts with, or phosphorylates and activates several transcription factors including c-Myc, c-Fos, Fra-1, and MEF2 family members to control cellular functions (Kato et al. 1997; Terasawa et al. 2003; Kondoh et al. 2006). As such activation of ERK5-MEF2

signaling is important for promoting cell proliferation (Kato et al. 1998), differentiation (Dinev et al. 2001), and neuronal survival (Shalizi et al. 2003).

1.4.1.2.2.1. ERK5 and MEF2

In addition to p38 MAPK, the serine/theronine kinase ERK5 is capable of directly phosphorylating the transactivation domain of MEF2 family members including MEF2A,-C, and -D, resulting in an increased transcriptional activity. However, ERK5 does not phosphorylate MEF2B (Kato et al.1997; Yang et al. 1998; Marinissen et al. 1999; Kato et al. 2000). Phosphorylation of MEF2D at S179 by ERK5 is required for enhanced transcriptional activity upon Epidermal Growth Factor (EGF) stimulation in transfected Hela cells. However, other group reported that phosphorylation at S179 did not induce ERK5-mediated enhancement of MEF2D transcription potential in response to ionomycin treatment in hybridomas. Additional studies reported that ectopically express ERK5 specifically up-regulates the activity of MEF2A and -C, but not of MEF2D in COS cells, suggesting the regulation of MEF2D by ERK5 may depend on cellular context (Pazyra-Murphy et al. 2009).

ERK5 was initially found to interact with the N-terminus of MEF2D in a yeast-two-hybrid screen (Yang et al. 1998). The regulation of MEF2 by ERK5 was first shown in Chinese hamster ovary (CHO) cells. ERK5 possesses a unique C-terminal transactivation domain, which mediates protein-protein interactions with MEF2 transcription factors and provides a potent coactivator function toward MEF2-driven transcription. Interestingly, ectopic expression of the C-terminal

coactivator domain of ERK5 is sufficient on its own to induce MEF2-dependent transcription (Kasler et al. 2000). Disruption of ERK5 causes cardiac developmental defects, observed in MEF2C knockout mice (Dinev et al. 2001; Regan et al. 2002). In T-cells, activation of ERK5 induces immediate-early transcription of the *nur77* orphan steroid receptor gene via MEF2 proteins (Kasler et al. 2000).

In non-neuronal cells, growth factor activation of ERK5 has been reported to contribute to cell proliferation, and differentiation. However ERK5 is activated by neurotrophic factors in primary neuronal cells and plays an important role in neurotrophin mediated neuronal survival (Shalizi et al. 2003). ERK5 is highly expressed in the brain during early embryonic development but declines as the brain matures (Liu et al. 2003). MEF2C is activated by neurotrophins, and the ERK5 signaling is required for neurotrophin stimulation of MEF2C transcription. Mechanism of ERK5-mediated survival involves MEF2 transcriptional regulation (Wang et al. 2009). ERK5 enhances the transcriptional activity of MEF2C by phosphorylating at S387, which is located in the transactivation domain of the protein (Kato et al. 1997; Yoon et al. 2005). Different studies have addressed the issue of whether ERK5 regulates MEF2 in neurons (Pazyra-Murphy et al. 2009). Using a brain-derived neurotrophic factor (BDNF) mediated survival model, it was shown that ERK5 protection of E17 cortical neurons may be mediated through MEF2 induced gene expression. ERK5 is activated by BDNF and regulates MEF2C-mediated gene expression in embryonic cortical neurons. Blocking MEF2 function also attenuated BDNF-induced ERK5 dependent survival. Overexpression

of a dominant-negative ERK5 mutant block the BDNF protection against trophic withdrawal in primary cortical neurons cultured from embryonic day (E17) but not P19 (Liu et al. 2003). Likewise the temporal survival effects of BDNF in cerebellar granule neurons seems to be mediated by an ERK5/MEF2 signaling pathway that induces transcription of neurotrophin (Shalizi et al. 2003). Overexpression of a constitutively active form of MEF2, MEF2C-VP16, attenuated the neuronal death induced by the dominant-negative ERK5. Together, these studies demonstrated that ERK5-mediated survival effect of BDNF requires the activity of its downstream target MEF2. Both ERK5 and MEF2 have been associated with neuronal cell survival following activation of neurotrophin (Cavanaugh et al. 2001; Pazyra-Murphy et al. 2009).

1.4.1.2.3. CDK5 and MEF2

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine cyclin-dependent kinase family member. It is highly expressed in the central nervous system (CNS) but activity of Cdk5 is largely restricted to post-mitotic neurons and requires its activator proteins p35/p39 or particular truncated forms, p25/p29 (Dhavan & Tsai 2001, Smith et al. 2003 & 2006). Cdk5 shares 60% homology with other members of cell cycle kinase family. However, Cdk5 is not directly involved in the cell cycle (Hellmich et al. 1992; Lew et al. 1992; Meyerson et al. 1992; Dhavan & Tsai, 2001). Cdk5 plays an essential role in the development of the CNS such as regulation of neuronal migration, axon growth, neurotransmission, and synaptic plasticity through phosphorylation of a large

number of substrates (Tang et al. 1995; Dhavan & Tsai, 2001; Gong et al. 2003; Tang et al. 2005; Qu et al. 2007; Ikiz & Przedborski, 2008; Lagace et al. 2008). During the past years, Cdk5 has received substantial attention as a result of its unique contribution in the neuronal apoptosis. Under pathological conditions, Cdk5 is strongly and consistently activated *in vivo,* while defects of Cdk5 is highly destructive in neurons after breakdown by Cdk5p35/Cdk5p25, which change cellular localization of Cdk5 that induces neuronal cell death eventually (Gong et al. 2003; Tang et al. 2005). The positive regulation of MEF2 has been well known in the CNS where it plays a critical role in neuronal survival. In contrast, oxidative stress appears to inhibit the pro-survival function of MEF2 in cortical neurons. Previous studies identified Cdk5 as a negative regulator of MEF2, phosphorylates and inactivates MEF2 in cerebellar neurons undergo neuronal apoptosis (Gong et al. 2003; Tang et al. 2005).

The Cdk5 phosphorylate conserved site in MEF2A, -C, and –D but this site is not found in MEF2B. Previous studies documented that Cdk5 phosphorylate at a conserved serine residue present in the transcriptional activation domain (Ser-408 of MEF2A and Ser-444 of MEF2D) (Gong et al. 2003). Oxidative stress and excitotoxic concentrations of glutamate escalate Cdk5 activity leading to Cdk5-dependent phosphorylation of MEF2 and inhibition of survival function. Moreover, MEF2 mutants that are resistant to Cdk5-mediated phosphorylation rescue MEF2-dependent transcriptional activity and prevent neurons from neurotoxin-induced apoptosis caused by excitotoxicity/oxidative stress (Gong et al. 2003). Recent study has provided evidence that neuronal activity withdrawal or neurotoxin stress

involve in gradual decline of MEF2 protein level in cerebellar granule neurons, which leads to neuronal apoptosis (Tang et al. 2005).

The complete mechanism by which Cdk5 phosphorylate MEF2 and inhibits MEF2 function is still unclear. But studies from different groups presented the possibilities that phosphorylation by Cdk5 may targeting MEF2 for cleavage and degradation in neuronal cells. When cortical neurons treated with N-methyl-D-aspartate (NMDA), a potent apoptosis-inducing reagent, led to caspase-dependent cleavage of MEF2A, C, and D (Okamoto et al. 2002). This data correlated to previous observations with activity withdrawal, caspase-cleaved fragments of MEF2 blocked MEF2-VP16-dependent transcriptional activation. Further, constitutively active form of MEF2 (MEF2-VP16) was neuroprotective against NDMA-stimulated apoptosis, however this effect was completely abolished by co-expression of caspase-cleaved fragments of MEF2 (Okamoto et al. 2002). Observations from one study identified a mechanism related to the caspase cleavage of MEF2 proteins. It appears that for MEF2 to be cleaved in fragments by caspase-3, and must be phosphorylated by Cdk5 (Tang et al. 2005). Studies in primary cerebellar granule neurons have shown that phosphorylation of MEF2A and -D by CDK5 inhibits MEF2 transcriptional activity, which also facilitates caspase-mediated cleavage and degradation of MEF2. Blocking Cdk5 activity by using either a dominant-negative Cdk5 or specific pharmacological inhibitor attenuated MEF2A and -D degradation. These observations suggest that Cdk5 and caspase dependent signaling are coordinated together to regulate MEF2 protein stability in

neurons, eventually controlling the neuronal survival upon neurotoxin stimuli (Tang et al. 2005).

MEF2C is neither phosphorylated nor cleaved by Cdk5 in granule neurons in response to excitotoxicity (Zhu & Gulick, 2004). Beside the Cdk5 role in neurons, another study on non-neuronal cells (COS7 and C2C12 cells) identified the Cdk5 site location within a transcriptional activation domain encoded by an alternative exon" γ domain" only exist in MEF2C, which is highly expressed in muscle, brain, and spleen. Furthermore, γ domain defines as an independent repressive domain which is phosphorylation-dependent neuronal and non-neuronal cells. Mutation of phospho-residue serine with alanine leads to profound induction of MEF2-dependent transcription, despite having no effect on MEF2 DNA-binding activity or stability. Interestingly, substantial portions of the γ-domain beyond the phosphorylation site are conserved in both MEF2A and –D isoforms, and demonstrate similar repressive effects on the transcriptional activities (Zhu & Gulick, 2004). These observation suggests that phosphorylation site of Cdk5 may influence the interaction of MEF2 with transcriptional regulators, most likely through recruitment of co-repressors class IIa HDACs.

It is worth noting that Cdk5 site in MEF2D is adjacent to the already identified sumoylation site (Gregoire & Yang, 2005). Phosphorylation-dependent regulation of MEF2 protein stability is certainly not restricted to neurons. Experiments performed in non-neuronal cells, showed that CDK5 acts in association with HDAC4 to stimulate MEF2D sumoylation at K439 residue by phosphorylating S444 (Gregoire et al. 2006). Opposing the activation of CDK5, calcineurin

dephosphorylates S444 and prevents sumoylation of K439, thus acting as modifier of MEF2's phosphorylation status, selectively removing inhibitory phosphatases (Gregoire et al. 2006). In cerebellar granule neurons, calcineurin (calcium-sensitive protein phosphatase) seems to be required to maintain MEF2 in a hypo-phosphorylated and active state. The interplay of K439 sumoylation and S444 phosphorylation consequently regulates MEF2 activity in certain contexts. Because of Cdk5 profound effects on MEF2 function in diverse tissue types, understanding the role of this novel phosphorylation site controlling MEF2 function in the nervous system may, in fact, provide insight into the regulation of MEF2 in tissue types other than neurons.

Biochemical studies have shown that phosphorylation of MEF2 at sites distinct from the Cdk5 site by unknown kinase (s) also regulates MEF2 stability in non-neuronal cells. For example S255 of MEF2A becomes phosphorylated when p38MAPK activity is enhanced. Mutation of S255 to aspartic acid destabilizes MEF2A and leads to its degradation in COS7 cells (Cox et al. 2003). It is not clear yet under what conditions, S255 is phosphorylated in neurons and degradation. In light of accumulating evidence that Cdk5 activity is observed in number of neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Takahashi et al. 2000; Grant et al. 2001). These observations raise the possibility that decreased function of the survival-inducing transcription factor, MEF2, Cdk5 contributes to the neuronal loss causing neurodegenerative diseases. These findings provide a novel regulatory mechanism of MEF2 activity by Cdk5-induced phosphorylation and

caspase-dependent degradation, suggesting regulation of MEF2 protein as more delicate and conspicuous process.

1.4.1.2.4. Casein Kinase 2 (CK2) and MEF2

Protein kinase CK2, also known as casein kinase II, is a highly conserved, multifunctional serine/threonine kinase. It is critically important for the regulation of variety of signaling cascades in eukaryotes that involve in cell proliferation, differentiation and survival/apoptosis (Montenarh, 2010). CK2 ubiquitously express in all tissues, particularly its amount and activity are highly elevated in tumor cells. In mammals, there are two paralog catalytic subunits, CK2α (A1) and CK2α' (A2), and one CK2β dimer, which together form the heterotetrameric holoenzyme and absolutely mandatory for embryonic development (Mazzorana et al. 2008; Dominguez et al. 2011). The catalytic subunits are distantly related to the CMGC subfamily of protein kinases, such as the cyclin-dependent kinases (CDKs). The CK2 is unique enzyme with some peculiarities, which are not found with most of the other protein kinases. CK2 is constitutively active and it can use ATP and GTP as phosphoryl donors (Mazzorana et al. 2008).

Protein kinase CK2 constitutively phosphorylates all MEF2 isoforms at a conserved serine 59 residue in the MADS-box domain (Molkentin et al. 1996). In *in vivo*, phosphorylation of this site by CK2 enhances DNA-binding and MEF2 transcriptional activity leading to enhance MEF2-dependent gene expression (Molkentin et al. 1996). Previously, there was another putative CK2 site detected in the alternative spliced exons of MEF2 isoforms, MEF2A,-C, and –D (Cox et al.

2003). But no evidence is observed in CK2-dependent direct regulation of MEF2 suggesting some crosstalk between CK2 and other signaling cascades. Interestingly, it has been shown that MEF2A to be phosphorylated at a consensus CK2 site, S289, in response to p38 MAPK signaling (Cox et al. 2003). This suggests a possible tissue-specific functional role of CK2 phosphorylation for this site which is located in alternately spliced exons of MEF2s. This also indicates the connection between p38MAPK signaling and the phosphorylation of MEF2A by CK2 (Cox et al. 2003).

CK2 is capable of regulating cell survival at multiple levels including DNA repair, activation of anti-apoptotic proteins and inhibition of pro-apoptotic proteins. This crosstalk with survival signaling cascades such as Wnt and PI3K/AKT/PKB signaling occurs in variety of tissue types (Ponce et al. 2011). Another recent study has indicated the important role CK2 in regulation of p38MAPK in keratinocytes differentiation (Isaeva & Mitev, 2011). Since p38MAPK mediated neuronal survival through MEF2 is well documented, it may be possible that CK2 involve in indirect regulation of MEF2 through p38MAPK in neurons and muscles. Further understanding of the regulation of MEF2 by CK2 may provide greater insight in identifying a mechanism of cell survival and CK2-dependent survival which makes this enzyme an important target for therapy.

1.4.1.2.5. Protein Kinase C (PKC) and MEF2

Protein kinase C (PKC) is a family of multifunctional isoenzymes, activated by ligand stimulation of transmembrane tyrosine kinase receptors. PKC play an essential role in signal transduction and intracellular crosstalk by phosphorylating at

serine/threonine residues as an array of substrates, including cell-surface receptors, enzymes, contractile proteins, transcription factors and other kinases (Ornatsky et al. 1999; Kang et al. 2012). PKCs regulate proteins indirectly by phosphorylating positive and negative co-regulators of protein expression or function. There are multiple PKC isoforms that are classified as conventional cPKCs (α, β and γ), novel nPKCs (η, ε, δ, and θ) and typical aPKCs (ι and ζ) (Mellor & Parker, 1998). Each of the isozyme is different in their pattern of specific tissue and subcellular distribution, function and $Ca2^{+}$/phospholipid cofactor requirements (Mellor & Parker, 1998). The novel Protein Kinase C (nPKCs) has been implicated in a wide range of important cellular processes such as regulating cell growth, homeostasis, and programmed cell death (Mackay & Mochly-Rosen, 2001). The nPKCs isozymes δ and ε have been shown to phosphorylate the transcriptional activation domain of MEF2A in cultured cells. The transcriptional activity of MEF2A enhances after phosphorylation by nPKC. However the exact phosphoacceptor sites targeted by PKC remain unknown (Ornatsky et al. 1999).

1.4.2. Acetylation

A well-characterized post-translational modification is acetylation, which involves the covalent linkage of an acetyl group from acetyl-coenzyme A to the ε-amino groups of lysine residues within the N-terminal tails of histones. The modification is regulated by two opposing families of proteins, histone acetyltransferase (HAT) and histone deacetylases (HDACs). Acetylation has

typically been associated with active transcription, whereas deacetylation of histones confers a repressed state. It is thought that acetylation facilitates transcription by opening up the chromatin structure through weakened histone-DNA interactions (Gregoire et al. 2007). The level of protein acetylation is balanced by lysine transferases and the complementary reversal is accomplished by histone deacetylases. This balance is perturbed in many tissues including muscle and brain (Mao & Wiedmann, 1999; Wu et al. 2001).

Protein phosphorylation is a key regulatory post-transcriptional modification necessary for normal cellular signaling and, therefore, of many cellular functions by regulating MEF2 in several tissue types. Beyond phosphorylation of serine and tyrosine residues, lysine acetylation has recently emerged as a critical modification regulating MEF2 functions (Kang et al. 2006; Gregoire et al. 2007; Angelelli et al. 2008). HDAC3, belong to class I HDAC, deacetylates MEF2 *in vivo*, represses MEF2 transcriptional activity and inhibits myogenesis. In contrast, the class IIa HDACs, HDAC4 and 5, repress MEF2-dependent transcription however, they cannot directly deacetylate MEF2 (Gregoire et al. 2007).

Previously, it has shown that p300 acetylates six lysine residues in the transactivation domain of MEF2C, both *in vivo* and *in vitro* (Ma et al. 2005). Acetylase p300 interacts with MEF2 and enhances MEF2 function by acetylation lysine residues at its C-terminal transcription domain. Mutation of these lysines affects MEF2 transcriptional activity and its synergistic effect with other transcription factors that ultimately inhibits myogenic differentiation (Ma et al. 2005). It is noteworthy that four major acetylatable lysines (K234/239/252/264) in

human MEF2C are also fully conserved in MEF2A, MEF2C, and MEF2D in variety of species. This suggests that MEF2 acetylation by p300 at these sites is a general mechanism conserved in vertebrates. These sites are functionally important, while overexpression of MEF2 mutants (non-acetylated) inhibit myogenic differentiation (Ma et al. 2005) **(Figure 12).**

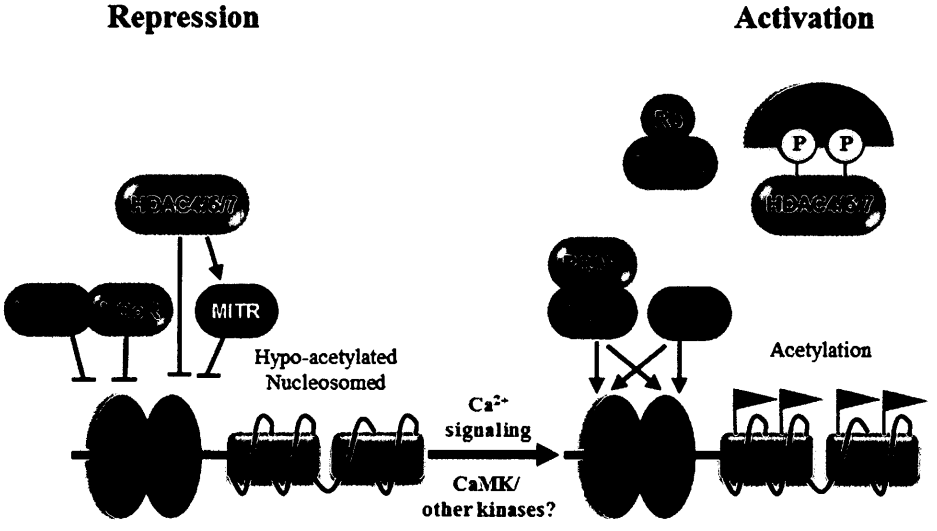

Figure 12. Schematic of model of HAT and HDACs. In the unphosphorylated state, class II HDACs are localized in the nucleus and associated with MEF2, resulting in repression of target genes. Phosphorylation of two conserved serines that flank the NLS of HDACs results in recruitment of 14-3-3 and dissociation from MEF2. Binding of 14-3-3 masks the NLS and activates a cryptic NES at the carboxyl terminus of HDACs leading nuclear export.

Calcium-dependent activation of calcineurin leads to dephosphorylation of Ser and Thr residues on MEF2 in neurons and muscle cells (Mao & Wiedmann, 1999; Wu et al. 2001). Dephosphorylation at S408 is particularly important for the ability to switch MEF2 from repressor to activator and is also required for activity to induce the switch at K403 from sumoylation to acetylation (Grégoire et al. 2006; Shalizi et al. 2006). It is conceivable that the MEF2 isoforms are similarly regulated by acetylation and play a key role in biological processes of T-cell development, cardiogenesis and neurogenesis.

1.4.3. Sumoylation of MEF2

Sumoylation is a novel post-translational mechanism where small ubiquitin-like modifier (SUMO) proteins are covalently attached to the lysine residues of target proteins through an amide bond (Gill, 2005; Hay, 2005). The SUMO is approximately 10 kDa in size, which is structurally related to ubiquitin and linked to histones. In vertebrates, there are at least three paralogues of SUMO proteins (SUMO1, -2, and -3). SUMO2 and -3 share greater than 90% sequence identity, and both are ~50% identical to SUMO1 (Gill, 2005; Hay, 2005). SUMO modifies many proteins that contribute in diverse cellular processes including cell cycle progression, subcellular transport, maintenance of DNA integrity, transcriptional regulation, and signal transduction (Bossis & Melchior, 2006). The growing number of extranuclear functions of neuronal sumoylation is reported to have critical implications in neurological disorders (Martin et al. 2007; Scheschonka et

al. 2007). Similar to acetylation, sumoylation targets lysine residues, but within a unique consensus sequence: ΨKxE (Ψ, large hydrophobic residue and x, any amino acid) which catalyze by a three set of enzymes, including E1-activating enzyme (Aos1/Uba2), E2 conjugating enzyme [Ubc9 (ubiquitin-conjugating enzyme 9], and E3 ligases. Further there are three types of SUMO E3 ligases identified as RanBP2, the PIAS (Protein Inhibitor of Activated STAT) proteins, and Pc2 (Gill, 2005; Hay, 2005). They exhibit different subcellular localization patterns and might enhance sumoylation of specific subsets of SUMO substrates *in vivo*. Like ubiquitination, sumoylation is a dynamic process and is actively reversed by SUMO-specific proteases, including SENP1, -2, -3 and -6, and is controlled by an enzymatic pathway (Gill, 2005; Hay, 2005; Bossis & Melchior, 2006). The functional consequences of SUMO attachment differ in great extent from substrate to substrate. Frequently SUMO alters interactions of substrates with other proteins or with DNA. SUMO can also act by increasing proteins stability through antagonizing ubiquitination The reversible covalent attachment of these small peptides modifies the target protein (Hay, 2005; Bossis & Melchior, 2006; Geiss-Friedlander & Melchior, 2007).

Sumoylation has emerged as an important regulatory mechanism for transcription factors and cofactors and, in general, inhibits their transcriptional activity in variety of tissue types (Riquelme et al. 2006). Series of studies suggest that sumoylation could play a pivotal role in controlling MEF2 transcriptional activity in muscle and neurons (Gregoire & Yang, 2005; Gregoire et al. 2006; Riquelme et al. 2006; Shalizi et al. 2006). Sumoylation of MEF2 is enhanced by

class IIa HDACs and by SIRT1-mediated deacetylation of the lysine acceptor for SUMO (Grégoire & Yang, 2005, Zhao et al. 2005). HDAC4 has a novel SUMO E3 ligase activity that regulates MEF2 sumoylation. Previous study has shown that HDAC4 binds the SUMO-conjugating enzyme Ubc9 and potently stimulates MEF2 sumoylation in both cultured cells and an in vitro reconstituted system (Grégoire & Yang, 2005, Zhao et al. 2005). In addition, C-terminal domain of MEF2C and MEF2D has been shown to be modified by SUMO2 and SUMO3 on a single lysine residue located at a consensus sumoylation motif which is conserved among MEF2 proteins. Sumoylation inhibits MEF2 transcriptional activity. SUMO protease SENP3 reverses this inhibitory effects and augments the myogenic activity of MEF2 (Gregoire & Yang, 2005). MEF2A also undergoes sumoylation primarily at a single lysine residue (K395) both under *in vitro* and *in vivo* condition. Mutation of K395 to arginine abolishes MEF2A sumoylation and enhances MEF2 transcriptional activity (Riquelme et al. 2006). Furthermore, sumoylation of MEF2C at K391 inhibits its transcriptional activity but does not block its DNA-binding activity (Kang et al. 2006). Interestingly, phosphorylation of S396 in MEF2C, a residue in close proximity to the major sumoylation site (K391), is known to be phosphorylated *in vivo* but enhances sumoylation of delta-N2-MEF2C *in vitro*. The S396A mutation reduces sumoylation of MEF2C *in vivo* and enhances the transcription activity of MEF2C in reporter assays (Kang et al. 2006). Previously, it has been documented that cdk5-induced phosphorylation at conserved S444 (4 amino acid downstream of a SUMO-targeted lysine residue) is required for

MEF2 sumoylation, and dephosphorylation of S444 by calcineurin reversed sumoylation of K439 (Gregoire et al. 2006, Kang et al. 2006).

Neuronal activity regulates the strength and number of synapses that are formed during neuronal development, and synapse formation involves contact between pre- and post-synaptic neurons (Shalizi et al. 2006). Recently, MEF2s have been identified as critical regulators of dendritic claw formation (Flavell et al. 2006). In developing hippocampal neurons MEF2 suppresses the number of excitatory synapses in a neuronal-activity and calcineurin-dependent manner. Neuronal activation leads to calcineurin activation and dephosphorylation of MEF2. Dephosphorylated MEF2 then leads to regulate various genes that restrict synapse number, such as activity-regulated cytoskeleton-associated protein (Arc) and synaptic Ras GTPase activating protein1 homolog (synGAP) (Flavell et al. 2006). Sumoylation of MEF2A strongly influences synapse formation through a phospho-regulated sumo-acetyl switch (Shalizi et al. 2006).

PIASx has been identified as an E3 for MEF2A sumoylation during synapse development *in vivo*. PIASx is a SUMO E3 ligase that represses MEF2-dependent transcriptional activity in neurons. Over-expression or knockdown of PIASx was shown to significantly enhance or inhibit dendritic claw formation respectively. This suggests a role for SUMO E3 ligases in brain development and neuronal plasticity (Shalizi et al. 2007). Previously, it has been reported that class IIa HDACs (HDAC4,5,7 and 9) also promote sumoylation of the transactivation domain of MEF2D in neuronal cells (Shalizi et al. 2007) The ability of class II HDACs to promote MEF2D sumoylation depends on the N-terminal repressor domain and it is

independent of the deacetylase motif (Lemercier et al. 2000; Gregoire & Yang, 2005). This may contribute repression of MEF2-dependent transcription by reducing the acetylation of histones at MEF2 target gene promoters and by deacetylating K403, a key SUMO residue in MEF2s. Deacetylation of K403 is correlated with modification of this residue by the SUMO moiety, which appears to stabilize MEF2 in the repressor state (Shalizi et al. 2006; Zhao et al. 2005).

On the other hand, during brain development, sumoylation at K403 residue represses MEF2A, and promotes postsynaptic neuronal differentiation. Activity-dependent calcium signaling leads to the activation of calcineurin and dephosphorylation of MEF2A at S408. This favours de-sumoylation of K403 and the subsequent acetylation of this residue, leading to MEF2A activation and inhibition of synapse formation (Shalizi et al. 2006). Thus, phosphorylation dependent switch between sumoylation (an inhibitory modification) and acetylation (an activating modification) comprise a novel regulatory mechanism to regulate the MEF2 activity which provides an understanding of the interplay between multiple post-translational modifications tightly regulating complex cellular processes.

1.5. Protein-protein Interaction

In all organisms, proper development, growth and function requires precise and integrated regulation of the gene expression. Often, regulation depends on the appropriate binding of proteins to DNA and upon protein-protein interactions. The function of MEF2 proteins is also regulated by their direct physical interaction with

diverse array of co-factors including activators/repressors, and adaptor/chaperone proteins that synergistically regulate MEF2 target gene expression in a variety of tissue types (Black & Olson 1998) **(Figure 13).**

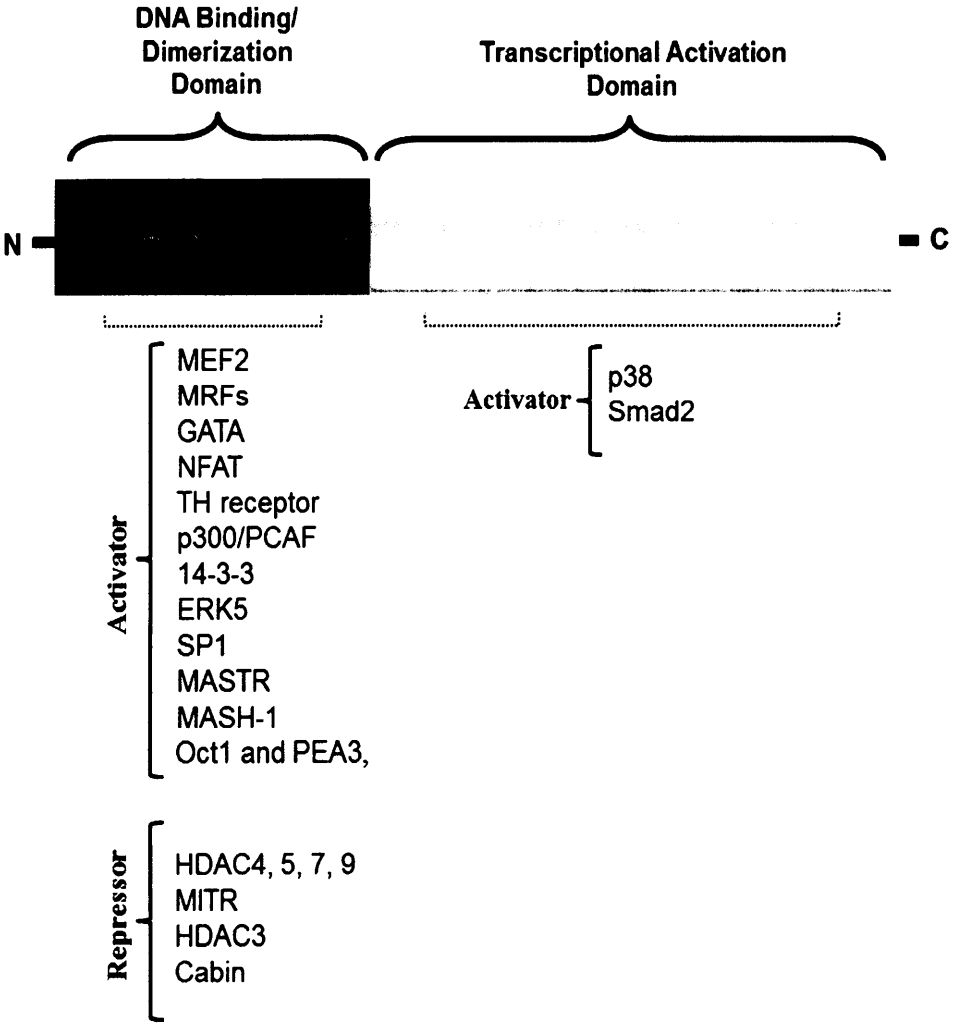

Figure 13: The schematic of MEF2 interacting partners (co-activators/co-repressors). The regulation of MEF2 is achieved through its interaction with diverse array of these co-factors in variety of tissues.

1.5.1. MEF2:MEF2 interactions

MEF2 members can homo- and heterodimerize with each other, but they cannot interact with other MADS-box containing factors, suggesting that specific amino acids residues within the MADS-box that establish dimerization interface are not conserved outside the MEF2 family (Black & Olson, 1998). In non-muscle cells MEF2A:MEF2D heterodimers are predominantly found to be transcriptionally inactive (Ikeshima et al. 1995; Black & Olson, 1998). In contrast, MEF2A:MEF2A homodimers are commonly formed in muscle cells and robustly activate muscle-specific gene transcription (Dodou et al. 1995; Ornatsky & McDermott, 1996; Black & Olson, 1998). Interactions between MEF2 members are mediated through the MADS-MEF2 domain of MEF2 factor. However the TAD domain of MEF2 is dispensable for the interaction with most of the known MEF2 coactivators. A truncated form of MEF2 containing only MADS and MEF2 domains acts as a dominant-negative transcription factor (Ornatsky et al. 1997), suggesting that gene expression requires more than DNA binding and dimerization of MEF2. The selection of genes regulated by MEF2 is normally determined by MEF2 interaction with other co-factors in various tissue types.

1.5.2. MEF2: HDACs & HATs interactions

MEF2 activity is tightly regulated by two families of chromatin-remodeling enzymes (HATs and HDACs). Histone acetylation by histone acetyltransferases (HATs) relaxes the structure of nucleosomes (Gregoire et al. 2007) whereas histone deacetylations by histone deacetylases (HDACs) promotes chromatin condensation,

and thereby act as transcriptional activators and repressors, respectively (McKinsey et al. 2001b; Grozinger & Schreiber, 2002; Verdin et al. 2003). HDACs regulate cellular processes in variety of tissue types, including skeletal muscle and cardiac growth, bone development, and neuronal survival by controlling gene expressions (Grozinger & Schreiber, 2002). The HAT p300, for example, has been shown to interact with the MADS domain of MEF2C, and to potentiate MEF2-mediated transactivation (Sartorelli et al. 1997). In mammals, there are four major classes of HDACs based on their homology to distinct yeast HDACs. Class I HDACs (HDAC1, 2, 3, and 8) are widely expressed and consist mainly of a catalytic domain, and are nuclear, believed to act predominantly at the chromatin level. Class II HDACs are divided into two sub-classes, IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6 and 10). Class III HDACs are referred to as sirtuins (SIRT 1-7) and class IV (HDAC 11). Members of Class I, II and IV HDACs share a common feature such as the dependence on zinc for their enzymatic activity, whereas class III HDACs are NAD+-dependent and members of class III HDACs are related to yeast Sir2, and are SirT1-7 (Wang et al. 1999). In addition, there is a truncated form of HDAC9, also called MEF2 interacting transcriptional repressor (MITR) that lacks intrinsic deacetylase activity, but it can recruit other HDACs or CtBP to MEF2 proteins (Sparrow et al. 1999; Bertos et al. 2001; Zhang et al. 2001a; Verdin et al. 2003). Growing evidence supports a therapeutic potential for HDACs against diseases such as neurodegenerative disorders and cardiac hypertrophy (Zhang et al. 2001a; Yang & Gregoire, 2005; Bolger et al. 2007) **(Figure 14A).**

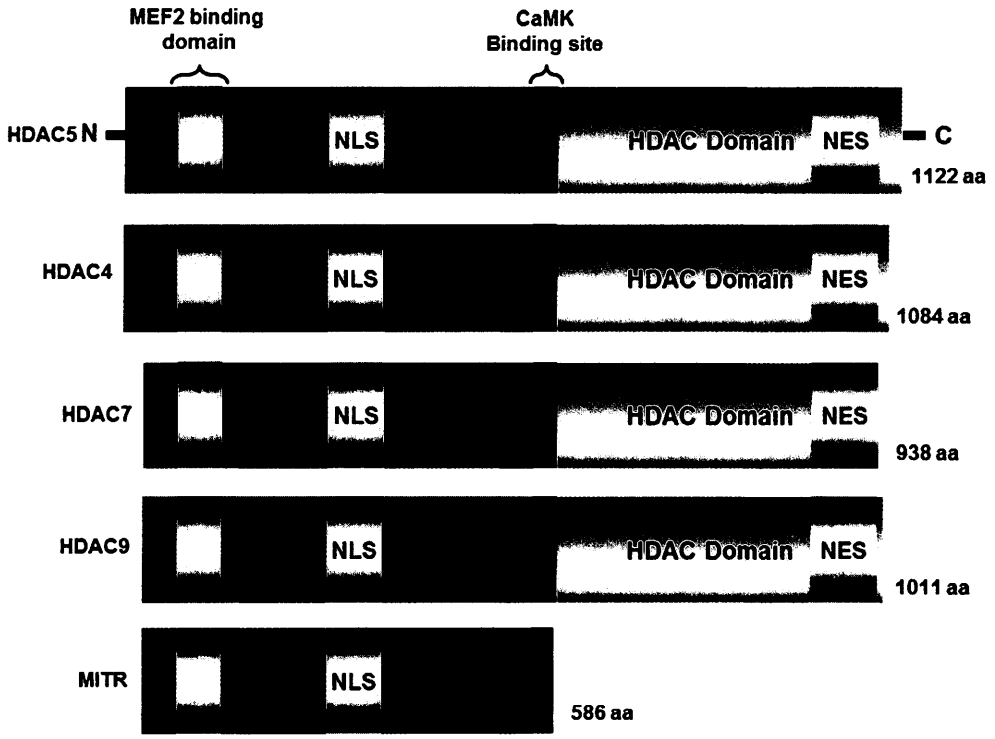

Figure 14A: The schematic diagram of class II HDAC family members. Members of class II HDACs have a bipartite structure, with a C-terminal catalytic (HDAC) domain and an N-terminal extension with a MEF2 binding domain. Conserved phosphorylation sites flank the nuclear localization sequence (NLS) and a nuclear export sequence (NES) is near the C terminus. MITR is a splice variant of HDAC9 that lacks an HDAC domain.

All class IIa HDACs (HDACs 4, 5, 7, and 9) are distinguished from other classes of HDACs. Class IIa HDACs exhibit three unique features. First, they are expressed in a tissue-specific manner and exert their transcriptional repressive function in diverse tissue types (Martin et al. 2009). Second, members of IIa HDACs contain a highly conserved 18 amino acid extension domain (MEF2

binding domain) at their N-termini that mediates binding to MEF2 proteins and regulating nuclear-cytoplasmic shuttling, while other classes of HDACs do not contain this domain and fail to directly interact with MEF2s (McKinsey et al. 2001a; Chan et al. 2003) **(Figure 14B).**

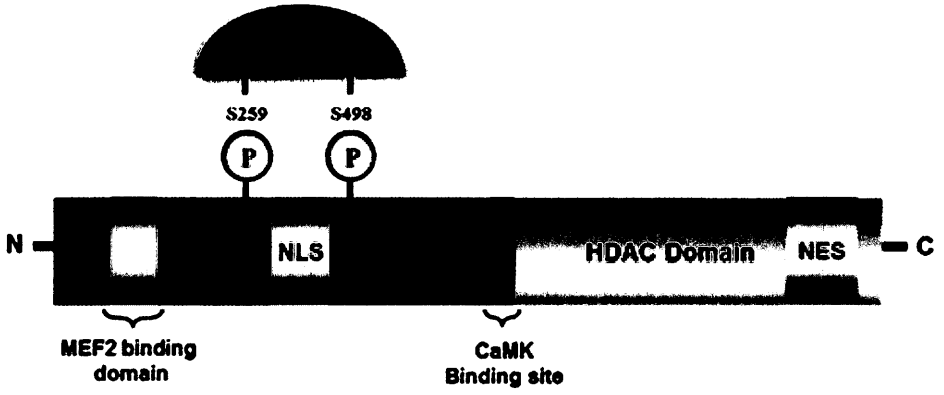

Figure 14B: The schematic structure of class II HDAC (histone deacetylase) protein. Class II HDACs have a bipartite structure, with a C-terminal catalytic (HDAC) domain and an N-terminal extension with a MEF2 binding domain. Conserved phosphorylation sites flank the nuclear localization sequence (NLS) and a nuclear export sequence (NES) is near the C terminus. Phosphorylation of two conserved serines that flank the NLS results in recruitment of 14-3-3. Binding of 14-3-3 masks the NLS and activates a cryptic NES at the carboxyl terminus.

MEF2 activity in muscle is inhibited under basal conditions by class IIa HDACs, which repress cellular gene expression by binding directly to MEF2 proteins in the nucleus (Miska et al. 1999). Third, phosphorylation of class IIa HDACs is a crucial event that determines whether they are localized in the nucleus or cytoplasm and, therefore, their ability to act as transcriptional co-repressors in

the nuclear compartment. The cellular trafficking class IIa HDACs are regulated by intrinsic nuclear import and export signals as well as binding sites for 14-3-3 proteins (Grozinger & Schreiber, 2000). HDAC4, 5, 7, and 9 contain series of conserved 14-3-3 binding sites located in the regulatory N-terminal domain. Binding of the 14-3-3 proteins stimulate the cytoplasmic retention or nuclear export of the class IIa HDACs in a phosphorylation dependent manner, which in turn regulates the activity of transcription factors such as MEF2, that regulates muscle, stress-responsive and survival genes (McKinsey et al. 2000a; Bertos et al. 2001; Verdin et al. 2003) **(Figure 15).**

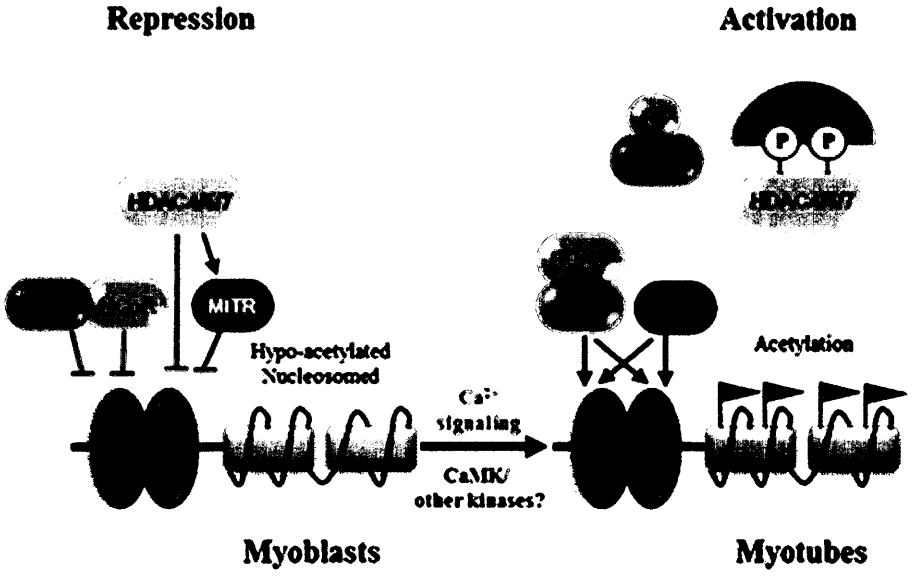

Figure 15. Schematic of model of HAT and HDACs in the control of muscle gene expression. In undifferentiated myoblasts, the activity of MEF2 is repressed by association with a variety of HDACs, resulting in deacetylation of

histones and transcriptional repression of muscle target genes. Initiation of differentiation, CaMK and possibly other kinases phosphorylates HDACs results in dissociation of HDACs from MEF2. Hyperphosphorylated Rb binds to HDAC1 in a competitive manner to dissociate HDACs from MEF2 leading histone acetylation. p300/PCAF and other coactivators with HAT activity are then recruited to muscle gene control regions through association with MEF2, with resulting activation of muscle transcription and myotubes formation (adapted from McKinsey et al. 2001).

Several signaling pathways, including Ca2+/calmodulin-dependent kinases (CaMKs) (McKinsey et al. 2000a), protein kinase D (PKD) (Vega et al. 2004), microtubule affinity-regulating kinases (Chang et al. 2005), and salt-inducible kinases (SIK) (Berdeaux et al. 2007) regulate phosphorylation of these 14-3-3 binding sites. Majority of class IIa HDACs demonstrate cell-type-restricted expression patterns and target selected physiological programmes (Bertos et al. 2001; Verdin et al. 2003, Majdzadeh et al. 2008) **(Figure 16).**

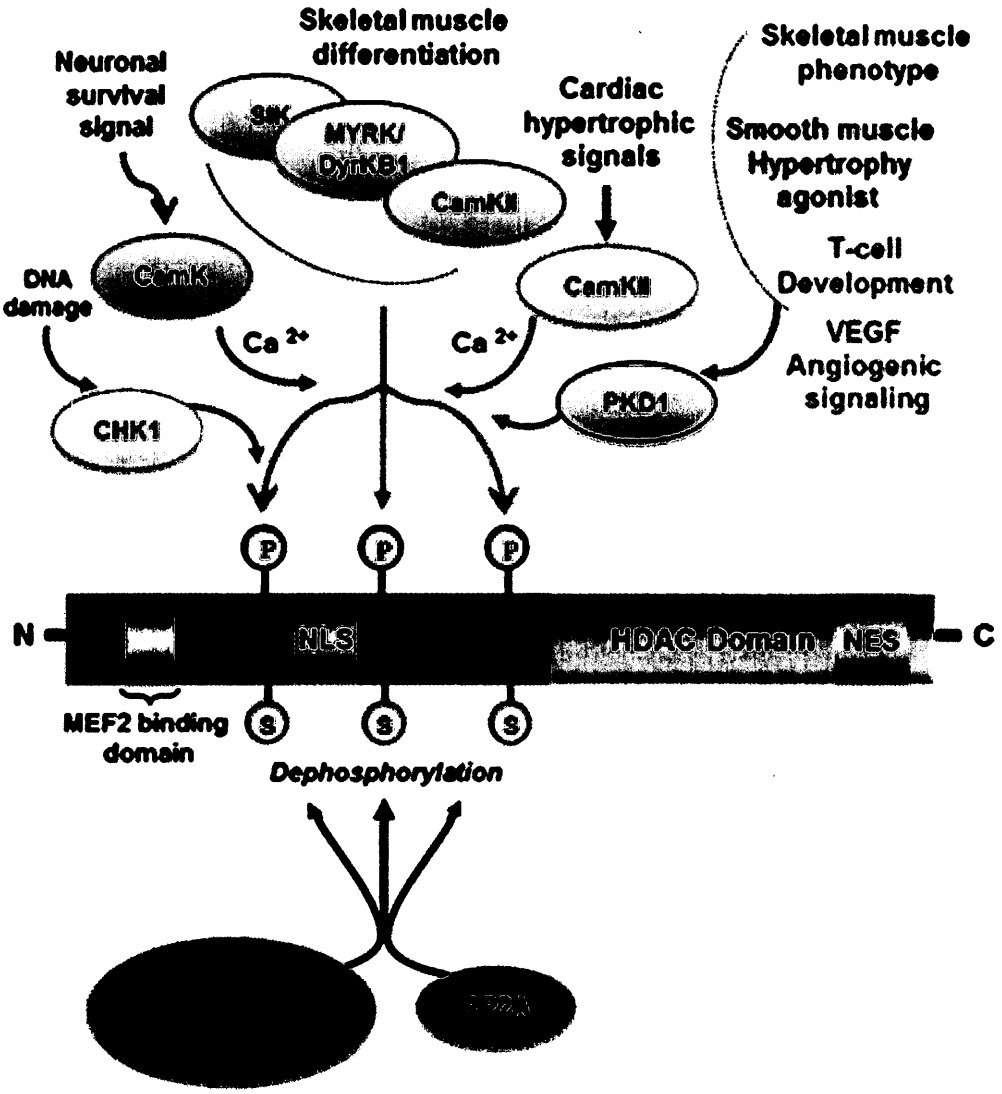

Figure 16. Model of class IIa HDACs activity regulated by multiple kinases/phosphatases in variety of tissue types. Class IIa HDACs have a C-terminal catalytic (HDAC) domain and an N-terminal regulatory domain. The phosphorylation sites are located in the N-terminal. Kinases/phosphatases that phosphorylate/dephosphorylate class IIa HDACs are shown with a description of the biological functions in diverse tissue types.

The interaction between MEF2 and HDACs was originally identified by yeast two hybrid screening (Sparrow et al. 1999; Lu et al. 2000a). Class IIa HDAC4 and -5 are predominantly expressed in the skeletal, cardiac, and smooth muscle, and the brain, within the same tissues where MEF2s expression are also found in highest levels. Interestingly, number of studies documented that HDAC4, 5, and 7 directly interacts with MEF2 and act as potent inhibitors of MEF2 dependent transcriptional activity (Lemercier et al. 2000; Lu et al. 2000b). Overexpression of both HDAC4 and HDAC5 suppresses skeletal muscle cell differentiation *in vitro* (McKinsey et al. 2000b), while expression of HDAC4 in muscle fibers is sufficient to induce muscle damage in mice (Miska et al. 1999). Previous studies in knockout mice have identified class IIa HDACs as key regulators of tissue growth and development. Mice lacking HDAC5 and HDAC9 show exaggerated hypertrophic growth of the myocardium in response to diverse stress stimuli (Backs & Olson, 2006). Mice deficient in HDAC4 show premature bone calcification, and mice lacking HDAC7 show embryonic lethality resulting from a failure to form tight junctions in the developing circulatory system (Martin et al. 2009). Both HDAC4 and HDAC5 show many similarities in control class IIa HDAC activity but differential localization of HDAC4 and HDAC5 has also been observed during differentiation of myoblasts into myotubes. HDAC4 is cytoplasmic in undifferentiated myoblasts and shuttle into the nucleus after myoblast fusion during differentiation (Miska et al. 2001; Zhao et al. 2001). However, HDAC5 localization exhibits an inverse pattern: it is nuclear in myoblasts and is exported to the cytoplasm during differentiation (McKinsey et al. 2001a; Zhao et al. 2001). The functional

significance of this difference is not clear. It is possible that HDAC4 and HDAC5 target different MEF2-dependent genes during the transition of myoblasts to differentiating myotubes. Previously, it has been shown that MEF2 proteins associate with histone deacetylase5 (HDAC5) in myoblasts, resulting in repression of muscle genes controlled by MEF2 sites (McKinsey et al. 2000a). The repression of particular MEF2 dependent genes by HDAC4 is required at the later stages of muscle differentiation/ or to maintain a terminally differentiated state of myotubes (Lu et al. 2000b). Association of class IIa HDACs and other co-factors with MEF2 promotes the formation of multi-protein repressive complexes on MEF2-dependent muscle genes such as myogenin (Potthoff & Olson, 2007). Previously, it was shown that HDAC4 and related members bind to MEF2 via the MITR homology domain and potently repress MEF2-induced gene transcription (Sparrow et al. 1999; Zhang et al. 2001b). Another study identified activation of HDAC4 in response to chronically reduced neural activity suppresses MEF2-dependent gene expression and contributes to progressive muscle dysfunction and also observed in neuromuscular diseases (Cohen et al. 2009). Previously, it was thought that inhibition of MEF2 transcriptional activity, through direct interaction with HDACs, prevents the myogenic genes expression prior to cells receiving appropriate differentiation cues. In proliferating myoblasts, MEF2 is present but transcriptionally silent. When cells are induce to differentiate, calcium-regulated protein kinases, such as PKD and various CaMKs convey signals from G protein-coupled receptors to the regulatory phosphorylation sites in class IIa HDACs in a

variety of tissue types, rescue MEF2-dependent target genes from HDACs inhibitory effects (Verdin et al. 2003; Paroni et al. 2004).

The interaction between MEF2 and HDACs (4 and 5) and its regulation by the CaMKs, has been well characterized in skeletal, cardiac muscle and in neuronal cells (McKinsey et al. 2002; Linseman et al. 2003a; Backs et al. 2006; Potthoff et al. 2007; Cohen et al. 2009). Activation of CaMK results in dissociation of MEF2 from these HDACs and unmasking of MEF2 transcriptional activity (Ginnan et al. 2012). Repression of MEF2 activity is specific for HDACs 4 and 5, but, it is not observed for other HDACs that lack the MEF2-interacting region. However, this repression can be relieved by over-expression of active CaMKs, whereas the transcriptional activity of MEF2 proteins can also be inhibited by CaMK inhibitors, such as KN-62 and KN-93 (Lu et al. 2000a; Linseman et al. 2003a). MEF2 can then associates with HATs and other positive regulators of muscle differentiation and activate myogenic genes. CaMK I and IV directly phosphorylate HDAC5 on conserved residues at Ser259 and Ser498. These sites are conserved in HDAC4 as Ser246 and Ser467, which creates a docking site for the intracellular chaperone protein 14-3-3 (McKinsey et al. 2000a; Vega et al. 2004). In skeletal muscle fibers, the signal-dependent regulation of HDAC4, but not HDAC5, seems to be under the control of CaMK II (Liu et al. 2005). The specificity among the different class IIa HDACs with respect to their responsiveness to upstream kinases is not fully determined yet. The binding of 14-3-3 to HDACs disrupts its interaction with MEF2, and subsequent export of the 14-3-3: HDACs complex from the nucleus to

the cytoplasm through a CRM1-dependent nuclear export process (Grozinger & Schreiber, 2000; McKinsey et al. 2000b) **(Figure 17).**

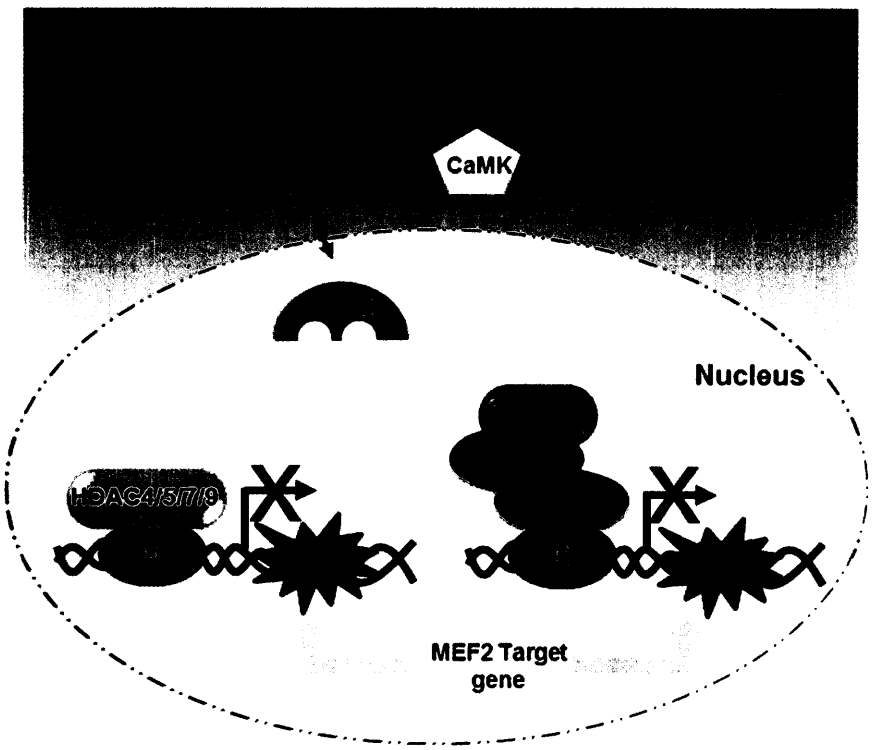

Figure 17. Regulation of class IIa HDACs activity. MEF2 factors bind to their target promoters and repressed by a variety of HDACs complexes. Class II HDACs, including HDAC4, HDAC5, HDAC7 and HDAC9, interact directly with the MEF2 DNA-binding domain. Class I HDACs, such as HDAC1 and HDAC2, can also be recruited to MEF2 through the corepressor Cabin1, inhibits MEF2 target gene expression in myogenic and neurgenic cells.

In addition to its chaperone role, 14-3-3 can also associate with MEF2D directly and enhance its activity (Grozinger & Schreiber, 2000). Phosphorylation-dependent association of class IIa HDACs with 14-3-3 has additional effects, such

as blocking the interaction between these HDACs and importin α, which prevents nuclear import and dissociation of class IIa HDACs from HDAC3, which would attenuate deacetylase activity (Grozinger & Schreiber, 2000; Grégoire et al. 2007). Most of the previous studies document that only class II HDACs are able to bind to MEF2 proteins, whereas Class I and III HDACs can not bind to MEF2 due to the lack of the 18 conserved amino acids that mediates the interaction. However, one of the class I HDAC proteins (HDAC3) can directly interact with MEF2D through the MADS-box domain (Grégoire et al. 2007). Moreover, HDAC3 can also bind to the acetyltransferases p300/PCAF to attenuate autoacetylation. Consequently, HDAC3 is able to repress MEF2-dependent transcription and inhibits myogenesis via the physical interaction and deacetylase activity. Whereas, the negative effect of HDAC3 was reversed by silencing HDAC3 expression with RNA interference resulted in enhanced MEF2 transcriptional activity and myogenesis. However, forced expression of HDAC3 had little effect on MEF2 activity in skeletal muscle cells (Grégoire et al. 2007). In contrast to HDAC4 and 5, HDAC3 efficiently deacetylated MEF2D *in vitro* and *in vivo* and this effect is specific to HDAC3, whereas other class I HDACs (HDAC1, 2, and 8) failed to act in similar manner (Grégoire et al. 2007).

The role of the HDAC-mediated regulation of MEF2 has begun to be explored in neurons that linked to regulation of neuronal survival as well as death (Li et al. 2001; Linseman et al. 2003). A substantial body of evidence indicates that the activity of MEF2 transcription factors play a critical role in neuronal survival. MEF2 family members, MEF2A and MEF2D are highly expressed and active in

cerebellar granule neurons (CGNs) promoting neuronal survival, particularly in response to depolarization-induced signals that is important during neuronal development (Li et al. 2001). In neurons, class IIa HDACs are identified to contain a MEF2 binding site, HDAC5 is the only member interacts with MEF2 in CGNs. Previously it was demonstrated that HDAC5 play a critical role in regulation of neuronal cell death. Overexpression of HDAC5 induces apoptosis in CGNs, as a result of inhibiting pro-survival role of MEF2 (Linseman et al. 2003). Localization of both HDAC4 and 5 appears to be calcium signaling dependent in cell culture. In CGNs, HDAC4 and 5 are mainly localized in the cytoplasm in the presence of depolarizing media (containing high potassium) but translocates to the nucleus under neuronal death conditions, such as withdrawal of depolarization media or excitotoxic glutamate conditions (Nakanishi & Okazawa 2006). By contrast, treatment with the neuronal survival factor BDNF suppresses HDAC4 nuclear translocation, whereas blocking CaMKs expression by pro-apoptotic CaMKs pharmacological inhibitors (KN-93) stimulates HDAC4 nuclear accumulation, mimics the effect which is accompanied by loss of MEF2 activity. These experiments suggest that CaMK induces neuronal survival by phosphorylating HDAC4, which leads to its cytoplasmic localization (Chawla et al. 2003; Bolger & Yao, 2005). HDAC5 binds to MEF2 in the nucleus and inhibit the expression of MEF2 regulated genes whose activities responsible for neuronal survival (Linseman et al. 2003) **(Figure 18).**

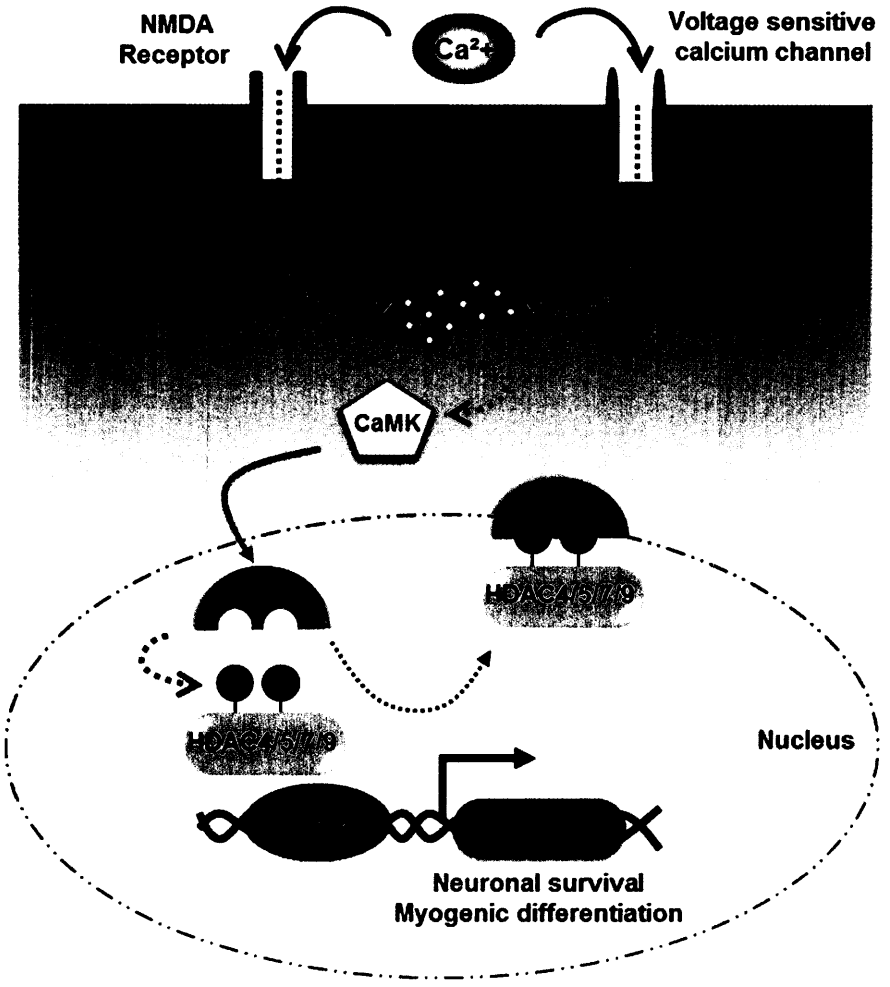

Figure 18. Regulation of class IIa HDACs activity in myogenic and neurogenic cells. In response to calcium signalling, class II HDACs are phosphorylated by activated calcium/calmodulin-dependent protein kinases (CaMKs). This results in recruiting the 14-3-3 family of chaperone proteins, which displace HDACs from MEF2 and promote HDAC export from the nucleus. After releasing from HDACs repressive complex, MEF2 become activated and activate target gene expression.

However, in hippocampal neurons, these HDACs act differently in response to depolarization activity, was shown to be sufficient for nuclear export of HDAC4 but not that of HDAC5 (Chawla et al. 2003). HDAC5 nuclear export was demonstrated to be induced following stimulation of calcium influx through NMDA receptors or L-type calcium channels (Chawla et al. 2003). Isozyme CaMKIIα is a brain-specific kinase and well-known for its role in depolarization-mediated survival of CGNs and in hippocampal long term potentiation, a cellular model of learning and memory (Linseman et al. 2003; Belfield et al. 2006). However, CaMKIIα is also expressed in cerebellum, where it has been implicated in CGN neurite outgrowth (Faison et al. 2002; Yang et al. 2008). These studies have shown that HDACs play a substantial role in inhibition of MEF2 dependent gene expression leading to neuronal cell death. Furthermore, overexpression of CaMKIIα rescued neurons from HDACs mediated cell death (Linseman et al. 2003; Bolger & Yao 2005); as a result of HDAC and 14-3-3 binding, HDACs are exported out of the nucleus (Bolger et al. 2007). The functional significance of the interaction between MEF2 family members and other class IIa HDACs in the context of neuronal survival is not known and needs further study. Previous studies has shown that overexpression of HDAC9 in muscle inhibits the upregulation of activity-dependent genes and chromatin acetylation in association with MEF2 and classI HDACs (Mejat et al. 2005). Moreover, HDAC9-null mice were demonstrated to be supersensitive to denervation-induced changes in gene expression. The splice variant of HDAC9 has been shown to contain a conserved MEF2 binding site functioning in cardiac

myocytes but no interaction between HDAC9 and MEF2 has been defined in CGNs (Mejat et al. 2005).

In addition, Protein Kinase C (PKC) signaling and its downstream effector, Protein kinase D (PKD) effectively block the nuclear export of HDAC5 in response to hypertrophic agonists in primary cardiomyocytes (Vega et al. 2004). PKD acts as a direct class IIa HDAC kinase. PKD has recently been reported to phosphorylate HDAC5 at the same sites which are phosphorylated by CaMK in skeletal muscle (Vega et al. 2004 ; Kim et al. 2008). The findings of PKD as HDAC kinase is critical to cardiac biology, while studies attempting to link HDAC5 regulation to CaMKs signalling in cardiac tissues have been unclear (Vega et al. 2004). PKD identified as a key regulator of skeletal muscle function and phenotype (Kim et al. 2008). Strikingly, levels of HDAC4 and HDAC5 phosphorylation were increased in the conditional PKD1 transgenic mice and correlate with elevated levels of MEF2 transcriptional activity (Kim et al. 2008). Abolishing the nuclear export of HDAC5 by inhibitors of PKC/PKD but not CaMK indicates the predominant role of the PKC/PKD pathway in regulating HDAC5 localization in heart. Ectopic expression of active PKD1 in the heart resulted in dilated cardiomyopathy (Vega et al. 2004). Furthermore, a novel and selective PKD inhibitor, bipyridyl PKD inhibitor (BPKDi), blocked the phosphorylation and nuclear export of class IIa HDACs, leading to the suppression of cardiac hypertrophy (Monovich et al. 2010). Interestingly, co-immunoprecipitation studies indicate that PKD, and not PKC, physically associates with HDAC5 while, PKC isoforms have also been identified to phosphorylate MEF2 *in vitro* and *in vivo* (Vega et al. 2004). PKDs are clearly

involved in regulating class IIa HDACs in cardiac tissues. Although, the exact mechanism of phosphorylation compared to PKD-induced HDAC export in muscle development is still unknown. Furthermore, a link between PKDs and class IIa HDACs in neurons has not been established.

Previous studies suggested that protein kinase A (PKA) also regulates MEF2 activity through HDAC-mediated mechanisms by promoting HDACs nuclear retention, as a result inhibiting of MEF2 activity in neuronal cells (Belfield et al. 2006). Series of studies in recent years, particularly in the McDermott laboratory, have revealed that class IIa HDACs isoforms, such as HDAC4 and HDAC5, act as signal-responsive repressors of nuclear MEF2 activity and MEF2-dependent target genes in skeletal and smooth muscle, and in hippocampal neurons (Du et al. 2008; Gordon et al. 2009; Perry et al. 2009; Salma & McDermott, 2012). The salt-inducible kinase 1 (SIK1) was originally identified as a serine/threonine protein kinase whose expression was enhanced in the adrenal glands of rats fed a high salt diet (Wang et al. 1999; Berdeaux et al. 2007). SIKs are also upregulated in response to ACTH signaling, and depolarization and kinase-induced seizures in adrenocortical tumor cells and in the nervous system, respectively (Feldman et al. 2000; Okamoto et al. 2004). In unstimulated adrenal cells, SIK1 is localized to both cytosolic and nuclear compartments; however, when adrenal cells are stimulated with ACTH, SIK1 is exported from the nucleus (Takemori et al. 2002). The nuclear shuttling of SIK1 is regulated by direct phosphorylation by PKA at Ser577; whereas mutation of this serine residue to alanine results in a nuclear distribution and constitutively active SIK1 activity (Takemori et al. 2002). Furthermore, previous

studies have revealed that SIK1 also phosphorylates class IIa HDACs specifically, HDAC4 and HDAC5 at Ser246/Ser467 and Ser259/Ser498 respectively and promote nuclear export of these HDACs (Berdeaux et al. 2007). Knockdown of SIK1 suppresses HDAC5 phosphorylation, whereas overexpression of SIK1 induced the nuclear export of an HDAC5-GFP fusion protein in C2C12 myoblasts culture (Takemori et al. 2009). In addition, forced expression of SIK1 *in vivo* was able to increase the amount of phosphorylated HDAC5 and decrease the necrotic foci number in a muscular dystrophy model (Berdeaux et al. 2007). Interestingly, SIK1 homologue (KIN-29) in *C. elegans* phosphorylates the class IIa HDAC (HDA-4) on one conserved residue in mammalian HDAC5 (van der Linden et al. 2007). In skeletal muscle, PKA phosphorylates SIK1 at Ser577 residue to inhibit its catalytic activity and reduce the amount of phosphorylated HDAC5. Another evidence suggests that SIK1 is an important regulator of HDAC4-mediated c-jun repression in vascular smooth muscle cells (VSMC) and forced expression of SIK1 promoted nuclear export of HDAC4 which leading to induce c-jun expression in cultured VSMCs (Gordon et al. 2009; Ginnan et al. 2012). SIK1, that is normally phosphorylate by PKA, co-expression of PKA, initiate rapid shuttling of HDAC4 into the nuclear region as a result increased repression of c-jun expression. However mutation of PKA phospho site in SIK1, serine 577 to alanine, increases c-jun expression. Therefore, these results indicate that SIK1 is an important HDAC kinase that regulates MEF2-dependent c-jun expression through PKA signaling in VSMCs (Gordon et al. 2009).

According to one study ataxin-1 is identified as a neurodegenerative disorder protein whose mutant form causes the spinocerebellar ataxia type-1 (SCA1). This acts as a repressor of MEF2-dependent transcription in cerebellar granule neurons by associating MEF2-HDAC4 transcriptional complex which compromised neuronal survival (Bolger et al. 2007). Interestingly, ataxin-1 binds specifically to HDAC4 and colocalizes with MEF2-HDAC4 in the nuclear inclusion bodies. Additionally, studies have revealed that phosphorylation at S776 is a critical modulator for ataxin-1 toxicity (Emamian et al. 2003; Chen et al. 2003) and phosphorylation of this site shown to recruit the phospho-binding protein 14-3-3, resulting in increased ataxin-1stabilization, aggregation, and toxicity (Chen et al. 2003). Remarkably, mutation of serine776 to alanine severely impairs the ability of ataxin-1 to obtain a prominent neurodegeneration phenotype (Emamian et al. 2003). The findings of this study provide evidence of functional interaction between ataxin-1 and MEF2-HDAC complex, suggested repression of MEF2 activity may contribute to ataxin-1-induced neurotoxicity in cerebellar granule neurons (CGN). Whereas, ectopic expression of MEF2 partially rescues cellular toxicity caused by ataxin-1 in CGN. Therefore, restoring MEF2 transcriptional activity, for example, by HDAC inhibitors could be beneficial for SCA1 patients (Bolger et al. 2007). Taken together, these findings suggested that the class IIa HDACs and MEF2 association play a significant role in the development of muscle and neurons.

1.5.3. MEF2 and Protein phosphatase 1 (PP1)

There are several co-factors that have been identified to directly or indirectly interact with MEF2 proteins and their contribution in regulation of MEF2 transcriptional properties such as kinases, Class IIa HDACs, discussed in above subsection. However, until very recently, the identity of the phosphatases as a MEF2 co-factor and effect of phosphatases dephosphorylation on their interacting partners remained unknown. Phosphorylation is reversible processes and many phosphatases are involved in dephosphorylation kinase activities in range of tissue types (Grozinger & Schreiber, 2000). The first evidence was obtained by using phosphatases inhibitor (calyculin A) in skeletal muscle, confirms the involvement of a serine/threonine phosphatase in the regulation of class IIa HDACs. Treatment with phosphatases inhibitor decreases the amount of HDACs in the nucleus of skeletal muscle fibers (Grozinger & Schreiber, 2002; Liu et al. 2005). Previously, it was shown that bidirectional synaptic plasticity at cerebellar parallel fiber (PF)–Purkinje cell (PC) synapses is under control of a kinases/phosphatases switch mechanism, which control the postsynaptically expressed long-term depression (LTD) or long-term potentiation (LTP) and similar mechanisms also observed at hippocampal synapses (Belmeguenai & Hansel, 2005). Protein phosphatases are classified into three families on the basis of phospho-amino acid specificity, structure and interaction with regulatory subunits (Ceulemans & Bollen, 2004). The family of phosphatases, protein phosphatase 1 (PP1) is one of the most conserved in eukaryotes. The family PP1 is composed of three ubiquitously expressed isoforms (α, β and γ) that regulate a variety of cellular functions (Ceulemans et al. 2002;

Cohen, 2002). Substrate specificity and localization of PP1 phosphatases is achieved through interaction with regulatory subunits (R-subunits) which typically contain the highly conserved 'RVXF'([K/R]-X-[V/I/L]-X-[F/W]) binding motif. PP1α phosphatase activity is regulated by direct phosphorylation of a C-terminal threonine residue (T320) by cyclin dependent kinase complexes or direct interaction with inhibitory R-subunits (Ceulemans et al. 2002).

Recently from our group, a novel physical interaction has been identified between the catalytic subunit of the protein phosphatase 1α (PP1α) and MEF2. Binding of PP1α to MEF2 occurs within the nucleus and shown to inhibit MEF2-dependent transcriptional activity, in part, by recruiting HDAC4 to MEF2A (Perry et al. 2009). Interestingly, PP1α phosphatase activity is not required for MEF2 repression and was originally thought to dephosphorylate class IIa HDAC4 to promote their nuclear import in both phosphatase-dependent and phosphatase-independent manner (Grozinger & Schreiber, 2000; Perry et al. 2009). Moreover, PP1α overrides the positive influence of calcineurin signaling on MEF2 regulation and MEF2-PP1α interaction leads to control nuclear retention of HDAC4 followed by recruitment of HDAC4 to MEF2 transcription complexes in skeletal muscle cells (Perry et al. 2009). This study also provided evidence that PP1α- interferes with the pro-survival effect of MEF2 in primary hippocampal neurons by repressing MEF2 function. Taken together, these findings reveal the first functional interaction between PP1α and MEF2 proteins and MEF2-mediated transcriptional repression with a phosphatase in skeletal muscle and hippocampal neurons. As such this

interaction between PP1α and MEF2 exhibit an important and novel aspect of MEF2 regulation in both tissue types.

1.5.4. MEF2: NFAT interactions

In vertebrates, nuclear factor of activated Tcells (NFAT) family consist of five isoforms NFATc1/2/c, NFATc2/1/p, NFATc3/4/x, NFATc4/3 and NFAT5/TonEBP (Rao et al. 1997; Graef et al. 2001; Macian 2005). All family members contain the DNA binding domain, however only NFATc1-c4 contains the Ca^{2+} sensor/translocation domain (Jain et al. 1995; Graef et al. 2001). The NFAT family of transcription factors functions as integrators of multiple signaling pathways by binding to chromatin in combination with other transcription factors such as MEF2 to regulate genes essential for many developmental processes. Evidence from previous studies that NFAT plays a role in vertebrate development came from NFAT kockout mouse. Targeted disruption of NFATc1 results in embryonic lethality with defects in cardiac valve formation (de la Pompa et al. 1998; Ranger et al. 1998). Deletion of NFATc2 causes hyperproliferation of lymphocytes (Hodge et al. 1996; Xanthoudakis et al. 1996), and also dysregulation of chondrogenesis (Ranger, Gerstenfeld et al. 2000). NFATc3 null mice have defects in myogenesis (Oukka et al. 1998; Kegley et al. 2001).

In T-lymphocyte, NFAT are most important co-activator links calcineurin signaling to MEF2, which is critical in the regulation of T-lymphocyte apoptosis (Blaeser et al. 2000, Youn et al. 2000). MEF2 factors act as integrators of calcium signaling. Variations in intracellular calcium (Ca^{2+}) concentration can alter MEF2's

phosphorylation status as well as the interaction with co-regulators in muscle (McKinsey et al. 2002). NFAT activation is dependent upon a rise in intracellular Ca^{2+}, which activates the serine/threonine phosphatase, PP2B/calcineurin (Clipstone & Crabtree 1992; Jain et al. 1993; Hogan et al. 2003). This phosphatase directly dephosphorylates several residues in the Ca^{2+} sensor/translocation domain of NFAT, resulting in nuclear import of NFAT. In contrast, the nuclear export of NFAT requires the sequential rephosphorylation of this domain by several kinases like GSK3β (Beals et al. 1997; Okamura et al. 2004). Previously, it has been shown that calcineurin activate MEF2 by recruiting members of the NFAT family of transcription factors and form complex that regulate MEF2 mediated gene expression (Molkentin et al. 1998; McKinsey et al. 2002). Upon dephosphorylation by calcineurin, NFAT translocates to the nucleus where it directly interacts with MEF2 family members, MEF2A and -D (Blaeser et al. 2000). Number of studies demonstrated that NFAT is involved in skeletal muscle fiber type acquisition (Chin et al. 1998, Wu et al. 2000). The direct interaction between MEF2-NFAT greatly stimulates MEF2-dependent genes expression by facilitating recruitment of p300/CBP to MEF2 response elements (Youn et al. 2000). However, NFAT also known for their co-activation of GATA transcription factors, where they induce cell hypertrophy (Wada et al. 2002). In addition, forced expression of calcineurin *in vivo* powerfully activated a MEF2-LacZ reporter gene in skeletal muscle and to a lesser degree in the heart (Wu et al. 2000).

NFAT is an important player in the developing as well as in adult CNS. MEF2 and calcineurin is highly expressed in the brain, and transgenic mice containing

NFAT reporters show that the brain is the organ having the highest levels of NFAT transcriptional activity (Plyte et al. 2001; Wilkins et al. 2004). The cooperative binding of NFAT with other transcription factors to form NFAT transcriptional complexes appropriate for neuronal development appears to be downstream of neurotrophin and netrin signaling pathways. Therefore, MEF2-NAFT physical association may play a critical role in neuronal development. In neurons, L-type calcium channel signaling modulates intracellular calcium levels to regulate the nuclear import and transcriptional activity of NFAT by activating the phosphatase calcineurin. The rephosphorylation and following nuclear export of NFAT is mediated by GSK3β, which contribute to the induction of NFAT transcriptional activity in neurons (Beals et al. 1997; Neal & Clipstone 2001; Sheridan et al. 2002). Modulation of Ca^{2+} levels through voltage gated Ca^{2+} channels might also allow NFAT transcription complexes to sense and integrate synaptic activity during the process of synaptogenesis. The calcineurin/NFAT signaling is important in neuronal axon growth and guidance during vertebrate development. Studies with triple NFATc2/c3/c4 mutant mice demonstrated that the organization of sensory neurons projection and commissural axons growth are both dependent upon NFAT activity (Graef et al. 2003). In addition to calcium signaling, neurotrophin (BDNF/NGF) also play an important role in NFAT transcriptional activity mediated axon growth in several neuronal populations, including cortical and hippocampal neurons (Groth & Mermelstein, 2003; Benedito et al. 2005). However, according to another study NFATc4 promoted neuronal survival in the developing cerebellum (Benedito et al. 2005). It may be suggested that genes in developing neurons are

regulated by integrating transcription factors, such as MEF2-NFAT transcriptional complexes, and involve in diverse neuronal pro-differentiation and pro-survival functions.

1.5.5. MEF2 and MRF proteins

Many promoters and enhancers are particular in their regulation due to specific transcription factors binding. It is thought that the specificity of transcriptional regulation is controlled by combinations of transcription factors. During skeletal muscle differentiation, muscle-specific genes are regulated by two groups of transcription factors, the MyoD and MEF2 families, which work together to drive the differentiation process. The transcriptional activity of MEF2 is highly sensitive to regulation of gene expression by post-translational modifications that modulate MEF2 mediated gene expression and interactions with co-factors (Black et al. 1998; McKinsey et al. 2002). The most studied interaction of MEF2 proteins is with MyoD; myogenic regulatory factors (MRFs). This plays an important role in the control of skeletal muscle development by enhancing the muscle-inducing activity of myogenic bHLH proteins. The MRFs belong to basic helix-loop-helix (bHLH) family of sequence specific DNA binding transcription factors. Members of the bHLH family have been shown to control determination and differentiation of a variety of cell types, including skeletal muscle, neurons, and hematopoietic cells (Black & Olson, 1998). There are four myogenic bHLH proteins, the MRFs, consist of Myogenic Differentiation-1 (MyoD), Myogenic Factor-5 (Myf5), Myogenin (MyoG), and Myogenic Regulatory Factor-4 (MRF4/Herculin/Mfy6) (Pownall et al.

2002). In the skeletal muscle lineage, the four MRFs compose of a regulatory pathway that establishes myoblast identity and control terminal differentiation. Ectopic expression of any members of MRF family into nonmuscle C3H10T1/2 fibroblasts in culture, each of these members can activate the entire program for skeletal myogenesis (Davis et al. 1987; Yu et al. 1996; Molkentin & Olson, 1996).

The MRFs heterodimerize with another class of ubiquitously expressed bHLH proteins known as E proteins (such as E12, E47), and binding to a consensus DNA sequence (CANNTG) commonly known as an E-box. This is found in the regulatory region of most muscle specific genes to induce muscle specific gene expression in co-operation with other transcription factors (Murre et al. 1989; Brennan & Olson, 1990; Etzioni et al. 2005). This leads to the differentiation of muscle progenitor cells to morphologically and biochemically distinctive skeletal myocytes (Olson et al. 1991). MEF2 proteins can potently synergize with MyoD-E12 heterodimers and capable of converting nonmuscle cells to differentiated myotubes, whereas these proteins cannot activate transcription in collaboration with E12 homodimers (Molkentin et al. 1995, Naidu et al. 1995, Ornatsky et al. 1997) **(Figure 19).** Mutation or substitution of the MyoD basic domain with the E12 basic domain allows interaction to occur but blocks the transmission of activation signal (Molkentin et al. 1995).

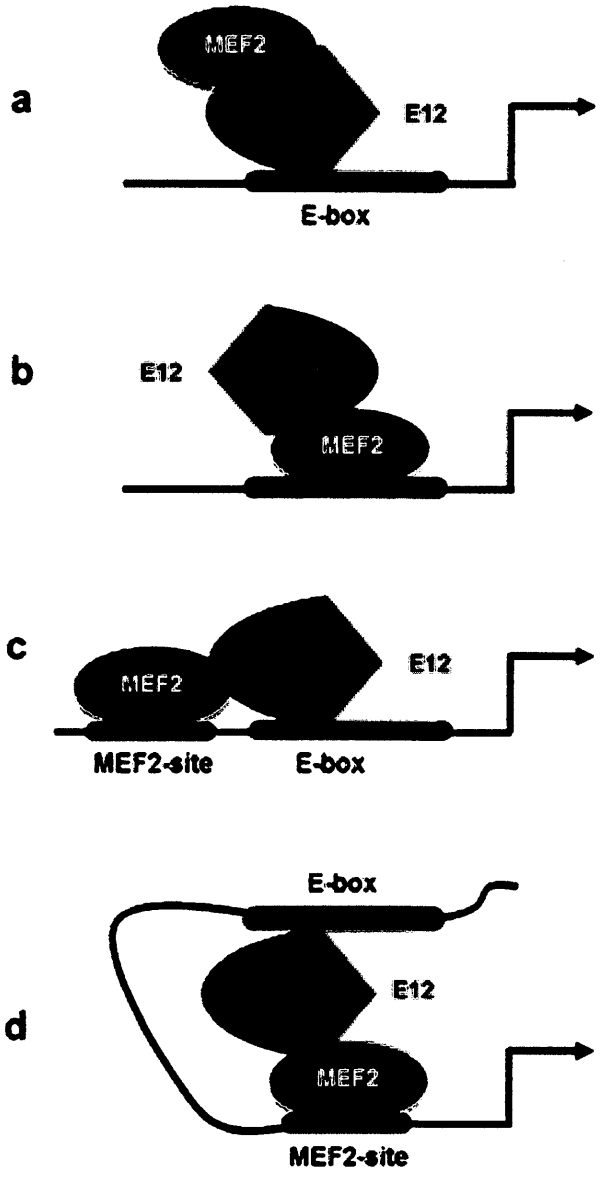

Figure 19. Schematic of MEF2-MRFs interaction. Four potential mechanisms for synergistic activation of gene expression. a) MEF2 heterodimerize with MRFs (MyoD/E12) bound to DNA. b) Recruitment of MRFs when MEF2 bound to DNA. c) MRFs and MEF2 heterodimerize by binding to adjacent sites on DNA. d) Binding of MRFs and MEF2 heterodimers to non-adjacent site on DNA and synergistically activate gene expression by protein-protein interaction (Adapted from Black & Olson, 1998).

There is accumulating evidence that MEF2 and MyoD associate through direct physical interaction to synergistically activate transcription and myogenesis when only one factor is bound to DNA (Kaushal et al. 1994; Molkentin et al. 1995; Molkentin et al. 1996). The bound factor is then capable of recruiting the other factor through protein-protein interactions. This interaction occurs through DNA-binding and dimerization domains (Molkentin et al. 1995). However, many muscle-specific promoters and enhancers contain MEF2 sites and E boxes in proximity to one another, suggesting that both classes of transcription factors may be bound to DNA at the same time while interacting with each other (Cheng et al. 1993; Yee & Rigby, 1993; Edmondson et al. 1992; Black et al. 1995; Naidu et al. 1995). Moreover, ectopic expression of MEF2 proteins with MRFs into C3H10T1/2 fibroblasts increases myogenic conversion whereas this myogenic conversion can be subdued by a dominant negative MEF2A protein lacking its transactivation domain (Ornatsky et al. 1997). Additionally, this dominant negative MEF2A was also documented to repress myotube formation in cultured myoblasts, suggesting that MEF2 is required for skeletal muscle differentiation (Ornatsky et al. 1997). MEF2C and myogenin can interact with each other while both are bound to DNA.

The structure of the MEF2C skeletal muscle promoter/enhancer is remarkably similar to that of the myogenin and MRF4 promoters, both of which contain MyoD- and MEF2-binding sites. Like MEF2C, myogenin and MRF4 are upregulated during myocyte differentiation, presumably through direct transactivation by bHLH and MEF2 factors. This type of crosstalk provides a powerful mechanism for amplification of both types of regulatory genes, thereby reinforcing and stabilizing

the transcriptional program for myogenesis. Alternatively, both MEF2 and myogenic bHLH factors bound to DNA may stabilize the protein-protein interactions between them to more efficiently activate transcription.

However, in neuronal cells, MEF2 physically interact with neurogenic basic helix-loop-helix (bHLH) transcription factor MASH-1, a protein implicated in the development of neurons, to regulate gene expression (Black et al. 1996; Skerjanc & Wilton, 2000). MEF2-MASH-1 interaction is neuron specific and with broadly expressed in brain which provides the adaptability of MEF2 function in CNS. This interaction is mediated through the MADS-MEF2 domain of MEF2 and bHLH binding region of MASH1 (Black et al. 1996; Mao & Nadal-Ginard, 1996). Further this interaction allows MEF2 and MASH1 to activate gene expression through their respective DNA-binding sites in a cooperative and synergistic manner, thereby providing functional mode that potentially expand its regulatory targets.

1.5.6. Interaction between MEF2 and other co-factors

It is clear that MEF2 function depends on its ability to recruit co-factors for appropriate activation of target gene. The HATs cyclic adenosine monophosphate (cAMP) response element binding protein (CREB)-binding protein (CBP) and p300 interact directly with the MEF2 domain. The site overlaps with the HDACs binding site (Sartorelli et al. 1997; DeLuca et al. 2003). Displacement of HDACs and recruitment of HAT activity following myogenic stimuli have been shown to enhance lysine acetylation in chromatin at MEF2 responsive genes, a common correlate of transcriptional activation (Zhang et al. 2002). In T-lymphocytes, MEF2

is required for apoptosis upon activation of the T-cell receptor (McKinsey et al. 2002). This process requires dissociation of the calcium-sensing receptor Cabin 1, which competes with p300 for MEF2 binding, from the MADS-MEF2 domain. Disruption of Cabin1 binding to MEF2 by calcium signaling leads to transcription of the pro-apoptotic nur77 gene in a p300 and MEF2-dependent manner (Youn & Liu, 2000). In addition to HATs, MEF2 interacts indirectly with the co-activator-associated arginine methyltransferase-1 (CARM1). The enzymatic activity of CARM1, which promotes histone arginine methylation, is requires for myoblast differentiation (Chen et al. 2002).

The interaction of MEF2 and CARM1 is dependent upon a nuclear receptor co-activator, glucocorticoid receptor interacting protein-1 (GRIP-1). GRIP-1 is a member of the steroid receptor co-activator (SRC)/p160 FAMILY of proteins that facilitate chromatin remodeling through the recruitment of histone acetyl and methyltransferases (Xu & Li, 2003). GRIP-1 targets MEF2 upon differentiation and enhances MEF2-dependent transcription (Chen et al. 2000). Conversely, stimuli that block muscle differentiation, such as transforming growth factor (TGFβ) activation of SMA- and MAD-homolog 3 (SMAD3) or the activity of cyclin-dependent kinases, disrupt the association of MEF2 and GRIP1 and prevent the GRIP1 dependent subnuclear targeting of MEF2 (Lazaro et al. 2002; Liu et al. 2004). Smad2/4 complexes, key mediators of the TGFβ signaling, have been shown to cooperate with MEF2 to initiate transcription in cultured C2C12 muscle cells (Quinn et al. 2001). An alternative coactivator of MEF2 dependent transcription is the peroxisome proliferators activated receptor γ (PPAR γ) coactivator 1 α (PGC-1

α). It is a master regulator of mitochondrial biogenesis and energy homeostasis and it promotes transcription through its ability to recruit chromatin modifying and RNA processing complexes (Puigserver & Spiegelman, 2003). In addition to MEF2A cofactor, expression of the PGC-1α gene is stimulated by calcium signaling and MEF2 dependent transcription. PGC-1α is not required for muscle formation but instead promotes fast-to-slow fiber type switching and enhances the expression of genes required for oxidative metabolism (Czubryt et al. 2003). A physical association between MEF2 and two SAP domain transcription factor, including myocardin (a cardiac-enriched isoform arising from alternative splicing) and MASTR, which robustly activates MEF2-dependent gene expression (Creemers et al 2006b). Interestingly, MEF2 can also cooperate with the broadly expressed transcritption factors SP1 to regulate the promoters of the neuronal N-methyl-D-aspartate (NMDA) receptor subtype1 (NR1) and trophic factor NT3, respectively (Grayson et al 1998; Krainc et al. 1998; Shalizi et al. 2003).

2. Function of MEF2 in myogenic & neurogenic cells

The robust expression of MEF2 transcripts and protein expression in myogenic and neurogenic tissues correlates with the strong MEF2 activity in diverse tissue types. Several lines of evidence have implicated MEF2 function in muscle progenitor cell specification and differentiation (Black & Olson 1998). Drosophila, *D-mef2* gene demonstrated an essential role of MEF2 in myogenesis and morphogenesis (Bour et al. 1995; Prokop et al. 1996). In addition to its functional

role in myogenesis, MEF2s are also involved in cardiac hypertrophy (Kolodziejczyk et al. 1999). The phenotypes of mef2s null mice showed distinct role of MEF2 in skeletal and cardiac muscle (Lin et al. 1997; Black & Olson, 1998; Naya et al. 2002; Phan et al. 2005; Kim et al. 2008).

Since the members of the MEF2 family (MEF2A-D) are critical during muscle differentiation and cardiovascular function (Black & Olson, 1998; Molkentin et al. 1998) but their individual roles within the central nervous system are still largely unknown. In addition to muscle, all members of the MEF2 family are highly expressed in brain tissues during embryogenesis suggest that these transcription factors also play important roles in the developing brain (Leifer et al. 1993, 1994; McDermott et al. 1993; Lyons et al. 1995; Lin et al. 1996). MEF2A and MEF2C expressions are restricted in cortex and cerebellum region during developing and adult neurons (Mao et al. 1999; Marinissen et al. 1999). Cortex contains a high level of MEF2C protein (Lin et al. 1996). In the mouse brain, MEF2A, C and D are expressed at high levels in multiple regions, including cortex, hippocampus and cerebellum (Lyons et al. 1995). MEF2A and MEF2D have been shown to play a specific role in cerebellar development and further involve to promote differentiation and survival of cerebellar granule neurons (Leifer et al. 1993, 1994; McDermott et al. 1993; Lyons et al. 1995; Lin et al. 1996; Mao et al. 1999; Marinissen et al. 1999; Salma & McDermott, 2012). Distinct patterns of expression during pre- and postnatal development suggest specific and distinct functions for each MEF2 protein at different stages of neuronal development. Furthermore, there

are indications that MEF2 proteins regulate acquisition of neuronal phenotypes (Ikeshima et al. 1995; Okamoto et al, 2000).

2.1. The role of MEF2 in neuronal cells

Over the last two decades significant progress has been made towards enhancing our understanding of the MEF2 functional role in CNS during embryogenesis, postnatal development, and adult tissue maintenance. Several lines of evidence suggest that MEF2 proteins are critically important for differentiation in post-mitotic neurons (Lyons et al. 1995; Li et al. 2001; Lam & Chawla, 2007; Genikhovich & Technau, 2011). In recent years, MEF2 protein has emerged as a key regulator in the development of the CNS (Kim et al. 2011; Salma & McDermott, 2012; Yin et al. 2012). Inhibition of MEF2 function in primary hippocampal and cortical neurons has been shown to induce neuronal cell death, suggesting that MEF2-dependent transcriptional regulation is necessary for neuronal survival (Mao et al. 1999; Okamoto et al. 2000; Kato et al. 2000; Shalizi et al. 2007; Yang et al. 2009; Perry et al. 2009; Lam et al. 2010; Kim et al. 2011; Salma & McDermott, 2012). Further the predominance of work on MEF2 function in the CNS has focused on the role of this transcriptional factor in controlling neuronal survival in response to a variety of extra- and intracellular stimulations (Shalizi et al. 2003; Linseman et al. 2003b; Wiedmann et al. 2005; Shalizi et al. 2006; Bolger et al. 2007; Fiore et al. 2009; Lam et al. 2010). In cerebellar granule neuron (CGN) survival depends on activity of MEF2 transcription factors. Depolarization-mediated MEF2 activity and CGN survival are compromised by

MEF2 inhibition. Both MEF2A and MEF2D undergo phosphorylation and caspase-mediated degradation during neuronal apoptosis (Li et al. 2001). Another study documented that ectopic expression of dominant-negative MEF2C cause apoptosis in cortical neurons. In dopaminergic neuron, modulation of MEF2 by cdk5 induced neuronal death (Smith et al. 2006). Additional evidence showed that MEF2 regulates activity dependent survival of granule neurons but overexpression of the MEF2 repressor histone deacetylase-5 (HDAC5) abolished this effect (Tian et al. 2010; Lam et al. 2010; Dietrich et al. 2012; Lyon et al. 2012). Knockdown of MEF2A using RNA interference (RNAi) markedly decreases the survival of granule neurons (Gaudilliere et al. 2002; Flavell et al. 2006). Moreover, depolarization-induced MEF2 activation is necessary for the survival of differentiating neurons (Gaudilliere et al. 2002) and MEF2 mediates activity-dependent survival of both cortical and cerebellar neurons (Mao et al. 1999). MEF2C is expressed in postmitotic differentiating neurons, but not in proliferating precursor cells in the cortex region, suggesting that MEF2 controls maturation of newly differentiated neurons (Mao et al. 1999). MEF2C transcription is stimulated by membrane depolarization in cerebellar neurons (Mao and Wiedmann, 1999). Another study has shown that loss of MEF2C in nestin-expressing neural stem/progenitor cells impairs neuronal differentiation *in vivo*. The neurotrophin comprise family of secreted proteins that has numerous functions in the nervous system development and plasticity (Poo, 2001). Neurotrophins (NGF and BDNF) promote the survival of distinct neuronal populations during brain development and their potential to promote recovery of neurons after injury in the mammalian adult

brain. Recent study found that BDNF activate MEF2C transcription in both neuron-like PC12 cells and in primary cultured cortical neurons which involve in neuronal plasticity (Lyons et al. 2012). ERK5 signaling contributes to neurotrophin stimulation of MEF2C transcription involves in neuronal survival. An intriguing feature of this effect is that ERK5 and MEF2D survival pathway is predominantly important for the survival of neurons that depend exclusively on neurotrophin support applied to distal axons (Liu et al. 2003). These observations suggest that the expression of different MEF2 isoforms during neuronal development in response to external stimuli can change the fine-tuning of transcription-dependent neuronal plasticity and MEF2 transcriptional inducibility of important activity-regulated survival genes (Tian et al. 2011). Another group demonstrated that neurotrophin-induced expression of anti-apoptotic gene (*bcl-w)* and MEF2D promote survival of developing sensory neurons (Pazyra-Murphy et al. 2009). However increased expression of MEF2D, in response to neurotrophin stimulation, eliminates synapse formations, but promotes neuronal survival. This indicates that MEF2 plays a dual role in maintaining number of synaptic formations, while simultaneously controlling the neuronal survival. Collectively, these findings suggest that MEF2 function is required for neuronal survival during the early stages of neuronal development and maturation. *In vitro* data strongly suggest important roles for specific MEF2 isoforms in neuronal survival, whereas the precise functions of the individual MEF2 isoforms in vivo remains to be defined.

Functions of the CNS rely upon synapses, the sites of communication between neurons and functional nodes in neural circuits. Synapses are complex structures,

changes in the structure and efficacy of synapses are the biological basis of neuronal functions such as learning and memory. Proper organization and activity of synapses define normal brain functions (Ruegg, 2001). MEF2 family members are highly expressed in the neurons where they regulate calcium-dependent transcriptional programs that are important for synapse and dendritic development (Flavell et al., 2006; Shalizi et al., 2006; Pfeiffer et al. 2010; Akhtar et al. 2012). Range of studies indicated that MEF2 protein is required for neurite growth, dendrite morphogenesis, and differentiation of post-synaptic structures (Flavell et al. 2006; Shalizi et al. 2006; Lam et al. 2007; Fiore et al. 2009; Kawashima et al. 2009; Tian et al. 2011; Akhtar et al. 2012; Chen et al. 2012). The expression of MEF2 proteins in the CNS is regulated temporally and spatially in an isoform-specific manner, and coincides with neuronal maturation. For example, cerebral cortical neuronal development is associated with changes in the expression of MEF2C (Leifer 1993 & 1994), whereas CGN maturation is coupled to enhanced expression of MEF2A and MEF2D (Lin et al. 1996). MEF2A and MEF2D are expressed in virtually all striatal neurons, including dorsal striatal and ventral striatal (nucleus accumbens) regions. The co-localization of MEF2A and MEF2D isoforms bind to DNA as heterodimers in striatal neurons (Pulipparacharuvil et al. 2008). Expression of MEF2A and MEF2D is found only in neurons but absent in astrocytes, indicating that MEF2 is restricted to neurons in the mammalian cortex and the cerebellum (Ikeshima et al. 1995). The predominantly neuronal expression of the MEF2 factors in the mammalian brain is particularly important in neuronal survival, synapse formation and maintenance (Flavell et al. 2006; Shalizi et al.

2006). MEF2 differential tissue distribution suggests that MEF2 proteins may be regulated in an isoform-specific manner and the regionally-specific differences of MEF2 involvement in neuronal differentiation and survival. RNA-interference-mediated knockdown of both MEF2A and D or activation in cultured hippocampal neurons results in increased excitatory synapse formation, or loss of synapses and dendritic spines, respectively. This suggests that activation of MEF2 restricts synapse numbers in hippocampal neurons (Flavell et al. 2006) (Figure 20). These alterations depend on the ability of the MEF2 isoforms to stimulate neuronal activity-dependent regulation of target genes expression (Flavell et al. 2008). MEF2 promotes post-synaptic dendritic morphogenesis that control neuronal survival. MEF2D expression is identified in both proliferating precursor cells and in differentiated neuronal cells that converts into two different population, neurons and glial cells but MEF2D expression increases in the nuclear region of neurons while decreases in the glial cells. MEF2D regulate neuronal development by controlling synapse formation and protecting newly differentiated neuronal cells (Shalizi et al. 2007). Interestingly, similar knockdown of MEF2A in developing cerebellar granule neurons *in vitro* or *in vivo* result in decline in the number of dendritic claws and synaptic formation (Shalizi et al. 2006; Pulipparacharuvil et al. 2008). Recent study documented that MEF2C is the major isoform involved in hippocampal synaptic function (Akhtar et al. 2012). Conditional deletion of MEF2C result in a marked increase in the number of excitatory synapses, accompanied by synaptic potentiation and decreased synaptic transmission, but with significant impairments in hippocampal-dependent learning and memory (Barbosa et al. 2008).

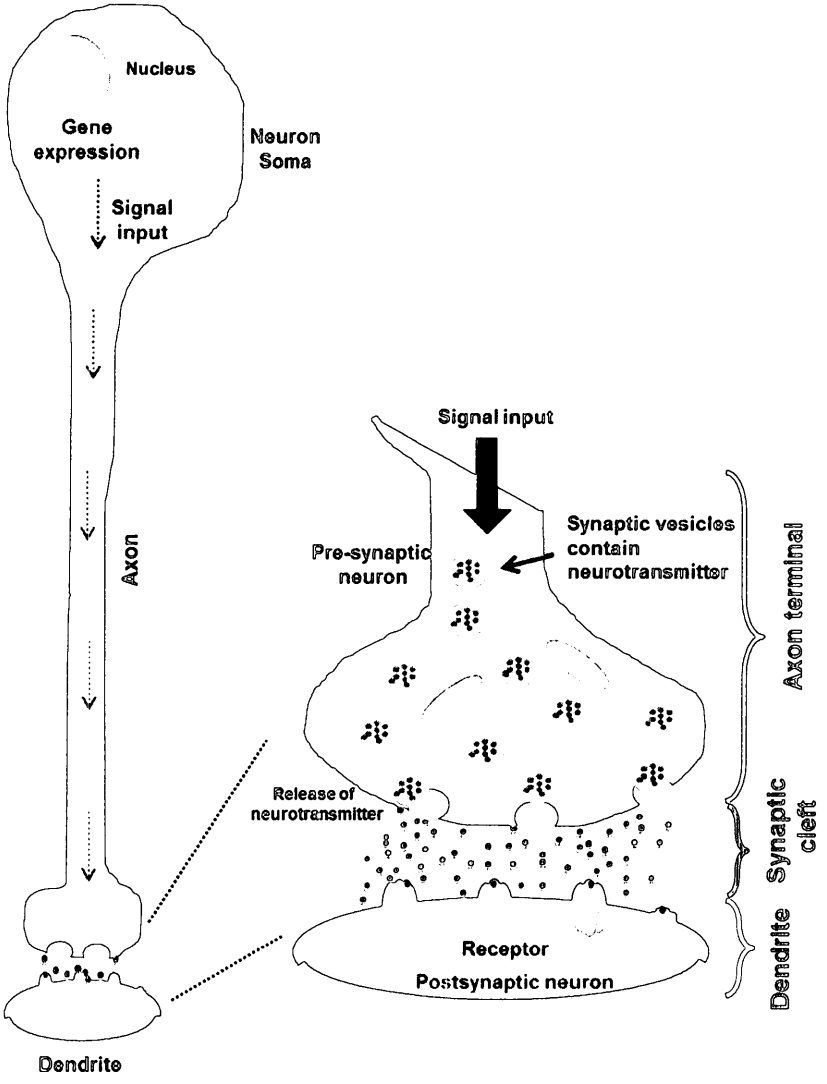

Figure 20. Schematic of synapse formation.
Synaptic activity is communicated through the nucleus to regulate activity-dependent gene expressions that regulate a range of neuronal functions in response to extracellular stimuli. Synapse formation (pre-synapse or signal sending neurons and post-synapse or signal receiving neurons) is important for neuronal development and plasticity.

In hippocampal and cerebellar neurons, MEF2A regulates the formation and maintenance of neurite and excitatory synapses by modulating the expression of synapse specific genes (Flavell et al. 2006). The MEF2A plays an important role in the formation of excitatory synapses in the hippocampus and the cerebellum, where it is localized exclusively in the nucleus. MEF2A plays an additional role in the lateral septum and bed nucleus of the stria terminalis (LS/BNST), amygdala, and paraventricular hypothalamus, where it is localized to axons and dendrites as well as nuclei and is primarily associated with inhibitory synapses (Neely et al. 2009).

Acute transcriptional activation of MEF2 causes a decrease in synapse number via induction of MEF2 mediated target genes, Arc and Homer1a which actively disassemble excitatory synapses formed onto MEF2 expressing neurons. Induction of Arc by MEF2 leads to internalization of postsynaptic AMPA receptors de-represses excitatory transmission. However, induction of Homer1a by MEF2 leads to the deconstruction of scaffolding proteins complexes at the synapse (Flavell et al. 2008). Although BDNF *(*brain derived neurotrophic factor*)* is also identified as a potential MEF2 target gene in neurons that are involve in limits synapses number but further studies are needed to exactly define the detailed molecular mechanism by which MEF2 activity involve in synapse formations and neuronal survival. Originally BDNF has been identified primarily in the context of synapse maturation and recent studies have shown that BDNF has diverse functions depending on its posttranscriptional modification and cellular localization, as well as the selection of receptor (Lyons et al. 2012). It is possible that under certain conditions BDNF and possibly some other MEF2 target genes may be selectively deployed to a subset of

synapses that will ultimately be retained rather than eliminated. Role of MEF2 in *Drosophila* is well known in muscle development. Recently MEF2 expression, recorded in clock neurons, reported as playing a role in circadian behavior. (Blanchard et al. 2010). So the knockdown of MEF2 means loss of circadian behavioral rhythms.

3. MEF2 target genes

MEF2 plays an important role in myogenic program by activation of muscle specific target genes which determine the transition of single cell to differentiate into multinucleated myotubes. Regardless different level of MEF2 activity, it has been demonstrated that MEF2 can independently affect different muscle genes. Some genes require higher MEF2 activity levels for their expression than others (Chen et al. 2012). In contrast to the wealth of information available on the regulatory targets of MEF2 in skeletal muscle, relatively little is known about the identities of the MEF2 controlled genes in neuronal cells. Therefore it is important to understand MEF2 role in neuronal cells by characterization of MEF2 target genes. It is also worthwhile to understand how hundreds of genes are regulated in a controlled program by a single transcription factor. MEF2 coordinate the complex process of development in diverse tissue types. In this study we have uncovered a novel MEF2 target gene in neuronal cells known as Krüppel-like factor 6 (KLF6). KLF6 gene expression is observed in neuronal and non-neuronal cells which dynamically responsive to extracellular stimuli. We found that KLF6 play critical role in neuronal survival and it is also involved in skeletal and cardiac muscle

development (Dionyssiou et al. 2013). Characterization of KLF6 may steps forwards our understanding of the role of MEF2 in the regulation of gene expression during skeletal muscle differentiation, cardiac and neuronal cells development. An emerging body of evidence implicates an important role for KLF6 factors in neuronal and cardiovascular development, but the role of KLF6 in muscle development is still need to be identified.

The Krüppel-like factor 6:

The Krüppel-like factor 6 (KLF6), also known as Core Promoter Binding Protein (CPBP)/GC-rich sites binding factor (GBF), is a zinc-finger transcription factor (Zf9) (Koritschoner et al. 1997). It belongs to the Krüppel-like factor (KLFs) family of gene regulatory proteins implicate in many biological processes, including proliferation, apoptosis, differentiation and development. The Krüppel-like factors (KLFs) are members of the zinc-finger family of transcription factors named after their similarity to the *Drosophila* body pattern-determining gap gene *Krüppel* (meaning "cripple") (Nusslein-Volhard & Weischaus 1980; Schuh et al. 1986). KLFs are closely related to the Sp family of zinc-finger transcription factors (Dang et al. 2000) and are critical regulators of phenotypic modulation and physiologic function. In 1993 the first mammalian KLF gene, KLF1 or *Erythroid Krüppel-like factor* (EKLF1), was cloned. To date, 17 members of the KLF family have been identified in mammalian cells and are known to be expressed in a broad range of tissue types. According to phylogenetic relationships and recent nomenclature are referred to as KLF1–KLF17. The majority of KLF family members are discovered

within the last 5 years (Suske et al. 2005; Pearson et al 2008). The 17 genes encoding different KLFs are found all over the human genome and identical to mouse genome which are also contain 17 *Klf* genes. These genes are evolved during gene duplications process. The high level of conservation of structure and function of KLF proteins in different species is a reflection of their ancient evolutionary history.

Initially, KLFs were named after the tissue in which they were highly expressed and detected first time such as erythroid Eklf (KLF1), lung Lklf (LKLF2), gut Gklf (KLF4), intestinal Iklf (KLF5) and KLF15 is kidney Kklf (Bieker, 1996; Shields et al. 1996; Turner & Crossley, 1999a; Pearson et al 2008). KLF9 was identified as a basal transcription element binding (BTEB) protein, with KLF5 (BTEB2) and KLF13 (BTEB3) as homologues (Kobayashi et al. 1995). KLF10 and KLF11 were identified as early genes induced by transforming growth factor β and they are also named as TIEG and TIEG2, respectively (Fautsch et al. 2008). Other KLFs family members are widely expressed including basic Bklf (KLF3) and ubiquitous Uklf (KLF6 and KLF7). During the last decade, the KLF family members have been known for investigation in human health and diseases (Ratziu et al. 1998; Black et al. 2001; Dong & Chen, 2009). These factors appear to exert important regulatory functions on many biological processes, including endothelial development (SenBanerjee et al. 2004; Parmar et al. 2006); hematopoiesis (Nuez et al. 1995; Turner & Crossley, 1999b; Matsumoto et al. 2006); cardiac remodeling (Shindo et al. 2002; Fisch et al. 2007); angiogenesis (Bhattacharya et al. 2005); neoplasia (Rowland et al. 2005; Wei et al. 2006);

gluconeogenesis (Gray et al. 2007); monocyte activation (Feinberg et al. 2005; Das et al. 2006) and determination of pluripotent stem cell fate (Takahashi & Yamanaka, 2006) (**Figure 21**).

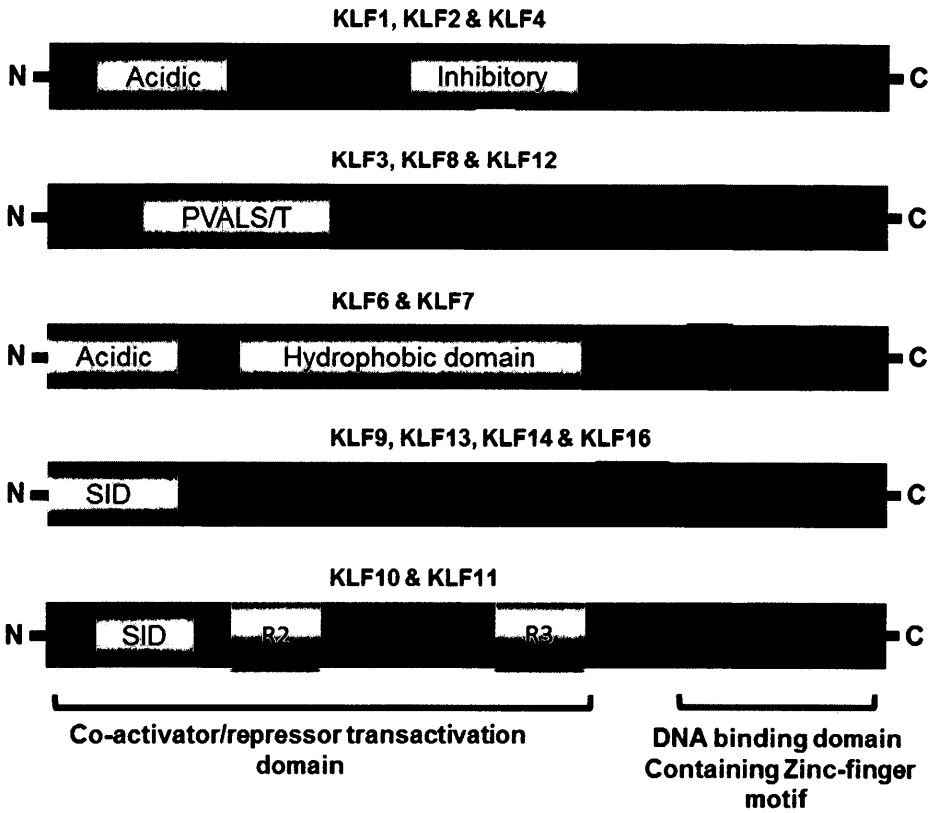

Figure 21: The schematic family members of Krüppel-like factor. KLF proteins are grouped according to common structural and functional domains. KLFs are highly homologous in their carboxyl-terminal DNA-binding regions, which contain three C2H2 zinc finger motifs.

Structure: There is significant conservation in several aspects among the KLF family members, for example, all KLFs are characterized by a highly conserved

DNA binding domain at the carboxyl terminus and recognize GC-rich DNA sequences that has a CACCC homology in its target gene promoters to mediate activation and/or repression of transcription (Miller & Bieker, 1993; Pearson et al. 2008). The unique feature of the KLF family is the presence of highly conserved classical three Cys2/His2 zinc fingers with more than 65 per cent amino acid sequence identity among the family members. Zinc fingers 1 and 2 contain 23 residues, while the third finger has only 21 residues. The Cys2/His2 zinc fingers present in the KLFs consist of two short beta strands followed by an alpha helix, two conserved cysteines and histidines coordinate a zinc ion in the classical Cys2/His2 zinc-finger domain (Philipsen & Suske, 1999) (**Figure 22**).

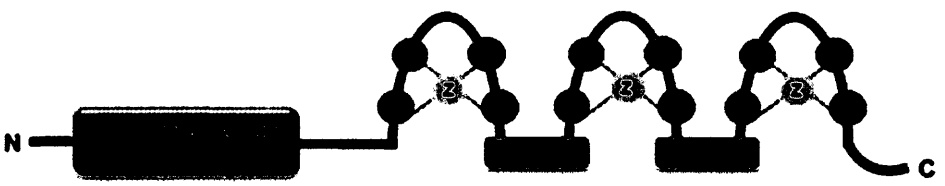

Figure 22: The structure of Krüppel-like factor. Shown are the three C-terminal C2H2 zinc fingers, each chelating a single zinc ion. The fingers are linked by the "TGERP"-like motif, which assists in binding to target DNA. The activation/repression domain is found at the N-terminus of the molecule.

In addition, the seven residue sequence in between the zinc-finger domains (TGE(R/K)P(Y/F)X) is highly conserved in KLF family members and this linker sequence is located to the extreme C-terminus of the proteins (Dang et al. 2000).

KLFs have unique N-terminal regions, which enable them to participate in diverse cellular processes including transcriptional regulation, cell proliferation, or differentiation (Cook & Urrutia, 2000; Sun et al. 2001; Kaczynski et al. 2003; Suske et al. 2005) (**Figure 22**).

Expression of Krüppel-like factor

KLF6 is evolutionary conserved and broadly expressed in numerous tissue types and at several developmental stages including the placenta, heart, lung, kidney, liver, and brain (in the forebrain and midbrain) (Ratziu et al. 1998; Blanchon et al. 2001; Fischer et al. 2001; Laub et al. 2001a; Ito et al. 2004; Matsumoto et al. 2006; Miele et al. 2008; Jeong et al. 2009). In the mouse, KLF6 is expressed in embryonic tissues at E10.5 and in undifferentiated mesenchyme surrounding the neural tube and brain vesicles by E11.5, with strong expression in the nervous system by E12.5 and low levels in the heart, ureteric bud, and lung buds (Fischer et al. 2001; Laub et al. 2001a). By E14.5, KLF6 is nearly undetectable except in the ventral horn at the level of the forelimbs. Subsequently, strong KLF6 expression is observed between E16.5 and E18.5 in the intestinal mucosa and in the fetal liver between E14 and E20 (Laub et al. 2001a; Ratziu et al. 1998). KLF6 is detected in the developing cornea of the 7-weeks old fetus. KLF6 mostly localize in the cytoplasm of cornea cells but accumulate more in the nucleus after birth (Nakamura et al. 2004 & 2007). Phylogenetically, KLF6 is most related to KLF7 (Laub et al. 2005). KLF6 and KLF7 have been segregated into distinct group based on structural and functional features. Thus both genes have comparable exon/intron organization in mammalians tissues. In addition to the high homology of the DNA-

binding domains, KLF6 and KLF7 share 47 residues at the N-terminus region providing similar transcriptional activation properties as the stimulation of the p21 gene promoter. KLF7 is highly expressed ubiquitously during development but at low levels in adult tissue. The main difference between KLF6 and KLF7 residue in a distinct expression pattern, KLF6 highest expression level is observed in placenta, while KLF7 high expression level is restricted to brain tissues, both in the peripheral nervous system (PNS) and CNS which reflects essential role of KLF7 in neuronal development. But KLF6 and KLF7 are both found to be highly expressed throughout the process of brain development (Laub et al. 2001b; Lei et al. 2005; Veldman & Bemben, 2007; Moore et al. 2009). Expression of KLF6 is observed in various regions in the forebrain, including the cerebral cortex, thalamus, hypothalamus and amygdale in the adult brain (Laub et al. 2001a; Jeong et al. 2009). KLF6 is highly expressed in the neurons of both the pyramidal and granule cell layers of the hippocampus. KLF6 signals are detected in the stratum radiatum and lacunosum-moleculare of the CA1 subfield. The phenotypes of KLF6 expressing cells in the normal brain are indicative of neurons. The KLF6 expression level in the pyramidal cell layer of CA1 and CA3 regions disappeared after epileptic seizures possibly due to neuronal cell death (Jeong et al. 2011). Expression of KLF6 in neuronal progenitor cells in the adult forebrain indicates functional role of KLF6 (Jeong et al. 2009). In terms of the vascular distribution of KLF6, there are several reports showing KLF6 expression in endothelial cells under physiological conditions and after vascular injury (Botella et al. 2002; Atkins & Jain, 2007; Andreoli et al. 2010; Garrido-Martín et al. 2012). Taken together, widespread

neuronal and endothelial distribution of KLF6 protein suggests that KLF6 plays specific roles in the physiological functions of the mature brain.

Researchers have reported that expression of KLF6 increases in various cells following the tissue injury. For example number of KLF6 positive cells increases upon renal ischemic insults especially in renal epithelial cells (Holian et al. 2008). KLF6 is expressed in hepatic stellate cells after liver injury induced by carbon tetrachloride, which is a model for hepatic fibrosis (Ratziu et al. 1998). Moreover, KLF6 expression is induced in vascular endothelial cells from the aorta and the carotid artery after ballooning (Kojima et al. 2000). Therefore, it is highly likely that KLF6 expression is directly linked to the mechanism which responds to tissue injuries. KLF6 expression is regulated in a number of physiological processes and the induced production of KLF6 may mediate some of the subsequent physiological responses to extrinsic/intrinsic stimuli. Certain KLFs show a tissue restricted expression pattern, but most are widely expressed. The mechanisms regulating expression of KLFs are incompletely defined, although it is becoming clear that they are often differentially expressed during differentiation. KLF2 is down-regulated upon activation of T cells. Similarly KLF4 shows changes in expression during B-cell development and KLF5 plays a major role in injury-induced cardiac remodelling (Shindo et al. 2002).

KLF9, a basal transcription element binding (BTEB) protein, is expressed in dentate granule neurons of the dentate gyrus (DG), a region of the mammalian brain in which neurogenesis occurs in adulthood. KLF9 is upregulated during the early

postnatal period and is expressed in dentate granule neurons during the late stage of maturation, when the cells are integrated into the hippocampal network (Scobie et al. 2009). Dentate granule neurons from KLF9−/− mice show delayed maturation, and adult KLF9−/− mice exhibit impaired differentiation of adult-born neurons. Thus, KLF9 is necessary for late-phase maturation of dentate granule neurons both in DG development and during adult hippocampal neurogenesis. KLF13 is highly expressed in adult hearts and is required for normal cardiac development (Lavallée et al. 2006). KLF15 is also highly expressed in adult hearts and in cardiac myocytes. Overexpression of KLF15 in cardiac myocytes suppresses the morphological changes and reduction in gene expression induced by hypertrophic stimuli (Fisch et al. 2007; Noack et al. 2012). The expression patterns of the individual KLFs vary during development and adulthood. Overlapping patterns of expression and function of KLFs suggest distinct functional role of the KLF family members, involving cardiac hypertrophy, hematopoiesis, adipogenesis and the pluripotency of stem cells.

Despite the similarities, the majority of KLFs seem to have unique tissue-specific roles in an *in vivo* setting. Some of these roles have begun to be elucidated primarily by *in vivo* experiments involving gene knockout. All KLFs knockout mice generated to date shows a lethal phenotype. KLF6 knockout mice die by embryonic day 12.5 and are characterized by markedly reduced hematopoietic differentiation in yolk sacs, placenta and liver. In culture, ES cells are less able to proliferate and differentiate in the absence of KLF6 (Matsumoto et al. 2006). KLF7 null mice die within 2 days after birth and show severe neurological defects resulting from

incorrect development of axonal pathways. These defects include disruption of the olfactory and visual systems, cerebral cortex and hippocampus. Further analysis of the KLF7 null mouse revealed that loss of the protein results in increased apoptosis of sensory neurons (Laub et al. 2005). KLF5 −/− mice die before E8.5 due to impaired cardiovascular development and adipogenesis (Oishi et al. 2005; Shindo et al. 2002). KLF2−/− mice are lethal, cardiac failure at E12.5 to E14.5 (Wani et al. 1998; Lingrel et al. 2012). KLF10 and KLF15 knockout mice are viable with bone defects, impaired skeletal development and aged cardiac defects (Subramaniam et al. 2005; Fisch et al. 2007; Wang et al. 2008). Ablations for several KLF gene family members indicate an essential role for these factors in the regulation of remarkable diverse functions.

Function of KLF6:

Many lines of evidence has shown distinct types of human cancers describing infrequent genetic alterations of KLF6 gene or even enhanced expression in some tumors. Studies has demonstrated that KLF6 function as a tumor suppressor in various cancers, such as in prostate and colon cancers, because of its ability to reduce cell proliferation through several biochemical mechanisms including regulation of cell cycle components, oncogene products and apoptosis. Specifically KLF6 appears to be growth suppressive, in part by up-regulating p21, a cyclin-dependent kinase inhibitor. However, up-regulation of p21 by KLF6 occurs independently of p53. Mutation within the KLF6 gene, decreased expression, and/or loss-of-heterozygosity is associated with the development of different human

malignancies. Thus, loss of KLF6 might lead to removal of cellular proliferation controlled mechanism (Narla et al. 2007; DiFeo et al. 2009). Additionally, increased evidence suggests that KLF6 is a bonafide target of several signaling cascades, which ultimately regulate decisions of cell survival and death. KLF6 interaction with c-Jun determines different cell outcomes such as proliferation control or apoptosis which depends on the external stimuli received by the cell.

Most KLFs act primarily as transcriptional repressors and/or activators according to specific circumstances. KLF6 is largely transcriptional activator but suppresses variety of genes (Jeong et al. 2011; Calderon et al. 2012). As an activator of transcription, KLF6 interacts with the core promoter element present in both TATA-less or TATA box-containing promoters (Koritschoner et al. 1997; Gehrau et al. 2005). In addition to interactions with other proteins, post-translational modifications (e.g. phosphorylation or acetylation) regulate the transactivating activities of different KLFs (Li et al. 2005a). KLF family members regulate fundamental cellular responses such as growth, apoptosis, angiogenesis and proliferation for example; overexpression of KLF5 is associated with cell proliferation, whereas KLF2, KLF4 and KLF6 are more consistently implicated in cell cycle arrest (Ghaleb et al. 2005). In diverse tissues, induction of KLF6 expression occurs rapidly during satellite cell activation. KLF6 has been shown to transcriptionally activate structural and cytokine genes including those encoding pregnancy-specific glycoprotein 5 (PSG5) (Racca et al. 2011), platelet-derived growth factor (PDGF) receptor (Kimmelman et al. 2004), collagen α1 (Botella et al. 2002), leukotriene C4 synthase (LTC4S) (Zhao et al. 2000), urokinase plasminogen

activator (uPA) (Kojima et al. 2000), transforming growth factor (TGF)-β1, and types I and II TGF-β receptors (Mgbemena et al. 2011). Urokinase plasminogen activator is a key enzyme implicated in tissue remodeling, tumor metastasis, and apoptosis. In addition, KLF6 physically interacts with KLF4 and together they activate the human keratin 4 promoters (Okano et al. 2000).

In addition, an association between KLF6 and transforming growth factor (TGF beta) has been reported in different tissues including liver, kidney and vascular cells (Kojima et al., 2000). In response to vascular injury, KLF6 interacts with Sp1 and cooperatively binds and transactivates the endoglin promoter (Garrido-Martín et al. 2012). Endoglin is an endothelial membrane glycoprotein involved in vascular remodeling and cardiovascular development that is up regulated in response to arterial injury. KLF6 has been demonstrated to play a role in endothelial cell motility (Das et al. 2006). Upregulation of matrix metalloproteinase-9 induces endothelial cell migration, a fundamental step in the process of vascular remodeling and repair (Garrido-Martín et al. 2012). Receptor activation leads to the disruption of Sp2/KLF6 repression complex on the matrix metalloproteinase-9 promoter via SHP (small heterodimeric partner). Taken together, these results strongly implicate KLF6 as playing a key role in vascular development, remodeling and response to injury. KLF6 gene products have also been identified in lower vertebrate and invertebrate organisms such as Luna, the Drosophila progenitor of the mammalian KLF6/KLF7 group, in which they appear to control cell differentiation during embryonic development (Slavin et al. 1999; Laub et al. 2001; De Graeve et al. 2003; Zhao et al. 2010). KLF6 also involve in

pre-adipocyte development by promoting adipocyte differentiation while inhibiting delta-like 1 (Li et al. 2005b). KLF6 was initially shown to be rapidly induced in activated hepatic cells, the key fibrogenic cell type during liver injury and repair, implicating this factor as playing a role in tissue injury (Ratziu et al. 1998). In addition, number of genes directly involve in the liver injury are transactivated by KLF6 in hepatic cells, including TGFβ1, type I and II TGFβ receptors, and collagen αI (Botella et al. 2002; Mgbemena et al. 2011). Induction of KLF6 occurs in distinct models of liver injury *in vivo* and *in vitro*, suggesting that the upregulation of KLF6 is important feature of liver cell development. The expression of KLF6 target genes play critical role in the process of hepatic fibrogenesis.

Despite significant progress in the understanding of KLF6 as a tumor suppressor factor and in cancer biology over the last two decades, the expression and function of KLF6 in the brain is only beginning to be elucidated. To date, there are only few reports published describing the expression and localization of KLF6 in the brain, however, it remains unclear how the expression of KLF6 changes after brain insults, such as epilepsy and ischemia. KLF7, which has 84% homology to KLF6 according to the phylogenetic classification, is found in neuronal precursors in the brain (Laub et al. 2001b). KLF7 knockout mice have deficits in neurite growth and axonal projections in the cerebral cortex and the hippocampus, suggesting the possible role of KLF6 and KLF7 in the differentiation or maturation of neurons and necessary for CNS development (Veldman et al. 2010; Caiazzo et al. 2011; Blackmore et al. 2012). In addition, KLF6 and KLF7 have been identified for their effects on axon growth in zebrafish retinal explants, where KLF7 was detected

together with KLF6, to be necessary for axonal outgrowth in retina (Veldman et al. 2007; Moore et al. 2011). However, ectopic expression of KLF6 and KLF7 in cortical neurons was not synergistic, suggested redundant role in their function (Moore et al. 2009). KLF6 and KLF7 that act as positive and negative regulators of axon outgrowth are coordinated to control the regenerative capacity of CNS neurons. Expression of KLF6 is detected in neuronal progenitor cells by positive PSA-NCAM signal in the specialized areas such as the rostro-migratory stream (RMS) and the subventricular zone (SVZ), where neuronal progenitors move toward the olfactory bulb by chain migration (Jeong et al. 2009). KLF6 has been reported to be localized in endothelial cells and neurons where KLF6 transactivates TGFβ1 and TGFβ receptors, as a result KLF6 may increases transcriptional activity of TGFβ1 in endothelial cells and TGFβ receptors in neurons. TGFβ signaling is known to associate with proinflammatory responses and neuronal hyperexcitability in the brain (Sanyal et al. 2004; Ivens et al. 2007; Levin & Godukhin, 2011). Various brain insults, including cerebral ischemia, subarachnoid hemorrhage and excitotoxicity, have been shown to markedly enhance the expression of HSP47 (a collagen-specific molecular chaperone) (Bui et al. 2009; Turturici et al. 2011; Bornstein & Poon, 2012). It has been known that HSP47 whose expression is activated by KLF6 in brain tissue, suggested that up-regulation of KLF6 after seizure could be associated with tissue remodeling following brain insult (Yasuda et al. 2002; Ho & Piquette-Miller, 2007; Dityatev, 2010; Jeong et al. 2011). Furthermore in astrocytes, HSP47 has been reported to be induced by TGFβ, which is also regulated by KLF6 and known KLF6 target gene (Yu et al. 2009). These

observations indicated that KLF6 may play critical role in brain inflammation and epileptogenesis through activation of HSP47 and TGFβ signaling. Taken together, it is suggested that KLF6 may involve in regulation of neuronal homeostasis such as regulation of neurotransmitter release or synaptic plasticity, by regulating type I and type II TGFβ receptors in the adult brain (Vivien et al. 1998; Sanyal et al. 2004; Levin & Godukhin, 2011). Furthermore, KLF6 may acts as a "molecular switch" regulating different function in health and disease (Kimmelman et al. 2004; McConnell & Yang, 2010; Moore et al. 2011).

The MEK5/ERK5/MEF2 pathway has been implicated as playing roles in the muscle cell fusion mediated by induction of KLF2 and KLF4. Studies indicate that KLF4, KLF2 and KLF6 may have overlapping functions in different tissues (Ruiz-Gomez et al. 1997; Kumar et al. 2005; Sako et al. 2009; Sunadome et al. 2011). In the future, it will be of critical importance to further develop and study animal models, such as transgenic and conditional knockout mice, to confirm the *in vitro* results in an *in vivo* system. The KLFs are emerging as important regulators of muscle biology with great interest to cardiovascular biology. The role of KLFs in skeletal muscle is significantly less developed than in cardiac or smooth muscle (Shindo et al. 2002; Cullingford et al. 2008; Himeda et al. 2010; Li et al. 2010; Sun et al. 2011). The initial observation was made in *Drosophila* kruppel protein as a critical determinant of myogenic fate (Ruiz-Gomez et al. 1997). KLF family members have been identified in developing or mature skeletal muscle cells including KLF6 but their regulation and exact role has not been identified yet (Sako et al. 2009; Himeda et al. 2010; Sunadome et al. 2011). Recently our group has

demonstrated that KLF6 is expressed in skeletal muscle where it involves in myoblast proliferation and survival in response to TGFβ signaling (Dionyssiou et al. 2013). Indeed, future studies are required to better define the spatiotemporal expression of the entire family of KLFs across skeletal and cardiac muscle both early and postnatal muscle development for our understanding the expression, regulation and functional significance of KLFs in muscle tissues. Therefore, a deeper understanding of the precise molecular mechanisms by which these factors are involved in muscle development will be of fundamental importance for a global understanding of gene expression and a variety of muscle pathologies.

Chapter II: Statement of Purpose

Chapter II: Statement of purpose

Myocyte Enhancer Factor (MEF2) is a key regulator of muscle differentiation. In recent years apart from acting as key regulator of muscle differentiation, the MEF2 proteins have also emerged as key molecules in the development of the CNS and in a variety of neuronal functions. For example MEF2s have been implicated in neuronal survival and differentiation. The regulation of MEF2 function is quite complex and coordinated at multiple levels including phosphorylation and protein-protein interaction. However, very little is known regarding regulatory mechanisms of MEF2, and functional role of MEF2 mediated target genes in the CNS. In fact, until recently there were no MEF2 target genes identified in neurons. ***Therefore, the main purpose of this study was to characterize signaling pathways and novel interacting partners that are involved in the regulation of MEF2 transcriptional activity and target-gene expression both in myogenic and neurogenic cells.*** As such the present studies have characterized a MEF2 target gene (KLF6) and a novel interacting partner (Strawberry notch1). Overall these studies will contribute to our understanding of functional role of MEF2 in muscle development and neuronal survival.

The following objectives will be examined to address the overall goal of this study.

Objective 1: To distinguish the possible role of MEF2 in neuronal survival and characterization of PKA signalling in hippocampal neurons (chapter III).

Objective 2: To identify novel MEF2 interacting partners (chapter IV).

Objective 3: To investigate the possible role of KLF6 in myogenic cells (chapter V).

These objectives are the main focus of these studies and will be discussed in detail in upcoming chapters.

Chapter III

Suppression of a MEF2-KLF6 survival pathway by PKA signaling promotes apoptosis in embryonic hippocampal neurons

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Jahan Salma and Dr. John C McDermott.
Experiments conducted by Jahan Salma

Rationale:

Previous work from our group documented that PKA represses skeletal muscle differentiation by directly phosphorylating MEF2D both *in vitro* and *in vivo* conditions. These observations characterize a potent inhibitory effect of PKA on the transactivation properties of MEF2D during myogenic differentiation program. MEF2D is abundantly expressed in hippocampal neurons and acts as a pro-survival factor but it is unclear how MEF2D is regulated by the PKA signaling in these neurons and how exactly the MEF2s promote neuronal survival. Since most studies have documented MEF2 survival role activity dependent manner in cortical and cerebellar granule neurons. But no evidence were found regarding MEF2 mediated target gene (KLF6) in neurons, involved in neuronal survival, regulated by PKA signaling. Therefore, we focused two major aspects in present studies: first, examining the molecular mechanisms of MEF2D regulation and second, characterization of MEF2 mediated target gene, KLF6, in hippocampal neurons involved in neuronal survival.

Suppression of a MEF2-KLF6 survival pathway by PKA signaling promotes apoptosis in embryonic hippocampal neurons

Jahan Salma[1,2,4] and John C. McDermott[*1,2,3,4]

Author affiliation:

[1]Department of Biology, York University, 4700 Keele Street,
Toronto,Ontario Canada M3J 1P3
[2]Centre for Research in Mass Spectrometry (CRMS), York University,
4700 Keele Street, Toronto, Ontario Canada M3J 1P3
[3]Muscle Health Research Centre (MHRC), York University,
4700 Keele Street, Toronto, Ontario Canada M3J 1P3
[4]Centre for Research in Biomolecular Interactions (CRBI),
York University, 4700 Keele Street, Toronto, Ontario Canada M3J 1P3

*Corresponding author:
J.C. McDermott, Department of Biology, 327 Farquharson,
York University, 4700 Keele Street, Toronto, Ontario, Canada. M3J 1P3
E. mail: jmcderm@yorku.ca
Phone: (416)-736-2100 ext.30389, Fax: (416)-736-5698,

Running title: PKA represses MEF2-KLF6 in Hippocampal Neurons

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Abstract

In the mammalian nervous system, regulation of transcription factor activity is a crucial determinant of neuronal cell survival, differentiation and death. The myocyte enhancer factor 2 (MEF2) transcription factors have been implicated in cellular processes underlying neuronal survival and differentiation. A core component of the MEF2 complex is the MEF2D subunit. Recently, we reported that cAMP-dependent protein kinase (cAMP/PKA) signaling negatively regulates MEF2D function in myogenic cells. Here, we assessed whether cAMP signaling converges on the pro-survival role of MEF2D in Sprague-Dawley rat embryonic (E18) hippocampal neurons. Initially, we observed that experimental induction of cAMP/PKA signaling promotes apoptosis in primary hippocampal neurons as indicated by TUNEL and FACS analysis. Luciferase reporter gene assays revealed that PKA potently represses MEF2D *trans*-activation properties in neurons. This effect was largely reversed by engineered neutralizing mutations of PKA phospho-acceptor sites on MEF2D (S121/190A). Krüppel-like factor 6 (KLF6) was

identified as a key transcriptional target of MEF2 in hippocampal neurons and siRNA mediated knockdown of KLF6 expression promotes neuronal cell death and also antagonizes the pro-survival role of MEF2D. These observations have important implications for understanding the pathways controlling cell survival and death in the mammalian nervous system.

Introduction

During development of the central nervous system (CNS), a critical balance exists between the molecular pathways that control neuronal cell death and survival (Contestabile, 2002). Neuronal apoptosis fulfills a crucial role for normal organization of the brain through the elimination of excess neurons and synaptic connections (Contestabile, 2002; Kano & Hashimoto, 2009; Eroglu & Barres, 2010). Conversely, abnormal apoptosis contributes to progression of neurodegenerative diseases such as Alzheimer's, and Parkinson's (Pfeiffer et al. 2010; Legradi et al. 2011; Chu et al. 2011). Thus, there is considerable interest in the functional characterization of pro-survival molecules in the CNS.

The transcription factor Myocyte Enhancer Factor 2 (MEF2) has been implicated as playing a pivotal role in neuronal survival as well as in the development, differentiation, and plasticity of the mammalian CNS (Leifer et al. 1994; Schulz et al. 1996; Heidenreich & Linseman, 2004; Pulipparacharuvil et al. 2008). The MEF2 transcription factor activity was originally identified in myogenic cells but has since been shown to function in multiple cell types including cardiac, skeletal and smooth muscle, T-cells and neurons (Ornatsky & McDermott, 1996;

Black & Olson, 1998; Zhu et al. 2005; Potthoff & Olson, 2007). MEF2 functions as a transcriptional regulatory complex that integrates inputs from various signaling pathways (Yang et al. 1998; Naya & Olson, 1999; Cox et al. 2000; Mckinsey et al. 2002). Expression of MEF2 in the brain is consistent with a role in neuronal differentiation and survival (Leifer et al. 1993; McDermott et al. 1993; Lyons et al. 1995; Lin et al. 1996). Disruption of molecular processes that control MEF2 transcriptional activity can indeed promote neuronal apoptosis (Li et al. 2001; Linseman et al. 2003; Butts et al. 2003; Bolger et al. 2007). The MEF2 family members (MEF2A, C and D) are expressed throughout the developing and adult cerebellum and hippocampus (Ikeshima et al. 1995; Lin et al. 1996). Thus, signaling pathways that control MEF2 activity may exert control over neuronal cell survival pathways in the CNS. Recently, the MEF2 complex has also emerged as a key regulator of synapse development in the CNS (Shalizi et al. 2006; Flavell et al. 2006 & 2008). RNA interference (RNAi) of MEF2A markedly decreases formation of synaptic structures in cerebellar granular neurons (Gaudilliere et al. 2002; Flavell et al. 2006).

The cAMP-dependent protein kinase A (PKA), an anciently conserved signaling molecule, regulates multiple biological processes (Belfield et al. 2006; Sands & Palmer, 2008). We reported an inhibitory effect of PKA on the *trans*-activation properties of MEF2D during myogenesis (Du et al. 2008). A role for PKA as a pro-survival kinase has been demonstrated in some cell types, although it has also been implicated in hippocampal neuronal apoptosis (Zhao et al. 2008). Thus, we explored a connection between MEF2 and PKA in neuronal cells. Here,

we report that repression of MEF2D by PKA signaling promotes neuronal apoptosis in hippocampal neurons. Also, we identify Krüppel-like factor 6 (KLF6) as a key downstream effector of the hippocampal MEF2 survival pathway.

Materials and Methods

Reagents and antibodies

Primary polyclonal antibodies were purchased from Santa Cruz Biotechnology; α-Actin (I-19) and α-KLF6 (R-173) sc7158 and α-β-tubulin III (Tuj 1) Sigma T3952, α-HDAC4 (ML-19), H9411 Sigma. Primary monoclonal antibodies α-β-tubulin III (T8660, Sigma) and α-MEF2D (610775, BD Biosciences). GFP(B-2) (Sc-9996 Santa Cruz Biotechnology). Rabbit α- β-tubulin III (Tuj 1) T3952 from Sigma. Normal mouse (sc-2025), rabbit (sc-2027), and goat (sc-2028) IgGs were purchased from Santa Cruz Biotechnology. FITC and TRITC-conjugated α-rabbit and α-mouse secondary antibodies were obtained from Sigma. Forskolin (F-3917), dbcAMP (D0260), H89-dihydrochloride hydrate (B1427), Trichostatin A (T1952), DAPI (D9542) and H_2O_2 (H0904) were purchased from Sigma for use in cell culture. All other reagents were obtained as indicated herein.

Plasmids

Expression plasmids for full-length pcDNA3-MEF2D, pMT2 MEF2A, pMT2 MEF2C pCMV β-galactosidase, and MEF2 reporter gene constructs have been described in previous publications (Du et al. 2008; Perry et al. 2009). The firefly luciferase reporter gene plasmid pGL3-4xMEF2-Luc was made with 4 copies of the MEF2 sites inserted. The expression vector of the catalytic subunit of PKA (pFC-

PKA) and expression vector for FLAG-tagged HDAC4 were previously described (Du et al. 2008; Gordon et al. 2009; Perry et al. 2009). The HDAC4-eGFP and HDAC4 L175A plasmids were a kind gift from Dr. X.-J Yang (McGill University). Expression plasmids for pcDNA3-Flag-Zf9 and pCIneo-KLF6 were a kind gift from Prof. Scott Friedman (Mount Sinai School of Medicine, New York, USA). The Krüppel-like factor 6 (KLF6) reporter constructs pROM6, pROM5, pROM4 and pROM3-Luc were generously provided by Dr. Nicolas. P. Koritschoner, Facultad de Bioquimica y Ciencias Biologicas, Universidad Nacional del Litoral, Santa Fe-Argentina.

Embryonic hippocampal neuronal cell culture and transfection

Primary hippocampal neurons were isolated from timed-pregnant (E18) Sprague-Dawley rat embryos (either sex) as described previously (Perry et al. 2009) in accordance with the Institutional Animal Care and Use Committee of York University, Toronto, Canada. The hippocampi were collected in dissociation medium and digested with trypsin, followed by trituration. Neurons were plated on pre-coated poly-D-lysine (Sigma), six-well tissue culture plates. Neurons were maintained in Neurobasal Medium (Invitrogen) supplemented with B27 (2ml/100 ml, Invitrogen) and L-glutamine (1ml/100 ml; Invitrogen), and penicillin (50 U/mL)/streptomycin (50 μg/ml) in a 37°C humidified incubator with a 5% CO_2 in air. After 24hours one-third of the culture medium was replaced with fresh medium and maintained for 7 days *in vitro* (7DIV) prior to transient transfection. In general primary hippocampal neurons were transfected by using calcium phosphate-

mediated transfection with indicated plasmids on day 6 or 7 in culture and assays were conducted 36 h post-transfection. Cells were replenished with fresh growth medium 24 h prior to transfection. The transfection efficiency was monitored by addition of a constitutively expressed GFP expression vector, typically yielding at least 50-60% transfected neuronal cells in each culture.

Cos7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, HyClone), 1% penicillin-streptomycin, sodium pyruvate, and L-glutamine in a humidified incubator at 37 °C and 5% CO_2 in air. Cells were seeded 1 day prior to transfection and transient transfections were performed using the standard calcium phosphate precipitation method. Cells were washed 16 hours post-transfection with phosphate-buffered saline (PBS) and harvested 48 hours after transfection followed by preparation of lysate for reporter gene assays (see below).

TUNEL assay

TUNEL (terminal deoxynucleotidyl transferase UTP-biotin nick end labelling) staining was performed using *In Situ* Cell Death Detection Kit (Roche) according to the manufacturer's instructions. Primary hippocampal neurons were harvested and washed twice with PBS. Cells were trypsinized (0.2%) and then fixed with 4% PFA in PBS for 1 h at RT. Cells were washed twice and permeabilized with 0.1% Triton X-100 for 4 min on ice followed by incubation with TUNEL assay reagent and PI for 1 h at 37°C. Cells were washed with PBS twice and TUNEL-positive cells were quantified by fluorescence microscopy.

Reporter gene assays

Primary hippocampal neurons (7DIV) and COS7 cells were transiently transfected using the calcium phosphate precipitation method. Reporter gene plasmids (1μg) and expression plasmids (1.5 μg) were transfected as indicated in figures. pCMV-β-galactosidase (1μg) was transfected as an internal control for monitoring transfection efficiency. The total amount of DNA for each experiment was kept constant by using empty vectors. Primary neuronal cells were seeded in 6-well plates for reporter gene assays. Neuronal cells were incubated with transfection reagent for 8 h. Cells were harvested after 36 hrs post-transfection.

Cos7 cells were plated in 6-well plates 1 day before transfection. Transient transfection was performed using standard methods and cells were washed twice with PBS after 16 h transfection and then harvested at 48 h post-transfection. Both β-galactosidase and luciferase activities were measured. Luciferase activity was assayed according to manufacturer's instructions (Promega), using a Berthold 9501 luminometer and Luciferase values were normalized to β-galactosidase values. All measurements were made in triplicate for at least three independent experiments with data presented as means ± standard error of the mean.

Western blot analysis

Protein extracts were prepared in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA [pH 8.0], 100 mM sodium fluoride, 10 mM sodium pyrophosphate) containing 0.2 mM PMSF and 0.5 mM sodium orthovanadate and protease inhibitor cocktail. Protein concentrations were

determined by Bradford assay (BioRad) with bovine serum albumin (BSA) as a standard. Equivalent amounts of total protein (15–20 μg) were diluted in sample buffer (sodium dodecyle sulphate-polyacrylamide)containing β-mercaptoethanol, boiled for 4-5min, and electrophoretically resolved by 10% SDS-PAGE gels, then electrophoretic transfer to an Immobilon-P membrane (Millipore Inc). Non specific binding sites were blocked with 5% milk in PBS for one hour at RT. Immunoblotting was carried out using appropriate primary antibodies in 5% milk (PBS), α-MEF2D (1:1000), α-KLF6 (1:1000), α-HDAC4 (1:1000) and α-Actin (1:1000). The blots were then incubated with the appropriate secondary horseradish peroxidase (HRP) antibody (BioRad) at 1:2000 with 5% milk in PBS for 1 hour at RT followed by Chemiluminescence detection of immunoreactive proteins as per the manufacturer's instructions (Amersham Biosciences).

Co-immunoprecipitation assays

Protein extracts were prepared from primary hippocampal neurons as described above. Immunoprecipitation was performed using the ExactaCruz kit (Santa Cruz), as per manufacturer's instructions. Precipitated proteins were separated by SDS page and immunoblotting of precipitated proteins was carried out as described above.

Flow Cytometry Analysis

Flow cytometry analyses were performed as previously described (Perry et al. 2009) using the Annexin V-FITC apoptosis detection kit (Sigma) following the manufacturer's instructions. Cell viability and apoptosis were measured by a

combination of Annexin V-FITC and propidium iodide (PI) staining. Primary hippocampal neurons were washed and briefly trypsinized, and then washed twice with cold 1xPBS. Cells were pelleted by centrifugation and re-suspended in 1x binding buffer followed by incubation with staining solution (Annexin V-FITC and PI) for 15 min in the dark at 4°C. The cells were re-suspended in 1x binding buffer. Samples were kept on ice during the entire procedure and analyzed immediately by flow cytometry. Ten thousand cells from each sample were scanned and analyzed by FACS Calibur flowcytometry (Becton Dickinson) using the standard configuration and parameters. Data acquisition and analysis was performed using the CellQuest software (BD). Necrosis and apoptosis were determined by PI (FL2) and Annexin V-FITC (FL1) fluorescence respectively.

Site-directed mutagenesis

PCR-based mutagenesis of the MEF2 *cis*-element contained within the KLF6 promoter (pROM6) was performed by insertion of double-stranded oligonucleotides containing the mutated sequences using the QuikChange site-Directed Mutagenesis kit (Stratagene, cat # 200518) according to the manufacturer's instructions. Mutated constructs were verified by DNA sequencing (York University Core Facility).

Immunocytochemistry

Hippocampal neuronal cells were seeded on pre-coated poly-D-lysine glass cover slips at a density of 0.5×10^5 cells/coverslip. After 7-9 days *in vitro,* cells were fixed in 4% paraformaldehyde in PBS for 10 min at RT and then permeabilized

with 0.3% Triton X-100 in PBS for 5 min. Cells were blocked with 10% goat serum in PBS for 30 min at 37°C and incubated overnight at 4°C with primary antibodies α-MEF2D, α-KLF6, α-HDAC4, and α-β-tubulin III (1:100) diluted in 1.5% goat serum (PBS). Cells were washed 3x with PBS for 10 min and then incubated with the appropriate TRITC/FITC-conjugated secondary antibodies (1:500) in 1.5% goat serum (PBS) for 2 hours at RT following DAPI (4',6-diamidino-2-phenylindole) staining for 15 min at RT. Cells were washed 3x with PBS and cover slips were mounted with DAKO mounting media (Dako) on glass slides. The fluorescence images were captured using a Fluoview 300 (Olympus).

siRNA gene silencing

Small interfering RNAs (siRNA's) targeting KLF6, HDAC4 and a nonspecific scrambled RNA were purchased from (Sigma). Primary hippocampal neurons were seeded for FACS analysis and Immunoblotting analysis. Cells were replenished with antibiotic-free Opti-MEM I (cat# 31985, Invitrogen) media 2-3 h prior to transfection. Cells were transfected with siRNA and scrambled RNA using Lipofectamine RNAiMAX reagent (cat# 13778, Invitrogen) according to the manufacturer's instructions. Cells were harvested after 48hrs post-transfection for western immunoblotting analysis to determine the efficacy of protein knock down or FACS analysis.

RT-PCR and Real-time quantitative PCR

Total RNA was isolated from primary hippocampal neurons (7/9DIV) using the RNeasy™ kit (Qiagen) followed by DNAse treatment (Qiagen) according to the

manufacturer's protocol. All RNA samples were assessed for quality by agarose gel electrophoresis gels. Reverse transcription (RT) was performed from equal amounts of total RNA using Superscript III reverse transcriptase (Invitrogen) and used for semiquantitative RT-PCR analysis for primer specificity. All Quantitative RT-PCR reactions were performed on cDNA using Power SYBR Green Mastermix (Applied Biosystem) and detected using a 7500 Fast real time PCR system, Applied Biosytems according to the manufacturer's protocol. All values were normalized to GAPDH (internal control) mRNA levels. Each experiment was done in triplicate and independently validated 3 times.

Results

cAMP signaling increase neuronal apoptosis

Previous studies have shown that activation of cAMP/PKA signaling in neuronal and non-neuronal cells can provoke apoptosis (Lomo et al. 1995; Myklebust et al. 1999; Zhao et al. 2008; Zhang et al. 2008). MEF2D acts as pro-survival factor and we showed previously that it is expressed in the hippocampal neurons (Perry et al. 2009). In skeletal muscle, cAMP/PKA signaling has been shown to inhibit MEF2D function and myogenic differentiation (Du et al. 2008). We therefore undertook the current studies to determine if the PKA-MEF2 pathway impinges on neuronal survival. Initially, we performed a survival assay to examine the effect of cAMP-PKA signaling in primary hippocampal neurons (7DIV) using the TUNEL method (Terminal deoxynucleotidyl transferase dUTP nick end labelling). A cell permeable cAMP analog was used to manipulate PKA activation

(dbcAMP). Treatment of primary hippocampal neurons (9DIV) with dbcAMP indicated a prominent increase of TUNEL positive cells when compared to untreated controls (Fig. 23A bottom panels). Total cell numbers for each treatment are indicated by DAPI staining, a nuclear marker (Fig 23A top panels). Quantitative analysis shows that cAMP induced an approximately five to six fold increase in neuronal apoptosis compared to the control condition (Fig. 23B). Hydrogen peroxide (H_2O_2) treatment was used in these assays as a positive control. Next, we assessed dbcAMP mediated apoptosis in hippocampal neurons by FACS analysis. Estimation of necrosis and apoptosis were determined by a combination of propidium iodide (PI) and annexin V-FITC fluorescence, respectively (Fig. 23C). Cells appearing in the lower right quadrant of the density plots (high level of annexin V-FITC and low level of PI), indicate an increase in the percentage of apoptotic cells in the cAMP treated cells (25.7%) compared to controls (11.4%) (Fig. 23C). Forskolin treatment, which enhances cAMP levels, also promotes apoptosis and a PKA inhibitor (H89) blocks this effect (Fig. 23D).

PKA signaling represses MEF2 transcriptional activity in hippocampal neurons

To obtain insight into whether the mechanism leading to apoptosis was through PKA inhibition of the pro-survival of MEF2D, we utilized a well known pharmacological inhibitor of PKA, H89, for one hour before treatment with FSK. We found that H89 blocked FSK effects and to some extent rescued neurons from apoptosis (Fig. 23D) and parallel reporter gene analysis demonstrated a reduction of

MEF2 activity in FSK and dbcAMP treated cells which was reversed by H89 treatment (Fig. 23E, F and G). As an indicator of promoter specificity we monitored the activity of a generic CMV enhancer/promoter reporter gene under the same experimental conditions (Fig. 23 H, I and J).

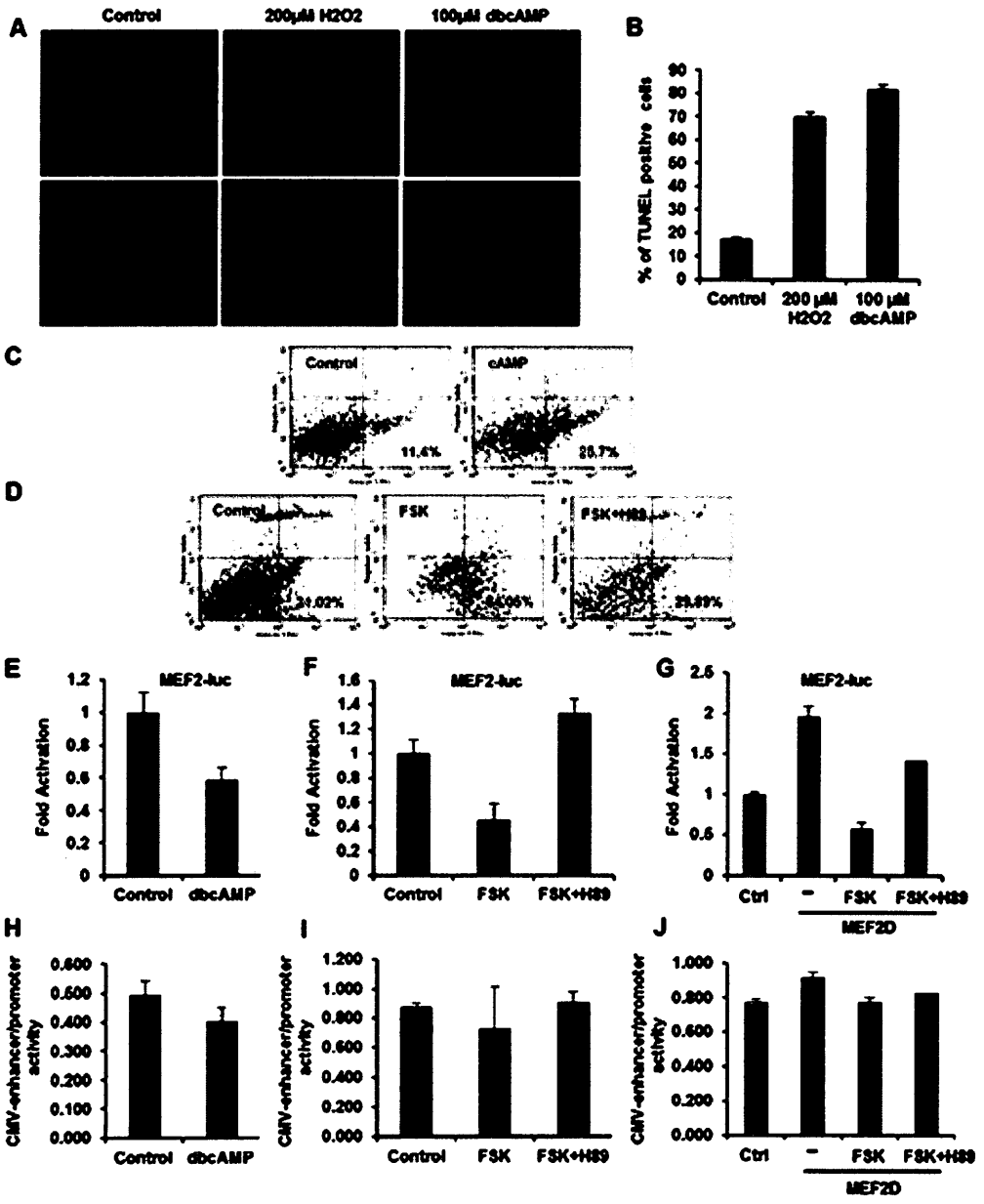


Figure 23. Activation of cAMP signaling induces apoptosis in hippocampal neurons. (A) Primary hippocampal neurons (9DIV) were treated with a cAMP analog (100 μM db-cAMPS, Sigma) for 6 hrs or 200 μM H_2O_2 (hydrogen peroxide) for 2 hrs as a positive control. Neuronal apoptosis was observed by using TUNEL assay. DNA fragmentation in apoptotic cells was visualized by terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labelling. Representative images were taken from ten randomly selected fields using fluorescence microscopy, both Total (DAPI positive, a nuclear marker) and TUNEL positive cells were counted. (B) Quantitation of these data represents the percentage of apoptotic cells in each condition. Data are expressed as mean +/- SEM from three separate treatments. (C) Primary hippocampal neurons were stimulated with 100 μM db-cAMP for 6 hrs. Percentage of apoptotic cells was determined by propidium iodide (PI) and annexin V-FITC fluorescence staining using flow cytometry analysis (FACS analyzer). (D) Primary hippocampal neurons were treated with 10 μM FSK alone and in combination with 10μM H89 for 6 hrs. Percentage of apoptotic cells were determined by propodium iodide (PI) and annexin V-FITC fluorescence staining using FACS analyzer. (E) Primary E18 hippocampal neuronal cells were transiently transfected with pGL3-4XMEF2-Luc reporter gene and pCMV-β-Galactosidase after 7 days in culture using the calcium phosphate precipitation method. 36 hrs after transfection, cells were treated with 100 μM db-cAMP or solvent (Control) for 6 hrs. MEF2 mediated transcriptional activity was determined by Luciferase and β-galactosidase assay as described in Materials and Methods. Luciferase values were normalized by β-galactosidase activity. Experiments were conducted at least three times, yielding comparable results. (F) Primary hippocampal neuronal cells were transfected with pGL3-4XMEF2-Luc reporter gene and pCMV-β-Galactosidase. Cells were treated with 10 μM FSK alone and in combination with 10μM H89 for 6 hrs (as indicated) (F and G). Data indicating the activity of a generic enhancer/promoter are indicated in H, I and J). Primary hippocampal neuronal cells were co-transfected with pGL3-4XMEF2-Luc reporter gene and wtMEF2D expression vector. MEF2 mediated transcriptional activition was determined by luciferase and β-gal assays.

These data illustrate no demonstrable effect of PKA on this promoter construct indicating that there is some specificity to the effects seen on the MEF2 dependent reporter gene. This specificity is also evident in the data shown later in Fig. 24A in which the message levels for a number of MEF2 target genes are unaffected by activation of PKA signaling whereas some, such as KLF6, clearly are affected. Together, these data indicate that the effect of PKA activation is not a generalized

down regulation of all transcribed genes. To further investigate the link between MEF2 transcriptional activity and PKA activation, primary hippocampal neurons (7DIV) were transiently transfected with a MEF2 reporter gene (MEF2-Luc) and increasing amounts of an expression plasmid encoding the catalytic subunit of PKA which results in constitutive cellular PKA activity (pFC-PKA). It was observed that PKA potently suppressed MEF2 dependent reporter gene activation in a dose dependent manner (Fig. 24A). Also, exogenous expression of wild type (wt) MEF2D increased MEF2 reporter gene activation and PKA repressed this activation (Fig. 24B). To further investigate whether PKA plays a direct role in neuronal survival/apoptosis, neurons were transiently transfected with empty vector or pFC-PKA followed by FACS analysis. Substantial increases in apoptotic cells were observed with PKA (15.42%) (Fig. 24C, right panel) when compared to control (0.83%) (Fig. 24C). In congruence with the results seen with dbcAMP and activated PKA, similar results were also seen with forskolin (FSK) treatment, which activates adenylate cyclase (data not shown). Taken together, these results demonstrate that cAMP/PKA signaling regulates MEF2 activity and also potentiates apoptosis in hippocampal neurons.

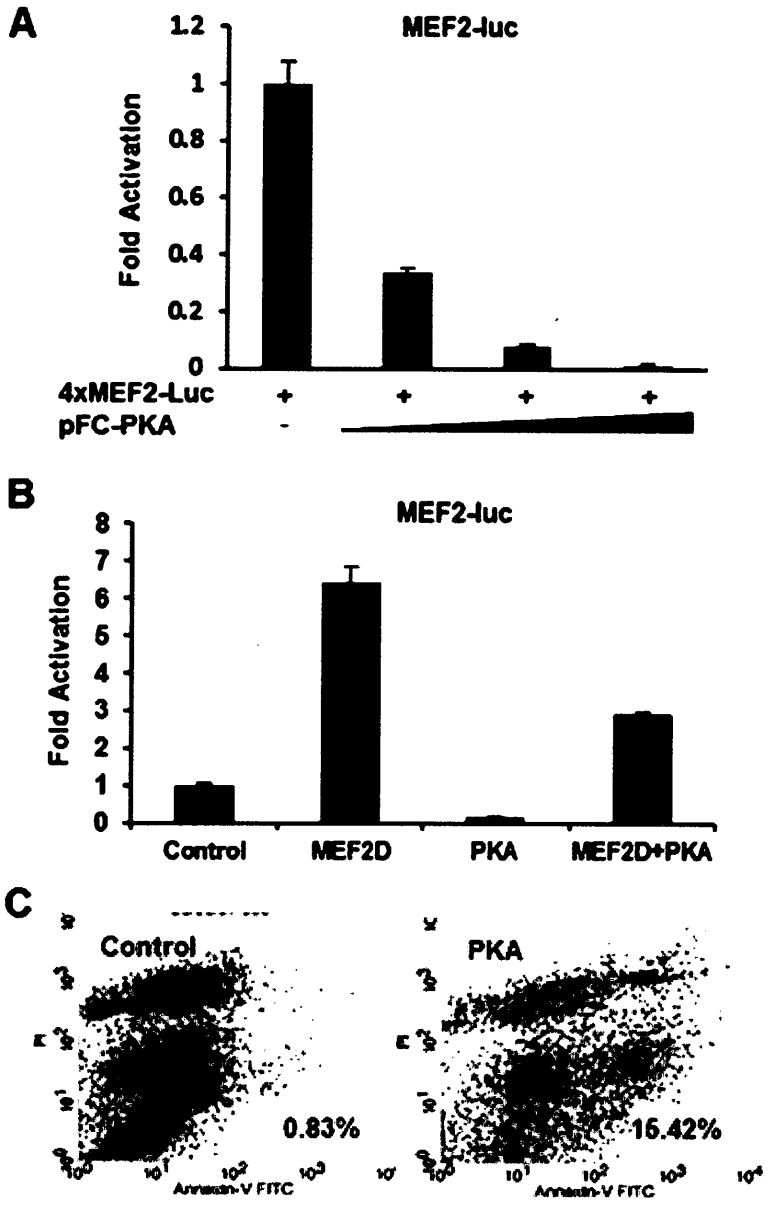


Figure 24. Exogenous expression of activated PKA suppresses MEF2's pro-survival role in hippocampal neurons. (A) Primary E18 hippocampal neuronal cells (7DIV) were transiently transfected with pGL3-4XMEF2-Luc reporter gene, pCMV-β-Galactosidase to normalize transfection efficiencies and increasing amounts of the catalytic subunit of PKA (pFC-PKA 250 ng-1μg). (B) Primary hippocampal neuronal cells were co-transfected either with empty vector or pcDNA3-MEF2D, in combination with or without pFC-PKA (as indicated). MEF2

mediated transcriptional activity was determined by pGL3-4XMEF2-Luc reporter gene and pCMV-β-Galactosidase assays. (C) Primary hippocampal neuronal cells were co-transfected either with empty vector or pFC-PKA (as indicated). 36 hours after transfection, primary hippocampal neuronal cells were stained with annexin V-FITC and propidium iodide (PI) (Annexin V-FITC apoptosis detection kit, Sigma). Necrosis and apoptosis were determined by flow cytometry analysis. (The percentage of apoptotic cells labelled with annexin V-FITC appeared in the lower right quadrant of the density plot, are shown in the bottom right corner of each panel).

MEF2's role in hippocampal neuronal survival

Our observations at this point suggested that PKA signaling might target MEF2's pro-survival role. Previously, we have documented that PKA directly phosphorylates S121 and S190 on MEF2D using mass spectrometry (Fig. 25A) and these sites were sufficient to confer repressive effects on the skeletal muscle differentiation program in response to cAMP signaling (Du et al. 2008). Moreover, neutralization of these phospho-acceptor sites by mutation to Alanine (A) rendered MEF2D resistant to PKA signaling and thus allowed rescue of differentiation when the PKA resistant MEF2D was transfected into cells even in the presence of cAMP signaling (Du et al. 2008). We therefore reasoned that this PKA resistant form of MEF2D might be capable of rescuing hippocampal neuronal cells from cAMP mediated apoptosis if the primary target of cAMP–PKA signaling is indeed MEF2D. Primary hippocampal neurons were transfected with empty vector or mutated forms of MEF2D S121/190A (neutralizing) and S121/190D (phospho-mimetic) with or without PKA at 7DIV (see Fig. 25A for schematic of where these phospho-acceptor sites are in relation to other domains).

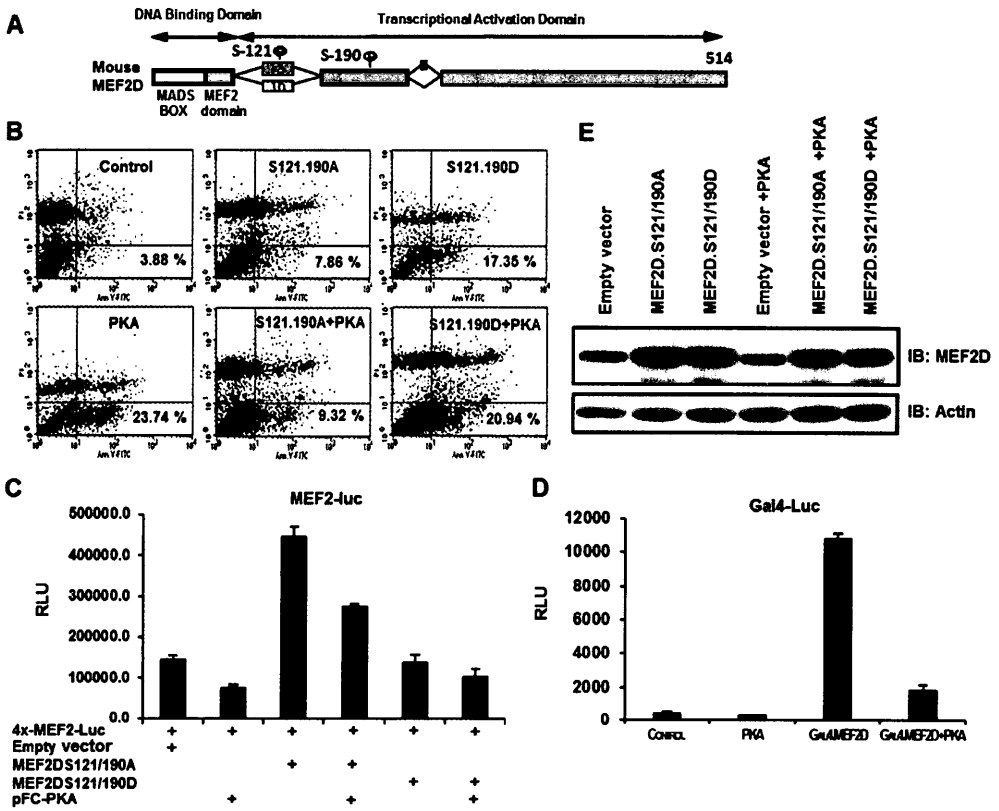


Figure 25. MEF2D-S121/190A confers resistance to PKA in hippocampal neurons. (A) Schematic of mouse MEF2D indicating PKA phospho-acceptor sites (B) Primary hippocampal neuronal cells (7DIV) were transiently transfected with empty vector or mutated forms of MEF2D S121/190A (neutralizing) and S121/190D (phospho-mimetic) with or without PKA. 36 hrs after transfection, cells were stained with annexin V-FITC and propiduim iodide (PI) (Annexin V-FITC apoptosis detection kit, Sigma). Neuronal apoptosis was determined by flowcytometry analysis (FACS analyzer). (C) Primary hippocampal neurons were transiently co-transfected at 7DIV with pGL3-4XMEF2-Luc reporter gene and pCMV-β-Galactosidase to normalize transfection efficiencies and with empty vector or double mutated forms of MEF2D S121/190A and S121/190D, in combination with or without pFC-PKA (as indicated). MEF2 mediated transcriptional activity was determined by Luciferase and pCMV-β-Gal assays. (D) Primary hippocampal neuronal cells were transiently co-transfected with 5XGAL4-Luciferase reporter vector, GAL-DBD or GAL4-MEF2D (87-507), with and without pFC-PKA and β-Galactosidase to normalize transfection efficiencies. (Data are the mean +/- S.E.M.; n=3). (E) Primary hippocampal neuronal cells (7DIV)

were transiently transfected with empty vector or mutated forms of MEF2D S121/190A (neutralizing) and S121/190D with or without PKA. Cells were harvested and lysates were prepared 36hrs later. Equal amounts of total protein were separated by 10%SDS-PAGE followed by immunoblot analysis using MEF2D monoclonal antibody (1:1000) to detect the expression levels of transfected constructs. Acitn (polyclonal, 1:2000) was used as a loading control.

Subsequently, FACS analysis was carried out using Annexin-V/PI to assess cell death. Interestingly, a decrease in apoptotic cells was observed when S121/190A was co-expressed with PKA compared to PKA alone in hippocampal neurons (Fig. 25B). Conversely, phospho-mimetic forms of MEF2D, S121/190D failed to alter PKA mediated cell death and, if anything, showed an increase in the percentage of apoptotic cells even in the absence of PKA when compared with the control condition (Fig. 25B). These data support the idea that a PKA resistant MEF2D (S121/190A) protects neurons from PKA mediated cell death. This effect is corroborated by reporter gene analysis in which a Gal4-MEF2D fusion protein is repressed by PKA and also that MEF2D S121/190A neutralizing mutation is much more resistant to the effects of PKA than wild type MEF2 (Fig. 25C and D). Expression levels of the various mutated and wild type MEF2D protein are shown in Fig. 25E.

Characterization of MEF2 target genes in Hippocampal neuronal cells

We next assessed the pro-survival effect of MEF2D and its inhibition by cAMP/PKA signaling by analyzing downstream MEF2 target genes identified in a

study in which a high throughput chromatin immunoprecipitation method coupled to massively parallel sequencing (ChIP-seq) was employed (Flavell et al. 2008). To directly examine whether the expression of KLF6 is targeted under conditions when MEF2 activity is repressed by PKA signaling, primary hippocampal neurons (7DIV) were treated with FSK or solvent for 6 hours to induce PKA signaling. After stimulation, total RNA was isolated using RNeasy™ kit (Qiagen) followed by quantitative real-time reverse transcription PCR (qRT-PCR) using Power SYBR Green Master mix. We carried out qPCR analysis of a number of these identified MEF2 target genes to identify which ones might be affected by PKA activation (Fig. 26A and B) and, from this analysis, a prominent responder was Krüppel-like factor 6 (KLF6). Other reported MEF2 target genes showed variable responses to FSK treatment suggesting that MEF2 might play a different role at these genes (Fig. 26A and B). Primary hippocampal neurons (7DIV) were also transfected with empty vector or the catalytic subunit of PKA (pFC-PKA) followed by qRT-PCR analysis. Again this showed that KLF6 mRNA level was suppressed by activated PKA (Fig. 26C). These results suggested that the decrease in KLF6 gene expression by PKA signaling might be through MEF2 inhibition.

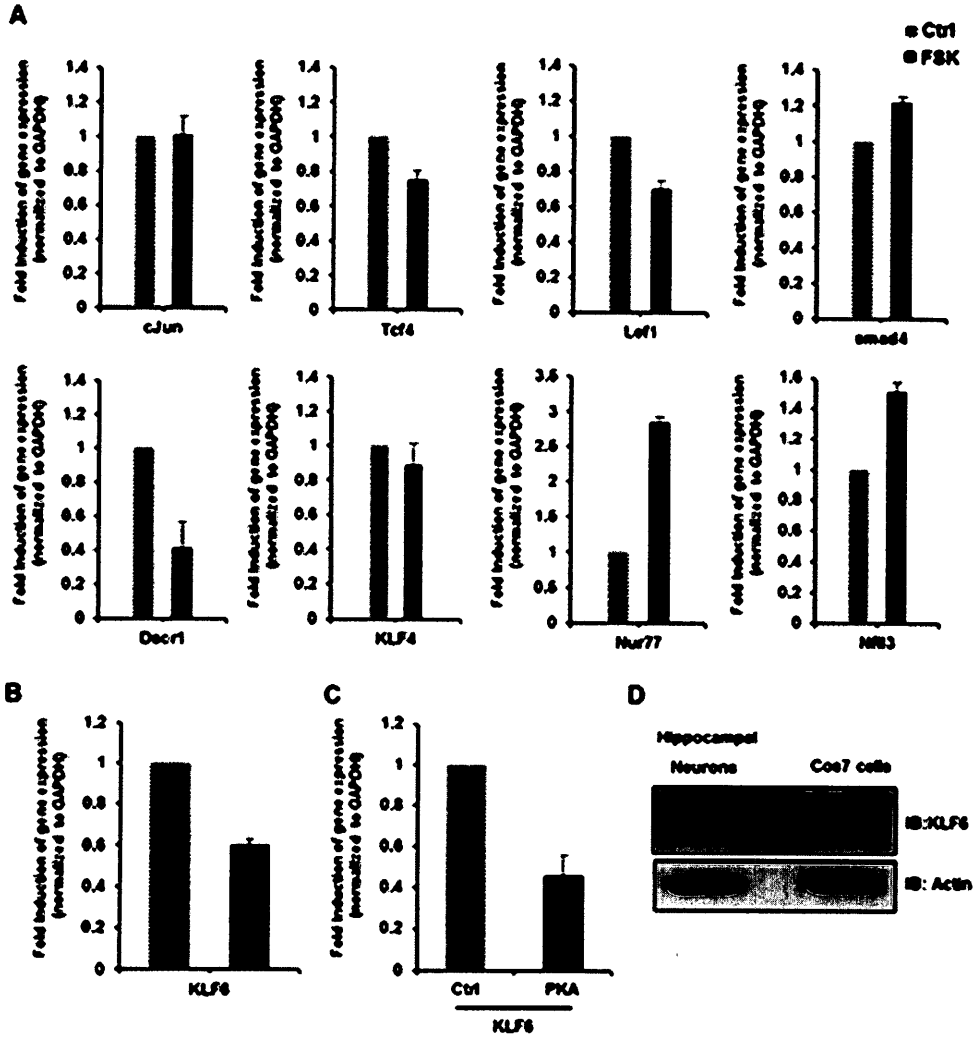


Figure 26. Krüppel-like factor 6 (KLF6) gene expression in hippocampal neurons. (A and B) Nine previously reported hippocampal MEF2 target genes were selected for qRT-PCR analysis. Primary hippocampal neurons (7DIV) were stimulated with 10μM FSK or solvent for 6 hours to induce PKA signaling. After stimulation, total RNA was isolated using RNeasy™ kit (Qiagen) followed by quantitative real-time reverse transcription PCR (qRT-PCR) using Power SYBR Green Master mix. Gene expression was analyzed using 7500 Fast real time PCR detection system as described in Material and methods. GAPDH housekeeping gene primers were used to normalize expression of target genes. (C) Primary hippocampal neurons (7DIV) were transiently transfected with empty vector or the catalytic subunit of PKA (pFC-PKA) followed by qRT-PCR analysis of KLF6 expression. (D) KLF6 protein is expressed in hippocampal neuronal cells. Cell lysates of primary hippocampal neurons and COS7 were prepared for

immunobloting analysis as indicated. Equal amounts of total protein were separated by 10% SDS-PAGE followed by immunoblot. KLF6 polyclonal antibody (1:1000) was used to detect the endogenous KLF6 protein. Actin (polyclonal, 1:2000) was used as a loading control.

KLF6 is highly expressed in various regions of the brain including the hippocampus (Fischer et al. 2001; Jeong et al. 2009). We therefore sought to define whether KLF6 might constitute an important downstream effector of the MEF2 pro-survival role in hippocampal neurons

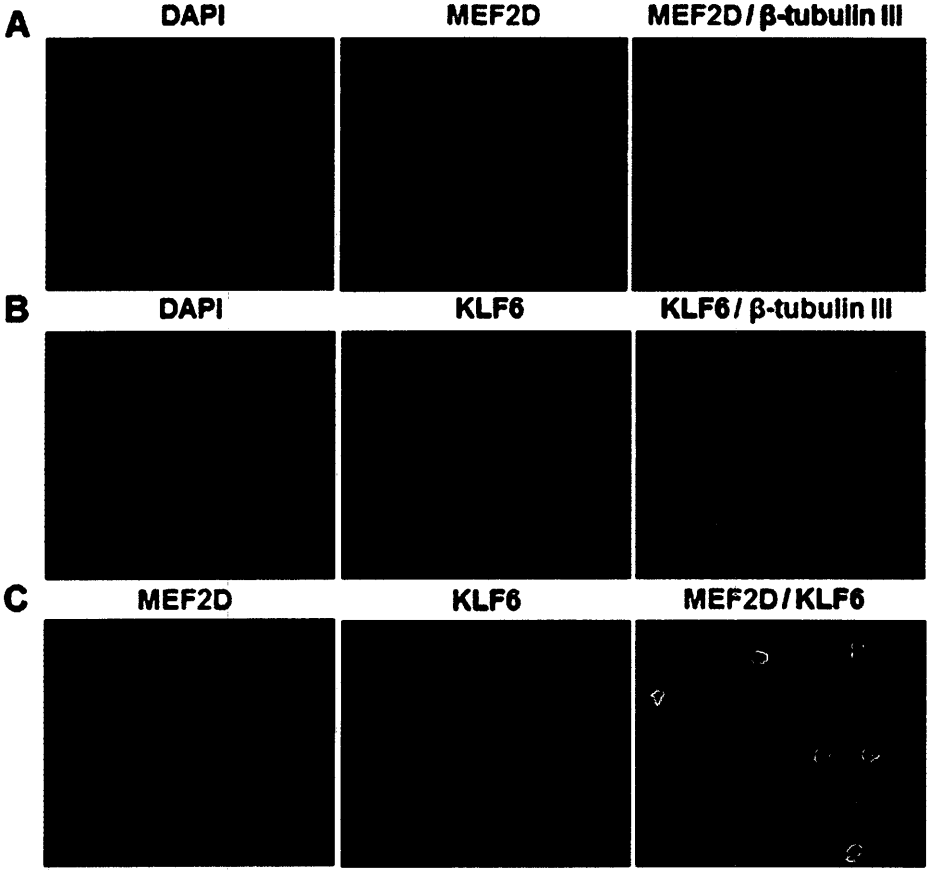


Figure 27. Cellular localization of MEF2D and Krüppel-like factor 6 (KLF6) in hippocampal neurons. (A) Primary hippocampal neuronal cells were fixed with

4% paraformaldehyde. Double immunofluorescence analysis was performed using primary antibodies to MEF2D (Green) and a neuronal marker beta tubulin III (Red). DAPI (4',6-diamidino-2-phenylindole) was used to identify nuclei (blue). The merged picture demonstrates localization of MEF2D (nuclear) and β-tubulin III positive cells (Red). (B) Primary hippocampal neurons express KLF6. Hippocampal neuronal cells were fixed and double immunofluorescence analysis was performed with primary antibodies to KLF6 shown in red and β-tubulin III shown in green. The merged picture demonstrates localization of KLF6 (nuclear) and β-tubulin III positive cells (green), counterstained with DAPI (Blue). (C) Double immunofluorescence labelling demonstrating KLF6 (red) and MEF2D (green) in primary hippocampal neurons. The merged picture indicates MEF2D positive cells are mostly positive for KLF6 and both proteins are predominantly nuclear.

Endogenous expression of KLF6 is detected in E18 hippocampal neuronal cells (Fig. 26D) and immunofluorescence labelling to observe the cellular localization of KLF6 and MEF2D in primary hippocampal neurons was also carried out. Primary hippocampal neurons, confirmed by β-tubulin III expression (red), were positive for nuclear MEF2D (green) (Fig. 27A). Neuronal cells also show strong nuclear localization of KLF6 (red) in β-tubulin III positive cells (green) (Fig. 27B). Nuclear co-localization of MEF2D and KLF6 in primary hippocampal neurons is evident (Fig. 27C). Interestingly, the co-localization of MEF2D and KLF6 that we have observed in primary cultured neurons is also reflected in data from the Allen Brain Atlas Resources in which in situ hybridization of KLF6 and MEF2D show strikingly similar patterns of expression in the hippocampal region (http://www.brain-map.org).

MEF2D regulates KLF6 promoter activity in Hippocampal Neurons

To further define the potential role of MEF2D in KLF6 gene regulation, we examined the previously characterized upstream KLF6 promoter (Gehrau et al. 2005) which contains a MEF2 *cis*-element, between -320 and -310 bp (Fig. 28A). This site is highly conserved in different species as depicted in Fig. 6B. The conservation of this *cis*-element suggests that MEF2 may play an evolutionary conserved role in KLF6 gene expression. To investigate MEF2D dependent regulation of the KLF6 promoter in hippocampal neurons, we utilized a number of KLF6 reporter gene constructs containing different fragments of the KLF6 promoter, pROM6 (-507 bp to +1 bp), pROM5 (-407 bp to +1 bp), and pROM4 (-344 bp to +1 bp), which contain the MEF2 *cis*-element, and pROM3 (-307 bp to +1 bp) which does not (schematic illustrations of KLF6 reporter deletion constructs are shown in Fig. 28C). All KLF6 fragments were cloned into the pGL3-basic reporter vector (pGL3-KLF6-Luc). Reporter gene assays were undertaken to assess MEF2D *trans*activation of the KLF6 promoter. Primary hippocampal neurons were transiently transfected with the KLF6 promoter constructs (pROM6 to pROM3) with and without wtMEF2D and pGL3-basic empty vector was used as a control. As shown in Fig. 28D, ectopically expressed MEF2D strongly induced pROM6 and pROM5 reporter transcriptional activity. MEF2D did not potentiate KLF6-pROM3 reporter activity which lacks the MEF2 binding site (Fig. 28 & E).

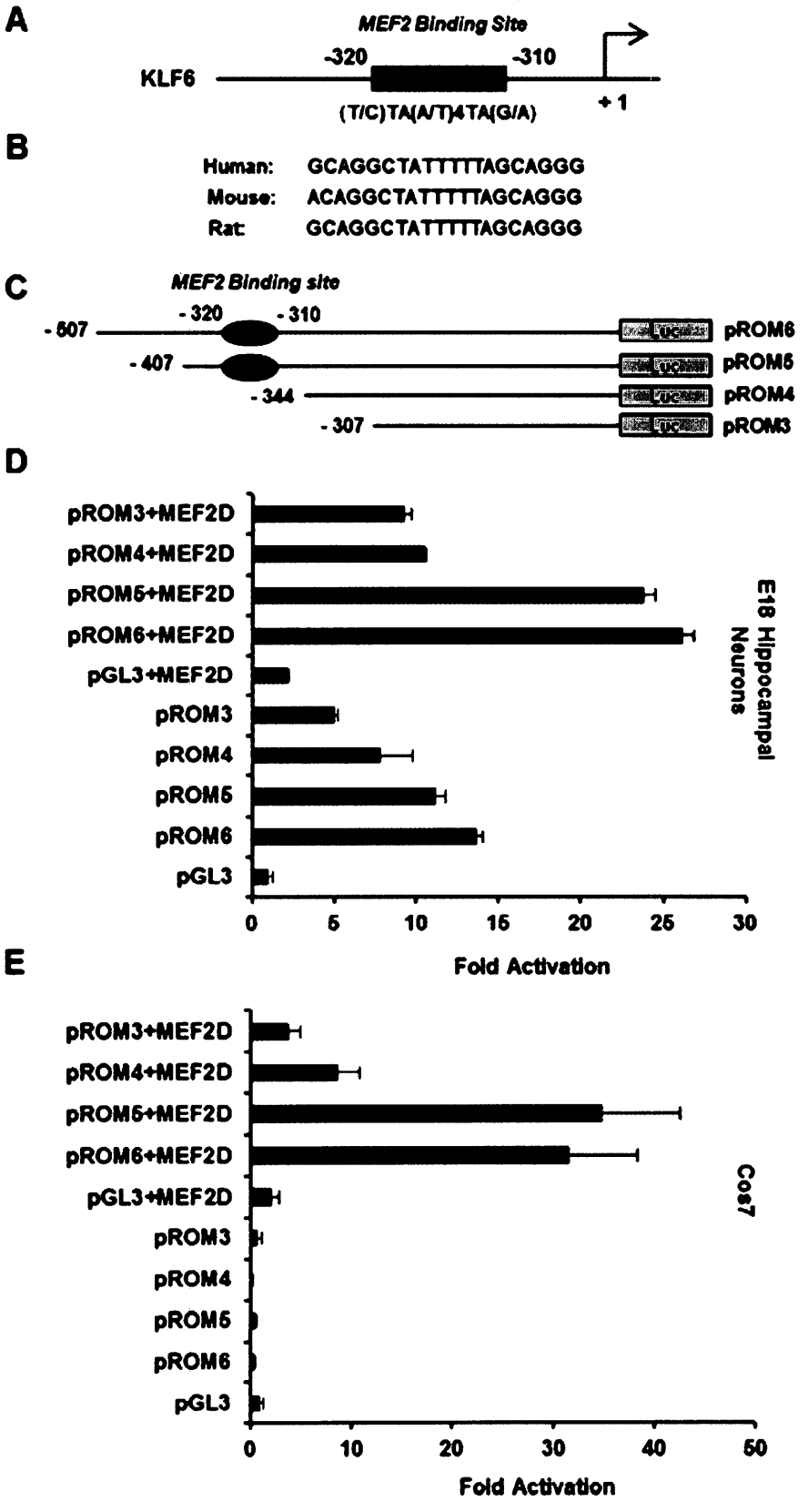

A
MEF2 Binding Site
-320
-310
KLF6
+1
(T/C)TA(A/T)4TA(G/A)
B
Human: GCAGGCTATTTTTAGCAGGG
Mouse: ACAGGCTATTTTTAGCAGGG
Rat: GCAGGCTATTTTTAGCAGGG
C
MEF2 Binding site
-320
-310
-507
-407
-344
-307
pROM6
pROM5
pROM4
pROM3
D
pROM3+MEF2D
pROM4+MEF2D
pROM5+MEF2D
pROM6+MEF2D
pGL3+MEF2D
pROM3
pROM4
pROM5
pROM6
pGL3
0
5
10
15
20
25
30
Fold Activation
E18 Hippocampal Neurons
E
pROM3+MEF2D
pROM4+MEF2D
pROM5+MEF2D
pROM6+MEF2D
pGL3+MEF2D
pROM3
pROM4
pROM5
pROM6
pGL3
0
10
20
30
40
50
Fold Activation
Cos7

Figure 28. Functional analysis of KLF6 promoter in hippocampal neurons. (A) Schematic illustration of upstream KLF6 promoter construct depicts location of MEF2 *cis*-element between -320 and -310 bp. (B) Sequence of MEF2 *cis*-element is highly conserved in the KLF6 promoter from human to mouse. (C) Schematic illustrations of KLF6 reporter deletion constructs used in reporter assays. All KLF6 promoter constructs were cloned into the pGL3-basic reporter vector (pGL3-KLF6-Luc) and were utilized in examining KLF6 promoter activity. (D) Primary hippocampal neuronal cells were transiently co-transfected with various constructs of the KLF6 promoter (pROM6 to pROM3), pCMV-β-Galactosidase was utilized to normalize transfection efficiencies. Cell extracts were prepared and analysed for luciferase and β-gal assays. (E) Cos7 cells were transiently co-transfected with various KLF6 promoter constructs (pROM6 to pROM3), pCMV-β-Galactosidase (to normalize transfection efficiencies) with and without wtMEF2D by using calcium phosphate method. pGL3-basic empty vector was use as a control. Cell extracts were prepared for luciferase and β-gal assays as described in Material and Methods. (Data are the mean +/- S.E.M n=3).

We wanted to precisely dissect the absolute requirement for the MEF2 *cis*-element in the KLF6 promoter and we therefore carried out site-specific mutation of the pROM6 MEF2 site. Primary hippocampal neurons were co-transfected with MEF2D and the pROM6 reporter construct containing the intact or mutated MEF2 binding site as shown in Figure 29A. MEF2D did not induce KLF6 reporter activity when the MEF2 binding site was mutated to a sequence that no longer fits the consensus binding site (Fig. 29B). Furthermore, increasing amounts of MEF2D induced a dose dependent increase in pROM6 reporter activity compared to the mut.pROM6-luc which was not activated by MEF2D (Fig. 29B). To further determine if other MEF2 isoforms (MEF2A and C) can alter KLF6 promoter activity, we performed reporter gene assays as described above using pROM6 reporter gene constructs containing the intact or mutated MEF2 binding site specific mutation. Increasing amounts of MEF2A induce pROM6 reporter gene activity compared to the mut.pROM6-luc reporter in which the MEF2 site is inactivated by

mutation (Fig. 29C). Conversely, although MEF2C is a reasonably strong *trans*-activator on synthetic and other natural promoters it had essentially no effect on pROM6 (Fig. 29D).

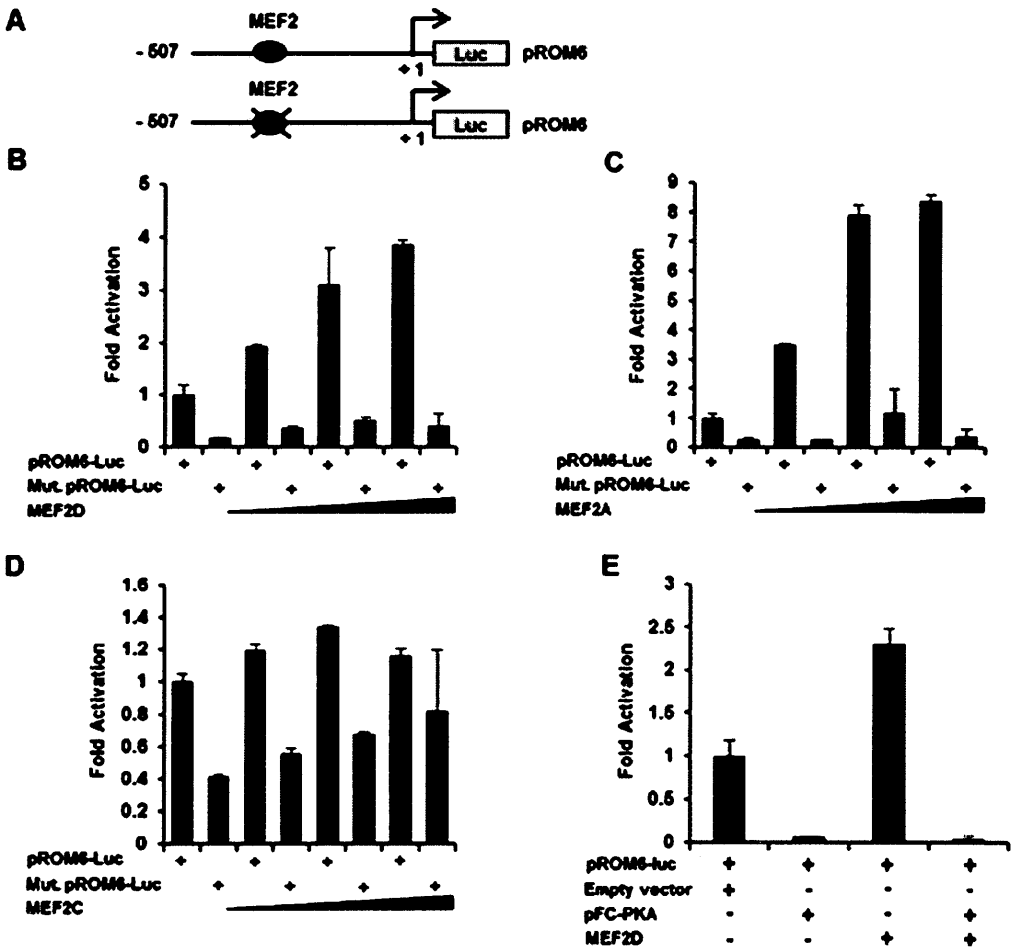


Figure 29. PKA represses KL6 promoter activity in primary hippocampal neurons. (A) Schematic illustrations of KLF6 promoter construct (pROM6) with intact MEF2 binding site or with MEF2 site mutated. (B-D) Primary hippocampal neurons were co-transfected with pROM6 reporter constructs and increasing amounts (250 ng to 1.5 μg) of wtMEF2D, MEF2A, and MEF2C plasmid

respectively. pCMV-β-Galactosidase was used to normalize transfection efficiencies. (E) Primary hippocampal neurons were transiently transfected with pGL3-KLF6-Luc reporter construct, pCMV-β-Galactosidase and wtMEF2D with or without pFC-PKA (as indicated). MEF2 mediated transcriptional activity was determined by luciferase and β-gal assays as described in Material and Methods (Data are the mean +/- S.E.M n=3).

There are differences between MEF2A, C and D in their ability to heterodimerize with each other, warranting further analysis of these observations. We next assessed whether PKA signaling targets the KLF6 promoter through the MEF2 *cis*- element. To investigate this, hippocampal neuronal cells were co-transfected with wtMEF2D and pROM6 (-507 bp to +1 bp) in the presence and absence of pFC-PKA. The activity of these reporter genes was markedly reduced by PKA. The wild type MEF2D was not able to reverse the inhibitory affect of PKA when ectopically expressed which is consistent with our previous studies showing that PKA repression trans-dominantly represses exogenously expressed MEF2D (Fig. 29E). Together, these data indicate that MEF2D, possibly in combination with MEF2A, is a potent transcriptional regulator of the KLF6 promoter and this promoter can be repressed by PKA signaling through its MEF2 *cis*-element.

Interestingly, we observed that exogenous overexpression of KLF6 can reduce the amount of cell death provoked by PKA suggesting it might play an important survival role downstream of MEF2 in hippocampal neurons (Fig. 30A and B). To further investigate MEF2 mediated regulation of KLF6 by PKA we performed reporter gene assays utilizing pGL3-KLF6-Luc (pROM6) and double mutations of MEF2D (S121.190A or S121.190D).

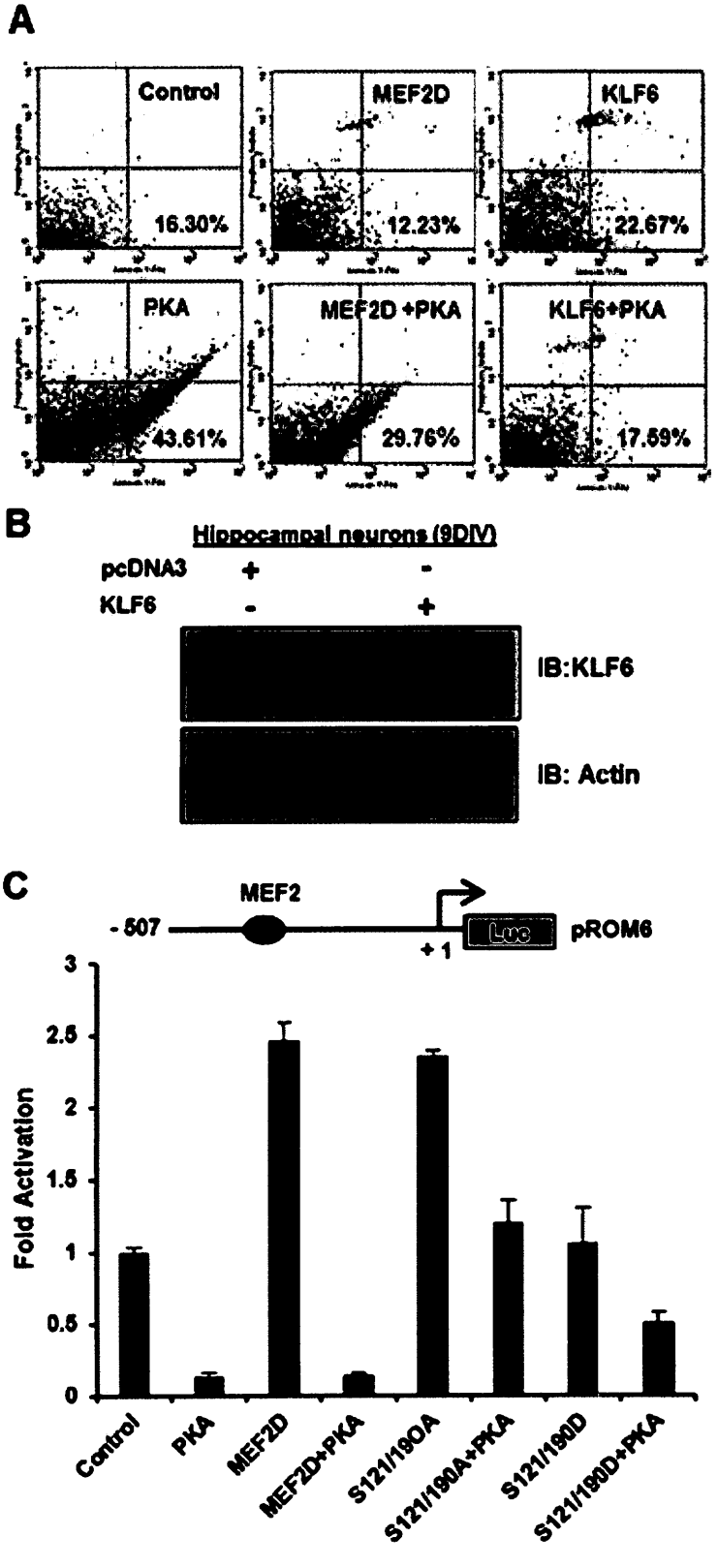

A
Control
16.30%
MEF2D
12.23%
KLF6
22.67%
PKA
43.61%
MEF2D +PKA
29.76%
KLF6+PKA
17.69%
B
Hippocampal neurons (9DIV)
pcDNA3 + -
KLF6 - +
IB:KLF6
IB: Actin
C
MEF2
- 507
+ 1
Luc
pROM6
Fold Activation
3
2.5
2
1.5
1
0.5
0
Control
PKA
MEF2D
MEF2D+PKA
S121/190A
S121/190A+PKA
S121/190D
S121/190D+PKA

Figure 30. Exogenous KLF6 expression promotes hippocampal neuronal survival. (A) Primary hippocampal neurons were transfected with expression vectors encoding wtMEF2D, wtKLF6 or empty vector with or without the catalytic subunit of PKA (pFC-PKA). 36 hrs after transfection, neurons were stained by annexin V-FITC and propidium iodide (PI) using Annexin V-FITC apoptosis detection kit, Sigma. Neuronal apoptosis was determined by flow cytometry analysis (FACS analyzer). (B) Detection of KLF6 protein expression. Primary hippocampal neurons were transiently transfected with empty vector or wtKLF6 expression vector. Cell extracts were prepared for immunoblotting analysis as indicated. KLF6 polyclonal antibody (1:1000) was used to detect the KLF6 protein level. Actin (polyclonal, 1:1000) was used as a loading control. (C) Cos7 cells were co-transfected with pGL3-KLF6-Luc reporter gene, wtMEF2D and MEF2D double mutants (S121/190A or S121/190D) with or without pFC-PKA. pCMV-β-Galactosidase was used to normalize transfection efficiencies. MEF2 mediated transcriptional activity was determined by luciferase and β-gal assays as described in Material and Methods (Data are the mean +/- S.E.M n=3).

Cos7 cells were co-transfected with wtMEF2D and MEF2D double mutants (S121.190A or S121.190D) with or without pFC-PKA. Consistent with our previous observations, the MEF2D double mutation S121.190A is partially resistant to PKA (Fig. 30C). However, as observed before (Du et al. 2008), phospho-mimetic mutations are still partially responsive to PKA suggesting an indirect mechanisms may be involved in MEF2 mediated KLF6 gene regulation.

PKA mediated MEF2 repression by HDAC recruitment

Previously, we have documented that HDACs (4 & 5) bind MEF2 with high affinity and, importantly, the mechanism by which PKA inhibits MEF2 transcriptional activity is due to two effects. One being a direct conformational change in the MEF2D protein caused by phosphorylation at Ser 121/190. The other

being an indirect mechanism by enhancing the nuclear content of HDACs which results in an increase in abundance of MEF2/HDAC repressor complexes (Du et al. 2008). The contribution of the latter mechanism varies depending on the opposing signals for nuclear HDAC content. In differentiating skeletal muscle there is a strong stimulus for HDAC nuclear extrusion which counteracts the nuclear retention effects of PKA whereas in other cell types we reasoned that PKA mediated nuclear retention of HDACs might account for more of the repressive effects on MEF2 activity (Du et al. 2008). A recent study has also documented that this mechanism occurs in cardiac myocytes (Backs et al. 2011). Interestingly, it has been documented previously that PKA signaling enhances HDAC localization to the nucleus in hippocampal neuronal cells (Belfield et al. 2006). We therefore assessed the contribution of HDAC nuclear retention to the inhibition of MEF2 activity and KLF6 transcription. To begin to elucidate the possible functional role of HDAC4, the endogenous protein level of HDAC4 was documented in primary hippocampal neurons (Fig. 31A). Next, we examined whether PKA signaling mediated any modulation of the cellular localization of endogenous HDAC4 protein. Primary hippocampal neuronal cells were treated with FSK followed by immunofluorescence labelling using polyclonal HDAC4 antibody. As shown in Fig. 31B, HDAC4 accumulated in the nucleus in response to FSK compared to that in the solvent treated cells. We further confirmed that PKA signaling promotes the nuclear localization of HDAC4 in hippocampal neurons by utilizing a GFP-HDAC4 fusion protein or GFP alone which was co-transfected with or without activated PKA (Fig. 31C). The GFP-HDAC4 localization data indicates that PKA increases

the nuclear levels of GFP-HDAC4 (Fig. 31C bottom panels) while having no effect on GFP alone (Fig. 31C top panels). These results are consistent with previous observations, suggesting that PKA signaling promotes the formation of HDAC4/MEF2 repressor complexes by increasing the levels of HDAC4 in the nuclear compartment leading to downstream repression of key MEF2 target genes involved in survival such as KLF6.

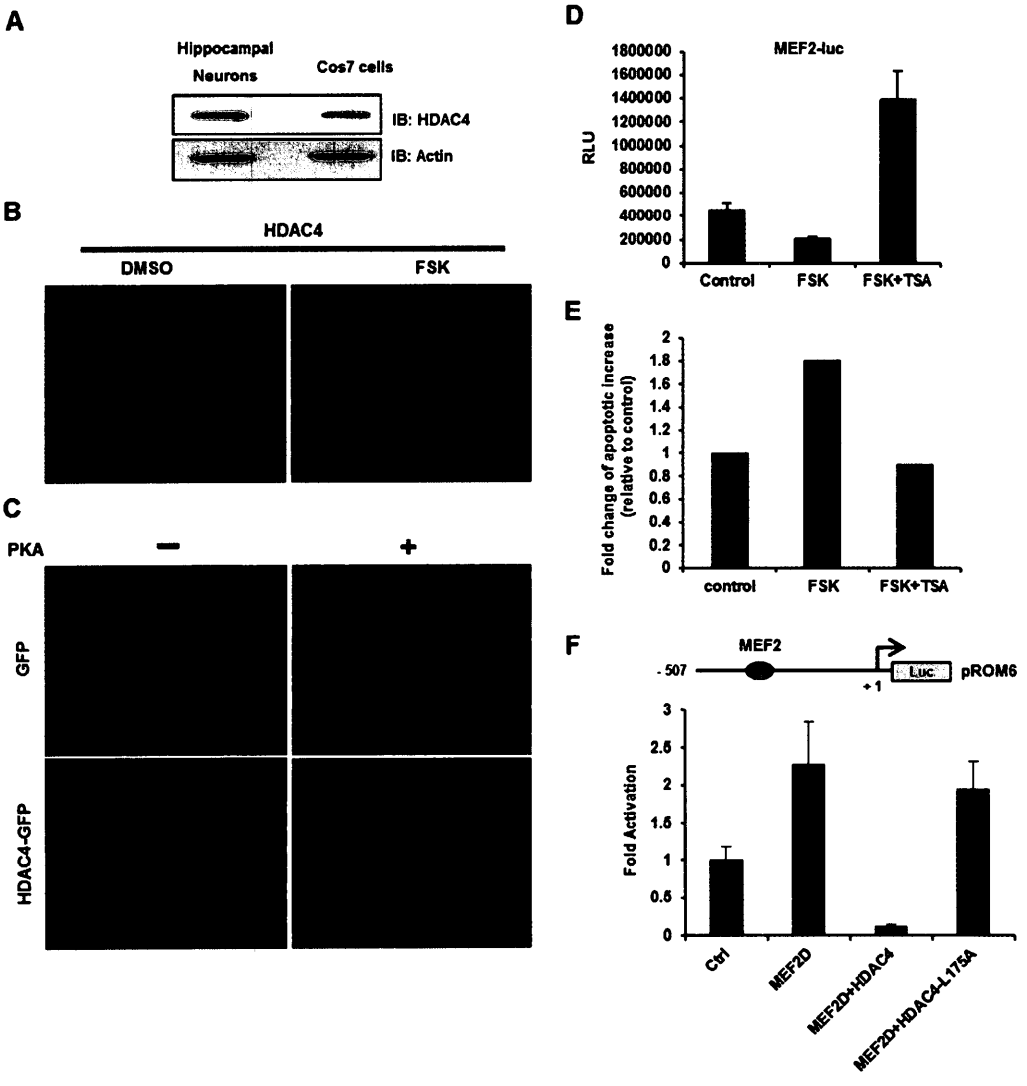
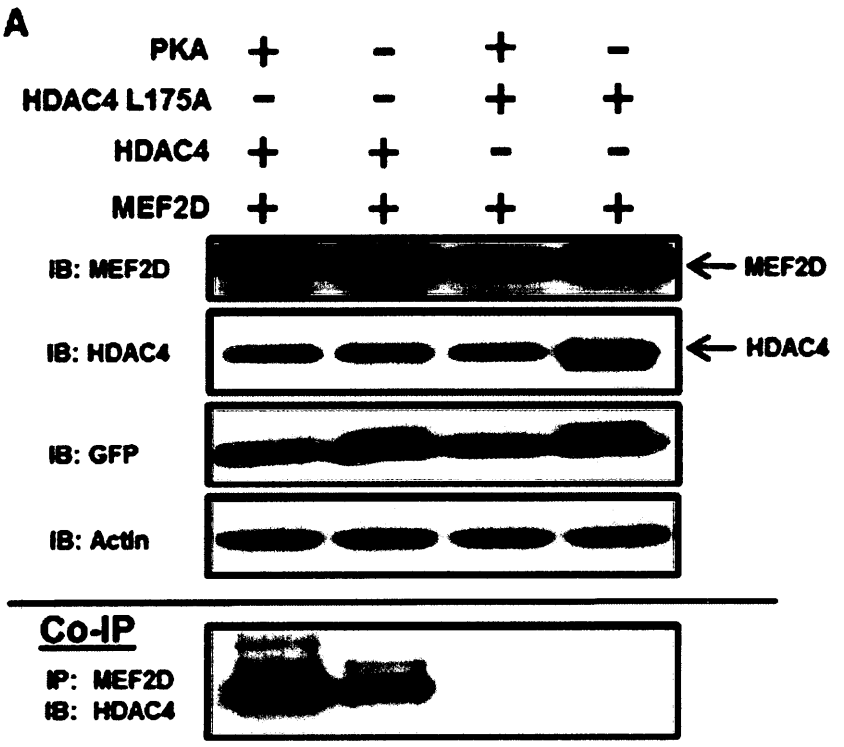


Figure 31. Increased nuclear localization of HDAC4 by PKA signaling in hippocampal neurons. (A) Cell lysates of primary hippocampal neuronal and Cos7 cells were prepared and equal amounts of total protein were separated by 10% SDS-PAGE followed by immunoblot analysis. HDAC4 polyclonal antibody (1:1000) was used to detect the endogenous HDAC4 protein. Acitn (polyclonal, 1:2000) was used as a loading control. (B) Primary hippocampal neuronal cells were treated with 10 μM FSK or solvent for 6 hrs followed by immunofluorescence labelling using polyclonal HDAC4 antibody. (C) Primary hippocampal neurons were co-transfected with GFP alone and GFP-HDAC4 fusion protein, with or without pFC-PKA. Hippocampal neurons expressing GFP alone showed a non specific distribution throughout the neuronal cell body including axon, dendrites and dendritic spines and this was unaffected by PKA. The shuttling of GFP-HDAC4 from cytoplasm to nucleus in response to PKA was visualized by fluorescence microscopy. (D) MEF2 reporter activity is repressed by PKA signaling and rescued by HDACs inhibitor trichostatin A (TSA). Primary hippocampal neurons were transiently transfected with pGL3-4XMEF2-Luc reporter gene and cells were treated with 10 μM FSK alone and in combination with 1μM TSA (as indicated). 36 hrs after transfection, cell extracts were prepared for luciferase and β-gal assays. (E) Primary hippocampal neurons were stimulated with 10μM FSK alone and in combination with 1μM TSA for 6 hrs. Percentage of apoptotic cells was determined by annexin V-FITC apoptosis detection kit, using FACS analyzer. Bar graph represents the changes in number of apoptotic cells. (F) Primary hippocampal neurons were transiently transfected with pGL3-KLF6-Luc reporter construct and wtMEF2D expression vector alone or combination with HDAC4 and HDAC4-L175A vectors (as indicated). MEF2 mediated transcriptional activity was determined by luciferase and β-gal assays as described in Material and Methods.

To further assess the extent of the HDAC4 contribution to PKA mediated MEF2 repression, we performed transcriptional reporter gene assays in conjunction with HDAC inhibition using trichostatin A (TSA). Primary hippocampal neurons were transfected with a MEF2 reporter gene and cells were treated with FSK alone and in combination with TSA. HDAC inhibition with TSA reduced the FSK effect and partially superactivates MEF2 transcriptional activity (Fig. 31D). Subsequent FACS analysis indicated that treatment with TSA also rescues neuronal cells from apoptosis (Fig. 31E). HDAC4 strongly represses MEF2 dependent transcriptional

activation of the KLF6 promoter and a mutation of HDAC4 (L175A) that has previously been shown not to interact with MEF2, has no effect (Fig. 31F). The lack of interaction between HDAC4-L175A and MEF2D was confirmed in co-precipitation assays (Fig. 32A). Also, the enhanced amount of HDAC4 co-precipitating with MEF2D when active PKA is co-transfected confirms our previous observations. To gain further insight into the role of HDAC4 in neuronal survival, we utilized siRNA to down regulate HDAC4 expression. First we tested the efficacy of HDAC4 silencing by western blot analysis. Primary hippocampal neurons (7DIV) were transfected with three independent HDAC4 siRNAs and a control scrambled siRNA (scRNA). As shown in Fig. 32B the reduction of HDAC4 protein level was observed in cells expressing siHDAC4 in contrast to cells expressing the scRNA. In order to examine whether HDAC4 silencing can induce hippocampal neuronal survival, primary hippocampal neurons were transfected with HDAC4-siRNA or scRNA. Neuronal apoptosis was quantified by flow cytometry. Depletion of HDAC4 protects neurons from apoptosis when neurons are treated with forskolin (Fig. 32C). These data indicate that the HDAC4-MEF2 interaction is an important component of PKA mediated MEF2 repression in hippocampal neurons.


A
PKA + - + -
HDAC4 L175A - - + +
HDAC4 + + - -
MEF2D + + + +
IB: MEF2D
MEF2D
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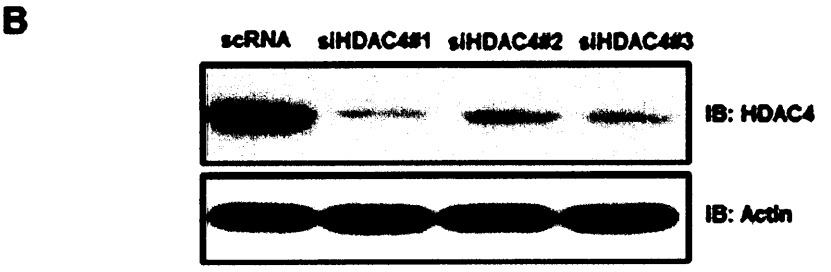
HDAC4
IB: GFP
IB: Actin
Co-IP
IP: MEF2D
IB: HDAC4


B
scRNA
siHDAC4#1
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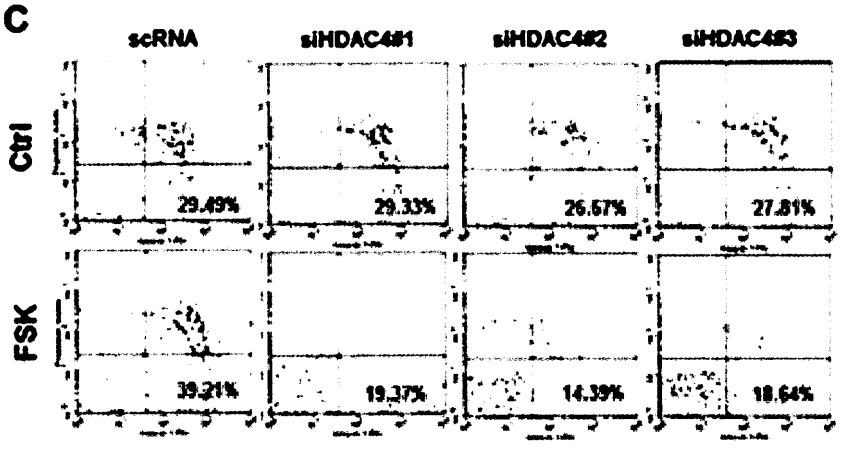
C
scRNA
siHDAC4#1
siHDAC4#2
siHDAC4#3
Ctrl
FSK
29.49%
29.33%
26.67%
27.81%
39.21%
19.37%
14.39%
18.64%

Figure 32. PKA induces physical association of HDAC4 with MEF2D in hippocampal neurons. (A) Primary hippocampal neurons were transiently transfected with indicated expression plasmids. Cell extracts were immunoprecipitated with anti-MEF2D followed by immunobloting with anti-HDAC4. Whole cell lysates were immunoblots with indicated antibodies. HDAC4 interacts with MEF2D in the presence of PKA and this association is lost with HDAC4 L175A with or without PKA. (B) For HDAC4 gene silencing in primary hippocampal neurons, three independent siRNAS were transfected along with a control scrambled RNA (scRNA). Cells were harvested and lysates were prepared 48 hrs later. Protein level of HDAC4 was analysed by immunoblot using HDAC4 polyclonal antibody (1:1000). Acitn (polyclonal, 1:2000) was used as a loading control. (C) Primary hippocampal neurons were transfected with two independent siRNAs and a control scrambled siRNA (scRNA). 48 hrs after transfection, cells were stained with annexin V-FITC and propodium iodide (PI) using Annexin V-FITC apoptosis detection kit, as described in Material and Methods. Neuronal apoptosis was measured using flow cytometry (FACS analyzer).

KLF6 protects hippocampal neurons from apoptosis

There is growing evidence that the KLF factors are involved in cell survival and we postulated that the activation of KLF6 could be an important downstream component of the MEF2D dependent pro-survival pathway. As shown earlier, KLF6 over expressing cells showed a substantially reduced percentage of apoptotic cells in the presence of PKA compared to PKA alone. To further address the role of KLF6 expression in hippocampal neuronal survival, we employed a loss of function assay using siRNA to down regulate KLF6 expression. First we assessed the efficacy of KLF6 silencing by western blot analysis. Primary hippocampal neurons (7DIV) were transfected with three independent KLF6 siRNAs and a control scrambled siRNA (scRNA). As shown in Fig. 33A. The reduction of KLF6 protein level was observed in cells expressing siKLF6 in contrast to cells expressing the scrambled siRNA. In order to further examine whether KLF6 silencing can induce

hippocampal neuronal apoptosis, primary hippocampal neurons were transfected with KLF6-siRNA or scrambled siRNA. Neuronal apoptosis was quantified by flow cytometry as described previously. Depletion of KLF6 enhanced neuronal apoptosis relative to the scRNA (Fig. 33B), suggesting that KLF6 functions as a pro-survival molecule in neuronal cells although this effect was not as penetrant as MEF2 inhibition. Taken together, the pro survival effect of exogenous overexpression KLF6 shown earlier and the modest but evident effects of KLF6 knock down on survival reveal that KLF6 may be an intrinsic component of the neuronal survival pathway mediated by MEF2.

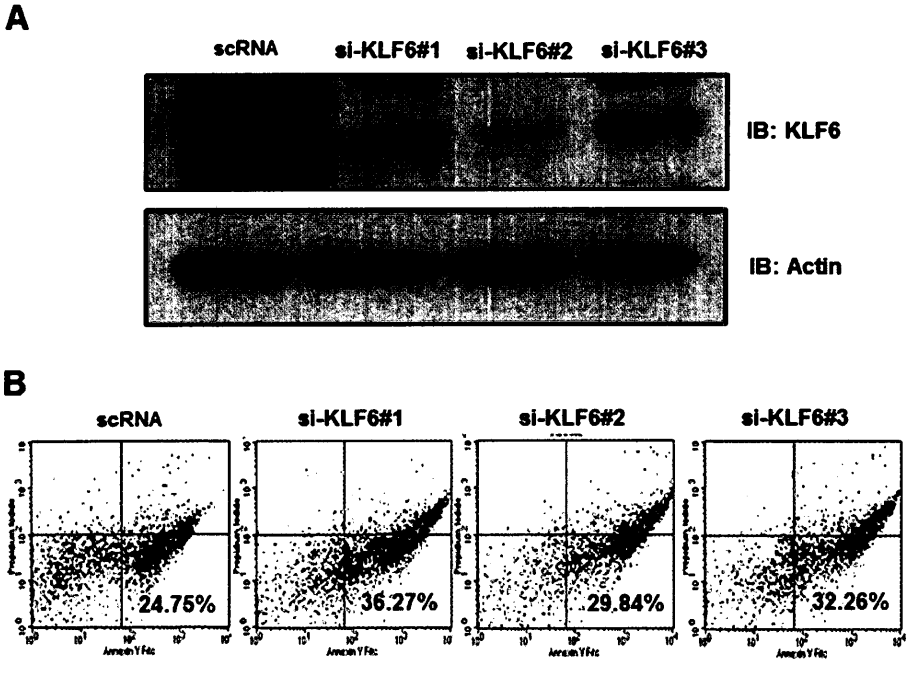

Figure 33. Silencing of KLF6 expression enhances apoptosis in primary hippocampal neurons. (A) For KLF6 gene silencing in primary hippocampal neurons, 3 independent siRNAS were transfected along with a control scrambled RNA (scRNA). Cells were harvested and lysates were prepared 48 hrs later. Equal

amounts of total protein were separated by 10%SDS-PAGE followed by immunoblot analysis using KLF6 polyclonal antibody (1:1000) to detect the KLF6 protein level. Acitn (polyclonal, 1:2000) was used as a loading control. (B) Primary hippocampal neurons were transfected with three independent KLF6 siRNAs and a control scrambled siRNA (scRNA). 48 hrs after transfection, cells were stained with annexin V-FITC and propodium iodide (PI) using Annexin V-FITC apoptosis detection kit, as described in Material and Methods. Neuronal apoptosis was measured using flow cytometry.

Discussion

Apoptosis has been implicated in the pathogenesis of various neurodegenerative diseases including Alzheimer's and Parkinson's disease (Bredesen et al. 1995; Gupta et al. 2006; Zhao et al. 2008). The cAMP-protein kinase A (PKA) signaling pathway regulates a variety of cellular functions and numerous important biological processes. More specifically, PKA signaling has been reported to be associated with the progression of Alzheimer's disease in the hippocampus (Martinez et al. 1999; Lim et al. 2005; Zhao et al. 2008). Modulation of gene expression networks underlying these pivotal cellular events ultimately leading to neurodegeneration is still in its infancy (Crews & Masliah, 2010). Furthermore, neurodegenerative diseases often exhibit alterations in many cellular processes such as increased oxidative and toxic stress, mitochondrial dysfunction, failure of synaptic activities and aberrant phosphorylation (Zhang et al. 2008). In this report, we demonstrate that a variety of perturbations of protein kinase A signaling induce apoptosis of primary hippocampal neurons through inactivation of the pro-survival role of the MEF2D transcription factor. Also, identification of

KLF6 as a key downstream component of the MEF2D survival pathway that is down-regulated by PKA signaling provides further insight into the physiology and pathophysiology of the nervous system.

Interestingly, a recent report has implicated KLF6 induction in the response to pilocarpine-induced seizures in the hippocampus (Jeong et al. 2011). In view of our data implicating MEF2D as a critical regulator of KLF6 expression, it will be of considerable interest to determine whether MEF2 activity is indeed induced in this pilocarpine-induced seizure model which mimics features of temporal lobe epilepsy (TLE) in humans. Since MEF2 had been implicated as a pro-survival molecule in the CNS, it is tempting to speculate that its induction may well occur in the physiologic response to the pronounced neuronal damage that results from excitotoxic stress. Also, in a model of zebra fish neural regeneration after optic nerve injury, KLF6 knockdown ablates regeneration due to the attenuated expression of KLF6 target gene, Tuba 1a (Veldman et al. 2010). KLF6 expression has been documented in multiple other adult brain regions apart from the hippocampus including the olfactory bulb, cerebral cortex, amygdale, thalamus, and hypothalamus (Jeong et al. 2009). Moreover, loss of heterozygosity on chromosome 10p in glioblastoma (Camacho-Vanegas et al. 2007) has implicated KLF6 as a potential tumor suppressor in this region and KLF6 expression has also been reported to be suppressed by transcriptional silencing in esophageal squamous cell carcinoma (Yamashita et al. 2002). The requirement for MEF2 for appropriate expression of KLF6 in all of these brain regions and potentially also in cancer models remains to be fully elucidated. In conjunction with the studies reported here,

a variety of experimental systems suggest a potential role of the MEF2-KLF6 pathway in neuronal survival and regeneration, future studies to further test this are therefore warranted.

As alluded to above, MEF2 activity has been implicated in a variety of contexts when neuronal survival is challenged and, based on available evidence, it is reasonable to assume that MEF2 plays a role in the orchestration of survival. Conversely, it is perhaps worthwhile to consider what happens to MEF2 activity when an insult is sufficiently severe as to overwhelm the survival response to cause cell death. One study has indeed reported that 4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) induction of neuronal cell death is correlated with cdk5 mediated phosphorylation of MEF2D (Smith et al. 2006) and a further study has documented that 6-hydroxydopamine (6-OHDA) mediated apoptosis in PC12 cells resulted in a marked reduction of MEF2D levels, an effect which was antagonized by treatment with roscovitine, a cdk5 inhibitor (Kim et al. 2011). As well as these studies, our studies presented here show that cAMP mediated PKA activation can lead to a potent repression of MEF2 activity which also results in enhanced neuronal apoptosis. Our previous studies (Du et al. 2008), along with these reported here, document a bipartite mechanism of MEF2D regulation by PKA through direct phosphorylation of S121/190 and enhanced assembly of a MEF2D/HDAC4 repressor complex. Strikingly, in view of these other studies that show a mechanistic link between MEF2D and neuronal survival, a recent study has documented a down regulation of MEF2D in nigral neurons in Parkinson's disease patients when compared to those of age-matched controls (Chu et al. 2011). The

implication of pro-survival MEF2 activity in a variety of neuronal damage models does suggest the possibility that MEF2 protects against a variety of cellular stresses and toxic insults. We have observed that overexpression of MEF2D does protect hippocampal neurons somewhat from H2O2 mediated cell death supporting this idea further (data not shown). Further delineation of the complex signaling pathways converging on MEF2 will be important in potentially identifying other cellular signaling pathways that modulate neuronal survival.

There are some studies clearly implicating cAMP in neuronal protection as well as in cell death and further clarification of this issue is warranted. It is possible that the duration and intensity of the signaling could be a factor in determining the cellular outcome. The effects of PKA/cAMP signaling on cell survival also likely depend on cell type, for example cAMP has been implicated in a neuroprotective role in cerebellar granule neurons (Wang et al. 2005). Conversely, our studies using a variety of perturbations of cAMP signaling (Forskolin, dbcAMP, exogenous expression of the active catalytic subunit of PKA) all indicate the same effect of promoting cell death in cultured primary hippocampal neurons. However, survival of neurons depends on a complex series of cellular and intracellular communication networks mediated by cross-talk between signal transduction cascades. Therefore it is entirely possible that modulation of the type of signaling (eg. short term versus chronic, high intensity versus low) may shift the balance resulting in a different cellular outcome.

In addition to the well defined survival role of MEF2 in neurons, MEF2 has also been implicated in activity dependent synapse elimination which fulfills a crucial role in the refinement of neuronal circuitry by altering synapse calibre and number (Flavell et al. 2006; Wierenga et al. 2006; Chandrasekaran et al. 2007; Turrigiano, 2008; Barbosa et al. 2008). Moreover, a recent study has reported that this MEF2 dependent excitatory synapse elimination is lost in hippocampal neurons from mice that are nullyzygous for an RNA binding protein (FMRP) whose function is lost in Fragile X syndrome, the most prevalent form of human autism and mental retardation (Pfeiffer et al. 2010). Lastly, and of relevance for our study, it was reported that cocaine administration inhibits striatal MEF2 activity through a cAMP dependent mechanism (Pulipparacharuvil et al. 2008) and cocaine mediated increases in dendritic spine density were dependent on MEF2 suppression. Consistent with this idea that MEF2 regulates dendritic morphogenesis, Shalizi et al. (2007) reported that PIASx, which functions as a MEF2 SUMO E3 ligase, represses MEF2 activity in neurons to orchestrate morphogenesis of postsynaptic dendrites. Thus, the regulation of MEF2 activity by PKA may also impinge on MEF2 dependent synapse modulation.

Clearly, the regulation of MEF2 activity and its downstream targets in the CNS by cAMP-PKA signaling requires consideration in terms of understanding the capability to dynamically alter synaptic connectivity and neural circuitry as well as neuronal survival during development, physiology and pathology of the mammalian CNS.

Chapter IV

An interaction with Strawberry notch1 (Sbno1) connects MEF2 to Notch signaling during myogenesis

Experimental design and writing of manuscript

Jahan Salma and Dr. John C McDermott

Experiments conducted by

Jahan Salma (all figures except figure 37B; Fig 41C & D)

Dionyssiou, M.G (Figure 37B; Fig 41C & D)

Eric Yang (Performed Mass spectrometry analysis)

Rationale:

The initial objective of this study was to identify MEF2 co-factors by using TAP (tandem affinity purification) tag protein purification system and modern mass spectrometry technology. Numerous studies have demonstrated the prominent role of MEF2 family members in orchestrating myogenic differentiation. The process of myogenesis is critical and tightly controlled by various myogenic factors. Previous studies have documented that activation of notch signaling inhibits myogenesis by induction of notch target genes. Notch signaling regulates the balance between maintenance of progenitor cells by inhibition of differentiation and facilitation of commitment to the muscle lineage. Notch signaling is known to block the expression and activity of the myogenic factors such as MEF2s but no exact mechanism of myogenic inhibition has been identified as yet. Interestingly, we identified a novel MEF2 interacting partner, strawberry notch 1(Sbno1), which has been characterized as a downstream effecter of Notch signaling pathway component. We, therefore, initiated studies to identify the mechanism of myogenic inhibition by notch signaling and characterize the detailed role of Sbno1 during skeletal muscle differentiation.

An interaction with Strawberry notch1 (Sbno1) connects MEF2 to Notch signaling during myogenesis

Jahan Salma[1,2,3,4], Mathew Dionyssiou[1,2,3,4], Eric Yang and John C. McDermott[*1,2,3,4]

Author affiliation:

[1]Department of Biology, York University, 4700 Keele Street, Toronto, Ontario Canada M3J 1P3

[2]Centre for Research in Mass Spectrometry (CRMS), York University, 4700 Keele Street, Toronto, Ontario Canada M3J 1P3

[3]Muscle Health Research Centre (MHRC), York University, 4700 Keele Street, Toronto, Ontario Canada M3J 1P3

[4]Centre for Research in Biomolecular Interactions (CRBI), York University, 4700 Keele Street, Toronto, Ontario Canada M3J 1P3

*Corresponding author:
J.C. McDermott, Department of Biology, 327 Farquharson, York University, 4700 Keele Street, Toronto, Ontario, Canada. M3J 1P3
E. mail: jmcderm@yorku.ca
Phone: (416)-736-2100 ext.30389, Fax: (416)-736-5698,

Running title: A novel MEF2 co-repressor Sbno1 inhibits myogenesis

Key Words: MEF2, Strawberry Notch 1, Notch signaling, Myogenesis

Abstract

Skeletal muscle development requires the coordinated expression of numerous transcription factors to control the muscle differentiation process by committed myoblasts into functional, contractile muscle. Myocyte enhancer factor 2 (MEF2) is essential for muscle differentiation. MEF2 interacts with various transcriptional cofactors/interacting partners and previous studies have shown that MEF2 proteins function combinatorially with cofactors to regulate transcription and muscle differentiation. In this study, we have identified a novel MEF2 interacting partner, Strawberry notch 1(Sbno1), which is known to be a downstream effector of Notch signaling. Notch signaling is an evolutionary conserved pathway that plays a critical role in cell fate decisions in proliferative cells including myogenic and neurogenic cells during development, but it is also involved in repair and maintenance in the adult after injury. In skeletal muscle, regulation of Notch signaling is involved in proliferation and cell fate determination of muscle stems cells during somite and muscle development. We, therefore, focused on characterization of the mechanism of skeletal muscle differentiation by Notch. First we investigated the role of Sbno1 in skeletal muscle proliferation and differentiation. We found that Sbno1 represses MEF2 trans-activation properties and plays a critical role in inhibition of skeletal muscle differentiation. Moreover, immunocytochemistry analysis revealed that Sbno1 may be involved in maintaining the "reserve" population of C2C12 cell differentiation. Thus, our data provide the evidence that a protein-protein interaction between Sbno1 and MEF2D results in interference with the myogenic program.

Introduction

The transcription factor myocyte enhancer factor 2 (MEF2) was originally identified in muscle tissues but has been shown to be expressed in a variety of tissue types although activity seems to be largely restricted to cardiac, skeletal, smooth muscles, T-cells and neurons (Ornatsky & McDermott, 1996; Zhu et al. 2005; Potthoff & Olson, 2007, Salma & McDermott, 2012). MEF2 has been implicated as playing a pivotal role in controlling cell proliferation, differentiation, and survival. Vertebrate MEF2 proteins are encoded by four genes MEF2 A, -B, -C, and -D (Yu et al. 1992; McDermott et al. 1993; and Breitbart et al. 1993), and a single MEF2 gene in *Drosophila* (Lilly et al. 1994; Nguyen et al. 1994). The organization of the MEF2 genes is identical within conserved regions, from *Drosophila to Homo sapiens.* This indicates that they evolved from a common ancestral MEF2 gene present in invertebrates (Breitbart et al. 1993). In *Drosophila*, genetic analyses have demonstrated the key role of MEF2 in terminal muscle differentiation (Bour et al. 1995). During embryogenesis, the MEF2 genes are expressed throughout developing cardiac and skeletal muscle lineages (Edmondson et al. 1994; Naya et al. 1999) and brain development (Leifer et al. 1994; Lyons et al. 1995) indicating MEF2's diverse role in cellular processes during development (Dodou et al. 1995). In adult tissues, members of the MEF2 family are critical regulators of skeletal muscle, cardiac hypertrophy, and activity-dependent remodeling of neuronal synapses (Ornatsky & McDermott, 1996; Flavell et al. 2008; Akhtar et al. 2012). Inhibition of MEF2 activity in cultured hippocampal neurons has been shown to

cause neuronal cell death, suggesting that MEF2-dependent transcriptional regulation is critical for neuronal survival (Salma & McDermott, 2012).

MEF2 belongs to the MADS-box (MCM1, Agamous, Deficiens, Serum response factor) super family of transcription factors (Black & Olson, 1998). The N-terminus of all MEF2 proteins is highly conserved, consisting of a MADS domain followed by an adjacent MEF2 domain which is required for high affinity DNA binding (T/C)TA(A/T)$_4$TA(G/A), homo- and hetero-dimerization, and co-factor interaction. The region C-terminal to the MADS/MEF2 domain is less conserved and contains domains required for *trans*-activation (TAD) and nuclear localization (NLS). The C terminus of MEF2 proteins are subjected to extensive alternative splicing and post-translational modifications (Black & Olson, 1998; Ornatsky et al. 1999; Gregoire et al. 2006). Thus, MEF2 allows the receipt of multiple inputs from various signaling pathways including calcium-dependent (Ghosh & Greenberg,1995; Mckinsey et al. 2002), MAPK-dependent (Yang et al. 1998, Naya & Olson, 1999; Zhao et al. 1999; Cox et al. 2000), ERK (Kato et al. 1997; Cavanaugh et al. 2001), and cyclic-AMP dependent signaling pathways that regulate MEF2 activity in wide range of cell types (Li et al. 1992; Du et al. 2008; Salma & McDermott, 2012).

The activity of MEF2 proteins is modulated by signal-dependent protein–protein interactions with a variety of other proteins in order to regulate diverse programs of gene expression. These proteins include transcriptional co-activators such as NFAT (Chin et al. 1998), Smads (Quinn et al. 2001), p300 (Sartorelli et al. 1997; Youn et al. 2000; Ma et al. 2005) and myocardin (Pipes et al. 2006) which

function in a cooperative manner with MEF2 factors. MRFs and MEF2 factors physically interact and synergistically potentiate MRFs mediated myogenic activities (Molkentin et al. 1995; Black & Olson 1998). The MEF2-HDACs association has been well characterized in skeletal and cardiac muscle and neuronal cells as a transcriptional co-repressor (Lu et al. 2000b; McKinsey et al. 2002; Perry et al. 2009; Salma & McDermott, 2012). Other MEF2 interactions have been reported with Sp1, PCAF, GRIP1, and MITR which enhances/represses MEF2 transcriptional activity (Morin et al. 2000; Park et al. 2002; McKinsey et al. 2001a; Ma et al. 2005; Creemers et al. 2006b). It is clear that MEF2 function depends on its ability to recruit such factors for appropriate target gene expression in variety of tissue types, underlying the MEF2 diverse roles in development and physiology of multiple organisms. Interactions between MEF2 factors and their interacting partners are critical determinants of cell growth, differentiation, and survival.

The notch signaling pathway is an evolutionary conserved mechanism in which cell–cell interactions influence distinct cellular fates in variety of tissues such as skeletal and smooth muscle, heart, and brain (Conboy & Rando, 2002; Rios et al. 2011; Pierfelice et al. 2011; MacGrogan et al. 2011; Gude & Sussman, 2012; Boucher et al. 2012). In mammals, there are four Notch receptors (Notch1–4) that interact with Notch ligands (DLL1, 3, 4 and Jagged1, 2) that are expressed on the surfaces of neighboring cells. The ligand-receptor interaction is followed by the sequential cleavage of Notch extracellular and intracellular domains by an ADAM protease and by γ-secretase, resulting in the release of the intracellular domain of Notch (ICND). The NICD (Notch intracellular domain) translocates to the nucleus

where it interacts with CSL transcription factor (CBF1{C promoter-binding factor 1} in human, Su(H) {Suppressor of Hairless} in *Drosophila*, LAG-1{Longevity-assurance gene-1} in *C.elegans*, RBP-J in mouse) cooperates with coactivator Mastermind-Like 1(MAML) to form a complex that induces transcription of multiple target genes including the well-characterized bHLH gene family, *Hes* and *Hey* (Kopan & Ilagan, 2009). Notch signaling pathway emerged as a critical regulator of myogenesis and muscle stem cell proliferation (Nye et al. 1994; Luo et al. 2005; Vasyutina et al. 2007; Buas et al. 2009; Wen et al. 2012). Notch signaling regulates the balance between maintenance of progenitor cells by inhibition of differentiation and facilitation of commitment to the muscle lineage (Kopan et al. 1994; Conboy & Rando, 2002). Notch signaling is essential for satellite cell activation in injured muscle (Luo et al. 2005). Deregulation of notch signaling contributes to severe pathological defects (Wilson & Radtke, 2006). Previous studies have documented that activation of notch signaling inhibits myogenesis by induction of notch target genes including *Hes* and *Hey.* These genes block the expression and activity of the myogenic determination and differentiation factors such as MRFs and MEF2s (Kopan et al. 1994; Shawber et al. 1996; Kuroda et al. 1999). Activation of Notch signaling in muscle cells by overexpression of Delta1 prevents MyoD expression during chick limb development resulting in inhibition of myogenesis *in vivo* (Delfini et al. 2000; Hirsinger et al. 2001). Ectopic expression of a constitutively active form of Notch 1 in myogenic cell culture or co-culture with notch ligand expressing cells inhibits myogenic differentiation (Kopan et al. 1994; Shawber et al. 1996; Kuroda et al. 1999; Lehar et al. 2005). Pharmacological

manipulations such as γ-secretase inhibitor or overexpressing Numb (a negative regulator of Notch), inhibit Notch signaling and subsequently promote cell differentiation by blocking the proteolytic cleavage of notch receptor (Conboy & Rando, 2002; Kitzmann et al. 2006; Moellering et al. 2009). Furthermore, overexpression of Notch target genes markedly inhibits of MyoD and MEF2 activity and suppresses myogenic differentiation (Kopan et al. 1994; Wilson-Rawls et al. 1999; Kuroda et al. 1999; Buas et al. 2010).

MEF2D is highly expressed in skeletal muscle and neuronal cells where it is required for skeletal muscle differentiation and neuronal survival respectively (Black & Olson, 1998; Du et al. 2008; Salma & McDermott, 2012). To further understand the diverse role of MEF2D in myogenesis and neurogenesis, we sought to identify new interacting partners of MEF2 using tandem affinity purification (TAP) coupled with mass spectrometric techniques. During the course of the present studies in mammalian cells, we detected and identified a novel interaction between MEF2D and strawberry notch 1 (Sbno1). Sbno1 is known as a downstream effector of notch signaling pathway which plays a critical role during embryo development of zebra fish and wing development of *Drosophila* (Coyle-Thompson & Banerjee, 1993; Majumdar et al. 1997; Nagel et al. 2001; Takino et al. 2010). Sbno1 was observed to be strongly expressed in the olfactory bulb, hippocampus, and cerebellum of the adult brain (Li et al. 2007; Takano et al. 2011). But to date, no evidence was found that Sbno1 regulates MEF2 activity through notch signaling in skeletal muscle or brain. However, it was documented previously that notch signaling involves modulation of MEF2 activity in myogenic cells (Wilson-Rawls

et al. 1999; Shen et al. 2006). A recent study in *Drosophila* identified a synergy between MEF2 and Notch which affects cell proliferation and metastasis (Pallavi et al. 2012). This led us to hypothesize that the Sbno1-Notch signaling pathway may play a role in cellular development such as myogenesis and neurogenesis through direct association with MEF2. We therefore examined the role of Sbno1-MEF2 interaction on muscle differentiation.

Materials and Methods

Antibodies and reagents

The following Primary antibodies were used: α-MEF2D from BD Biosciences. α-MEF2A rabbit polyclonal antibody was prepared in the lab as describe previously (Perry et.al. 2009).α-Actin (I-19), α-GFP (B-2), α-MyoD (C-20), α-Myf-5 (C-20), α-MCK, and α-SBNO1 (H-240) antibodies were purchased from Santa Cruz Biotechnology. Monoclonal antibodies α-MyHC (MF20) and α-myogenin (F5D) were purchased from Developmental Studies Hybridoma Bank. Normal mouse (sc-2025), rabbit (sc-2027), goat (sc-2028) IgGs and ImmunoCruz™ IP/WB Optima reagents were purchased from Santa Cruz Biotechnology. FITC and TRITC-conjugated α-rabbit and α-mouse secondary antibodies and DAPI (D9542) were obtained from Sigma. Antibodies used to detect Notch ligand (α-delta 1, H-20), Notch receptor (α-Notch 1, M-20), and Notch target gene (α-Hes1, H-140) were obtained from Santa Cruz Biotechnology. Gamma secretase inhibitor was purchased from TOCRIS. Expression plasmids for full-length pcDNA3-MEF2D, pMT2 MEF2A, pCMV β-galactosidase, MEF2 and Krüppel-like factor 6 (KLF6)

reporter gene constructs have been described in previous publications (Du et al. 2008; Perry et al. 2009; Salma & McDermott, 2012). pcDNA4/TO/TAP empty vector and pcDNA4/TO/TAP-MEF2D, pcDNA4/TO/TAP-MEF2A were previously described in detail (Du et al. 2008).

Cell culture and differentiation assay

C2C12 myoblasts and Cos7 cells were obtained from the American Type Culture Collection and cultured as described previously (Perry et al. 2009; Salma & McDermott, 2012). Cells were maintained in high-glucose Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, HyClone), sodium pyruvate, L-glutamine, and 1% penicillin-streptomycin (GM: growth medium). Cells were incubated at 37° C in 5% CO_2 humidified incubator. Transient transfections were performed using the standard calcium phosphate precipitation method. Cells were harvested and lysed 48hours after transfection according to experiments or twenty four hours after transfection, cells were transferred to DM.

Proliferating C2C12 cells were grown to 80-90% confluence, and induced for differentiation by switching from GM (serum withdrawal) to differentiation medium (DM: DMEM containing 5% horse serum supplemented with 1% penicillin–streptomycin) for 48, 72, 96, and 120 hours. Cells were kept in DM, replaced every 2 days and monitored for appropriate morphology (multinucleated myotube formation). C2C12 cells normally started differentiating 48–72 h after the serum withdrawal.

Co-culture of C2C12 and Delta 1 expressing cells

Parental OP9 stromal cells and cells expressing Delta1 (Dll1-Notch ligands) were a kind gift from J. C. Zuniga-Pflucker (University of Toronto, Ontario, Canada). C2C12 cultures were carried out as described previously (Du et al. 2008; Perry et al. 2009). OP9 monolayers were prepared one day in advance in 10cm/6 well culture plates, and C2C12 myoblasts cells were plated in 10cm/6well plates on monolayers of OP9 stromal cells that had been plated on the previous day. One day later, when cells were ~80-90% confluent, C2C12 differentiation was induced by replacing the culture medium with differentiation media, DMEM 5% horse serum (DM). The γ-secretase inhibitor (final concentration: 1μM) or dimethyl sulfoxide (DMSO) carrier was added to selected cultures, and the DM was replaced every 2 to 4 days. C2C12 cells were incubated for 48, 72, 96, and 120 hours in DM. To monitor differentiation (multinucleated myotubes), MF20 staining was performed and viewed with a fluorescence microscope.

Reporter gene assays

C2C12 cells were transiently transfected using calcium phosphate precipitation method. Cells were seeded at a density of 12.5×10^3 cells/well in 6-well plates 1 day prior to transfection. Transcription reporter assay plasmids (1μg) and expression plasmids (1.5 μg) were transfected as indicated in figures. pCMV-β-galactosidase (1μg) was transfected as an internal control for transfection efficiency. The total amount of DNA for each experiment was kept constant by using empty vectors. Cells were washed 16 hours post-transfection with phosphate-

buffered saline (PBS) and harvested 36-48 hours after transfection followed by β-galactosidase and luciferase assays according to manufacturer's instructions (Promega), using a Berthold 9501 luminometer. All measurements were made in triplicate for at least three independent experiments with data presented as means ± standard errors of the means.

Western blot analysis

Protein extracts were prepared in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA [pH 8.0], 100 mM sodium fluoride, 10 mM sodium pyrophosphate) containing 0.2 mM PMSF and 0.5 mM sodium orthovanadate and protease inhibitor cocktail. Protein concentrations were determined by Bradford assay (BioRad) with bovine serum albumin (BSA) as a standard. Equivalent amounts of total protein (15–20 μg) were diluted in sample buffer (sodium dodecyle sulphate-polyacrylamide) containing β-mercaptoethanol, boiled for 4-5min, and electrophoretically resolved by 10% SDS-PAGE gels, then electrophoretic transfer to an Immobilon-P membrane (Millipore Inc.). Non specific binding sites were blocked with 5% milk in PBS for one hour at RT. Immunoblotting was carried out using appropriate primary antibodies in 5% milk (PBS), α-MEF2A (1:1000), α-MEF2D (1:1000), α-MyoD (1:1000), α-Myf5 (1:1000), α-Sbno1 (1:1000), α-MCK (1:1000), α-MyHC (1:5), α-Myogenin (1:5), α-GFP (1:1000), and α-Actin (1:1000). The blots were then incubated with the appropriate secondary horseradish peroxidase (HRP) antibody (BioRad) at 1:2000 with 5% milk in PBS for 1 hour at RT followed by Chemiluminescence detection of

immunoreactive proteins as per the manufacturer's instructions (Amersham Biosciences).

Co-immunoprecipitation assays

Protein extracts were prepared from COS7 and C2C12 cells as described earlier. Immunoprecipitation was performed using the ExactaCruz kit (Santa Cruz), as per manufacturer's instructions. Protein complexes were immunoprecipitated with 3 μg of IP antibody or normal rabbit/mouse IgG, 40 μl of suspended IP matrix (50% slurry) (Santa Cruz) and 500 μl of PBS by incubation at 4 °C overnight on a nutating platform. The beads were washed 3Xwith NETN wash buffer (0.1% NP-40, 150 mM NaCl, 1 mM EDTA, and 50 mM Tris–HCl pH 8.0). Equal amounts of total protein (500 μg) were diluted with NP-40 lysis buffer containing protease inhibitor to a final concentration of 1 μg/μl, incubated with IP matrix at 4 °C overnight on a nutating platform. The beads were washed 3X NETN wash buffer and boiled in sample buffer for 4-5min. Precipitated proteins were separated by SDS page and immunoblotting was carried out as described above.

Immuno-fluorescence Analysis

C2C12 myoblasts were seeded at a density of 1×10^5 cells when they reached a confluence of 80-90% differentiation was induced the next day. The cells were fixed at MB, 48, 96 and 120 hours after start of differentiation, and processed for immunofluorescent detection as described previously (Salma & McDermott, 2012). Cells were incubated overnight at 4°C with primary antibodies α-MEF2D, α-Sbno1,

and α-MyHC (1:100) and then incubated with the appropriate TRITC/FITC-conjugated secondary antibodies (1:500) for 2 hours at RT following DAPI (4',6-diamidino-2-phenylindole) staining for 15 min at RT. Cells were washed and cover slips were mounted with DAKO mounting media (Dako). All images were captured using a fluorescent microscope.

MF-20 Staining

MF-20 staining was performed to detect myosin heavy chain (MHC) expression, C2C12 cells cultured in DM for various time points were washed 3x with PBS (pH7.4 and fixed with 95% methanol at -20 °C for 10 min. Following a 30 min blocking using 5% milk in PBS at 37 °C, cells were incubated at room temperature (RT) with MF-20 (primary antibody) diluted in blocking buffer (5% milk PBS) for 1 hour. After incubation, the cells were washed 3x with PBS and incubated for 60 min at RT with Horseradish peroxidase (HRP)-conjugated α-mouse secondary antibody. The cells were washed 3x with PBS and incubated in DAB staining solution (0.6 mg/ml DAB, 0.1 % H_2O_2 in PBS) to detect MyHC. The nuclei were counter-stained with haematoxylin. Images were obtained with a Carl Zeiss microscope.

Tandem Affinity purification (TAP)

C2C12 cells (1×10^5) were transiently transfected with (25 μg of DNA per 100 mm dish) either pCDNA4/TO/TAP-MEF2A and TAP-MEF2D or empty vector. The purification scheme and tandem affinity purification (TAP) vector system was

described previously (Du et al. 2008). Cells were lysed by quick-freeze-thaw in IPP150 lysis buffer with protease inhibitors. The cell lysate was incubated with rabbit immunoglobulin G (IgG) resin (Sigma) overnight on a rotator. After washing the resin with IPP150 buffer, Tagged proteins were eluted by cleaving with AcTEV protease (Invitrogen), then incubated with calmodulin resin (Stratagene) in IPP150 calmodulin binding buffer supplementing with Ca^{++} for 1 h at 4°C. Proteins were eluted using either 2 mM EGTA following liquid chromatography tandem MS (MS/MS) analysis or SDS sample buffer and boiled for 4 min at 95°C then separated by 10% SDS-PAGE. Proteins were visualised using Gelcode Blue (Pierce).

Results

Identification of novel interacting partners of MEF2

The function of MEF2 proteins is highly regulated by their multiple interacting partners (acting as co-activator/co-repressor) to regulate target gene expression in a variety of tissue types. This suggests that gene expression requires not only DNA binding of MEF2 but also interaction between two or more proteins as critical determinants of cell growth, survival, and differentiation. Previously our lab identified endogenous MEF2 interacting partner HDAC4 (a co-repressor) from cultured Hela cells by using a tandem affinity purification (TAP) technique (Puig et al. 2001; Cox et al. 2002). This technique is based on generic two step affinity purification under physiological/native conditions which has proven successful in

the identification of multi protein complexes for subsequent analysis by mass spectrometry (Rigaut et al. 1999).

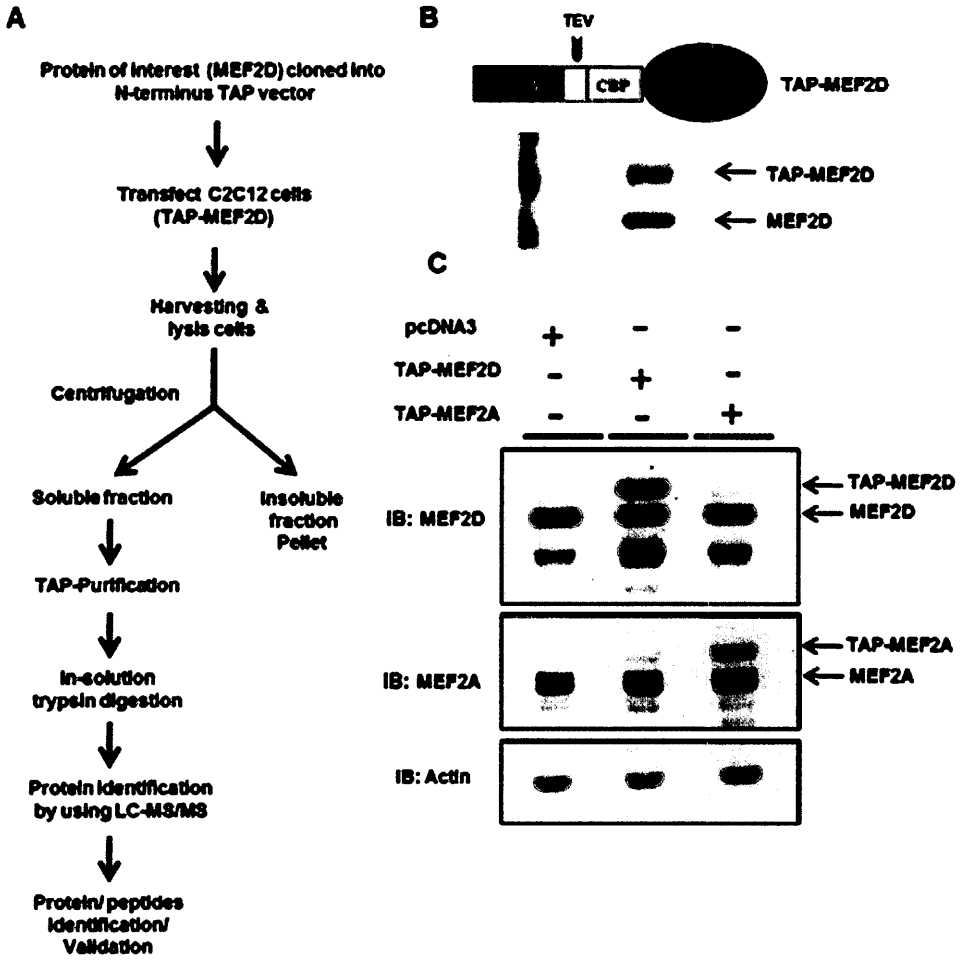

Figure 34. Strategy for Affinity purification and protein-protein interaction identification.

(A) Typical workflow for sample preparation, in-solution digestion, and mass spectrometry based identification of a protein. **(B)** Schematic representation of TAP-tagged fusion protein. TAP complex purified from C2C12 separated by denaturing protein gels and stained with coomassie blue. TAP-tag approach allows more stringent washing preventing the loss of protein complexes. **(C)** C2C12 cells were transiently transfected either with empty vector or TAP-MEF2D/TAP-MEF2A vectors. Cells extract were prepared for immunobloting analysis as indicated. Equal amount of total proteins were separated by 10%SDS-PAGE. MEF2D monoclonal

antibody (1:1000) and MEF2A polyclonal antibody (1:1000) was used to detect the protein levels. Acitn (polyclonal, 1:1000) was used as an equal loading control.

The combination of two purification steps provides gentle binding and washing conditions to preserve the protein-protein interaction as well as to remove the majority of non-specific protein contaminants (See material & Methods). This level of purification also provides high selectivity and unambiguous identification of interacting proteins (Figure 34A).

The primary interest of this study was to identify novel co-regulators/interacting partners of MEF2 for better understanding of MEF2 diverse role in variety of cellular programs. Therefore, we employed TAP technique to purify protein complexes from mammalian cells (C2C12) in combination with state-of-the-art Liquid chromatography mass spectrometry (LC-MS/MS). C2C12 myoblasts (MB) were transiently transfected either with pcDNA4/TO/TAP-MEF2D or empty vector by using calcium phosphate precipitation method followed by TAP purification, in-solution trypsin digestion and subjected to LC-MS/MS analysis (Figure 34A). Expression level of tagged proteins was examined by coomassie staining and immunoblotting to confirm the tagged protein and its associated partners that can be detected from the crude cell extracts (Figure 34B & C). As a positive control TAP-MEF2A was used to confirm TAG protein expression, identified previously by Cox et al. 2002 (Figure 34C). From MS/MS analysis, numerous interacting partners of MEF2 were identified along with MEF2D when sample purified with TAP-MEF2D was compared with control sample. One of the prominent interacting partner was Strawberry Notch homolog1 (Sbno1) detected *in*

vivo complex with MEF2D. Previously Sbno1 has been reported as a downstream component of the Notch signaling pathway (Coyle-Thompson & Banerjee, 1983; Majumdar et al. 1997). Notch signaling pathway is crucial for the proper development of diverse cell types and tissues and is shown to inhibit muscle differentiation (Nye et al. 1994; Giebel, 1999; Wilson-Rawls et al. 1999; Yoon, 2005; Takano et al. 2010 & 2011; Pierfelice et al. 2011; Rios et al. 2011; Boucher et al. 2012). Therefore, we decided to characterize the interaction between Sbno1 and MEF2D. There may be possibility that notch signaling plays an important role in muscle differentiation through the activation of Sbno1 which may involve in regulation of myogenic factors (MEF2D) during similarity.

Sbno1 physically interacts with MEF2D

To begin to elucidate the possible functional role of Sbno1 in mammalian cells, we first validated the physical interaction between Sbno1 and MEF2D by performing immunoprecipitation assays. COS7 cells were transiently transfected with expression plasmids of Sbno1 and MEF2D alone and both plasmids together. Cell extracts were subsequently immunoprecipitated with anti-Sbno1 antibody and immunobloted with anti-MEF2D monoclonal antibody. A prominent MEF2D detection was observed when Sbno1 is ectopically co-expressed with MEF2D, confirmed a specific interaction between Sbno1 and MEF2D (Figure. 35A, Co-IP panel, compare lane 4). MEF2D and Sbno1 was not detectable in pcDNA3 transfected lysates (Figure 2A, upper panels, lane 1) but their expression were seen only in input-lysates when transfected alone or together (Figure 35A, upper panels,

lanes 2, 3 & 4). GFP was used as a marker for transfection efficiency and actin as a loading control of total protein (Figure 35A, Actin & GFP blots). Detection of faint bands of MEF2D from lysates not over-expressing was due to low levels of endogenous MEF2D expression in cos7 cells (Figure 35A, MEF2D blot lanes 1 and 2). Endogenous Sbno1 protein level was not observed when pcDNA3 ectopically expressed in COS7 cells and whole cell lysate (Figure 35B, lanes 1 and 3) compare to ectopically expressed Sbno1 in cos-7 shown in Figure 35B, lane 2, further confirming that there is no endogenous Sbno1 in these cells which might interact with MEF2D.

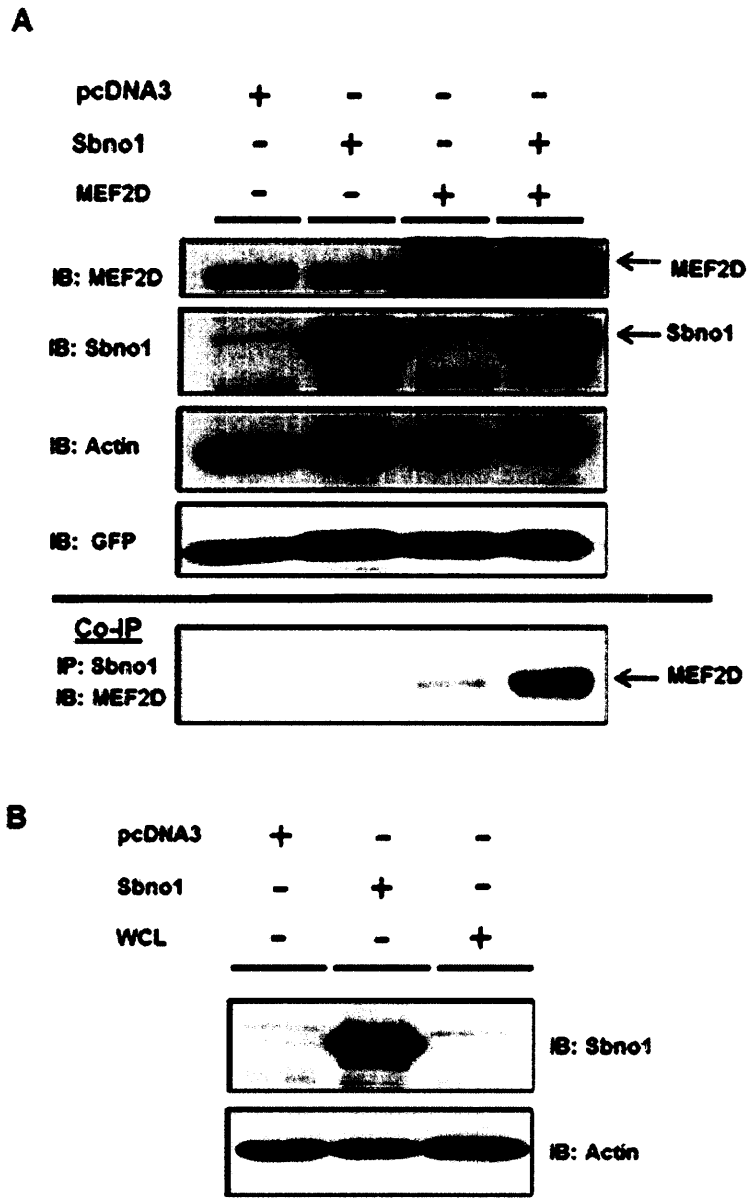

Figure 35. Physical Interaction between Sbno1 & MEF2D.
(A & B) COS7 cells were transiently transfected with empty vector and combinations of Sbno1 and MEF2D expression vectors (as indicated). Cells extract were prepared for coimmunoprecipitation and western blot analysis. (A) For coimmunoprecipitation 500 μg of total protein extracts was used for analysis, and for immunoblotting, 20 μg of total cell extract was diluted with NP-40 lysis buffer to a final concentration of 1 μg/μl. Protein complexes were immunoprecipitated with 2 μg Sbno1 polyclonal antibody or 2 μg normal rabbit IgG, and 20 μl matrix

beads (50% slurry) ImmunoCruz (Santa Cruz) by incubation at 4 °C overnight on a rotating platform. The beads were washed with three changes of wash buffer. Beads were boiled in sample buffer, and protein complexes were resolved by SDS-PAGE and immunoblotted as indicated in figure 2A, bottom panel. Western blot of MEF2D monoclonal (1:1000) and Sbno1 polyclonal antibody (1:1000) as indicated in figure 2A, upper panels. Acitn (polyclonal, 1:1000) and GFP (1:1000) were used for loading control and transfection efficiency respectively. **(B)** Cos7 cells were transiently transfected either with empty vector or Sbno1 expression vector (as indicated). Cells extract were prepared for western blot analysis. Sbno1 did not detected from cos7 cells when whole cell lysate (WCL) was used in western blot analysis of Sbno1.

In order to further verify the direct interaction between Sbno1 and MEF2D, we initiated experiments to test evidence–whether both proteins localize together in myogenic cells by using immunofluorescence assay. Figure 36A shows that Sbno1 (in red) and MEF2D (in green) specifically and strongly localized in the nuclear regions (Figure 36A, top panels) and co-localization of both proteins is visible when their respective images were merged together (Figure 36A, bottom panel). Cell nuclei were counter-stained with DAPI (in blue). These observations revealed that Sbno1 and MEF2D co-localized together within the nucleus of proliferating myoblasts. This co-localization may possibly, by targeting MEF2, have a functional role in skeletal muscle differentiation.

We additionally wanted to confirm, whether Sbno1 and MEF2D physically interact in myogenic context. More direct evidence of the physical interaction between these two proteins was obtained by co-immunoprecipitation analysis in C2C12 cells (proliferating myoblasts) as shown in figure 36, panels B & C.

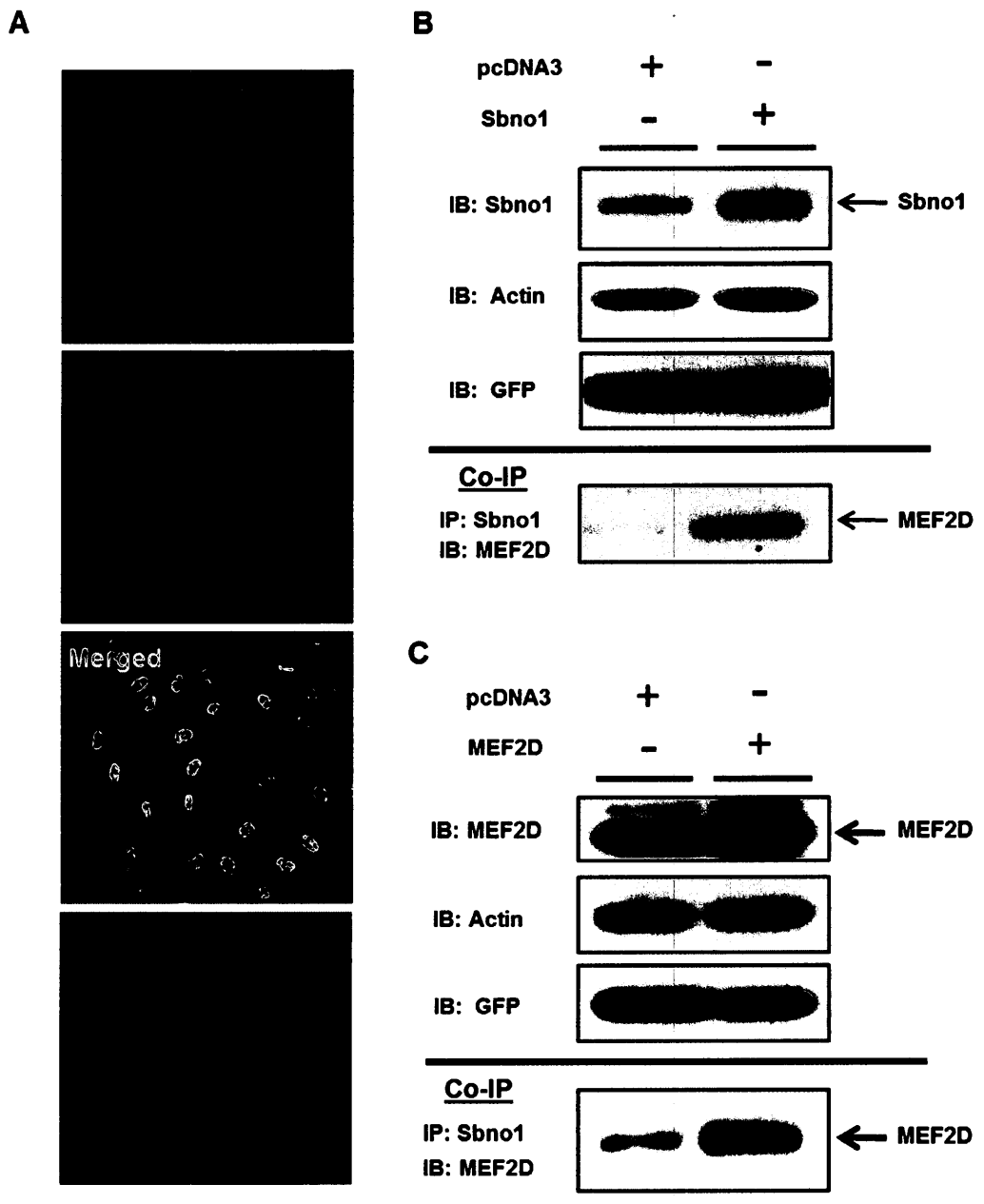

Figure 36. Cellular localization of Sbno1 in myogenic cells.
(A) Immunofluorescence analysis of C2C12 cells were performed at proliferative myoblast stage. Cells were fixed in 4% paraformaldehyde followed by double immunofluorescence analysis. Primary antibodies to anti-Sbno1 (rabbit) shown in red were merged with anti-MEF2D (mouse) labeled with green, revealed co-localization of both proteins in nuclei (yellow) in the myoblasts. Nuclear DAPI

staining is shown in Blue. All images were acquired from same field using a confocal laser scanning microscope. **(B & C)** C2C12 cells were either transfected with empty vector or expression vectors of Sbno1/MEF2D and GFP. Cell extracts were prepared for co-immunoprecipitation (Co-IP) analysis as indicated in bottom panel of B & C. Westernblots show the expression levels of MEF2D and Sbno1. Actin was used as a loading control and GFP as a marker for transfection efficiency.

C2C12 cells were transiently transfected with either Sbno1/MEF2D alone or empty vector. GFP was co-transfected as a transfection marker. Detection of endogenous MEF2D was observed when cell extracts were immunoprecipitated with anti-Sbno1 and immunobloted with anti-MEF2D antibody revealed endogenous interaction between Sbno1 and MEF2D which occurred in a native cellular environment (Figure 36, Co-IP panels B & C, lane 2). Collectively, these results further confirm a novel protein-protein interaction between Sbno1 and MEF2D identified by LC-MS/MS analysis in current studies.

Sbno1 inhibit MEF2 dependent transcription

Next we precisely examined the direct functional role of Sbno1 on MEF2 regulation, C2C12 cells were transiently transfected with MEF2-dependent luciferase reporter gene (MEF2-Luc) and an increasing amounts of an expression plasmid of Sbno1. This experiment clearly demonstrated that Sbno1 inhibited MEF2-mediated transcriptional activity of a MEF2-dependent reporter in a dose dependent manner when co-transfected with MEF2D, providing evidence for the involvement of Sbno1 in the regulation of MEF2 activity (Figure 37A).

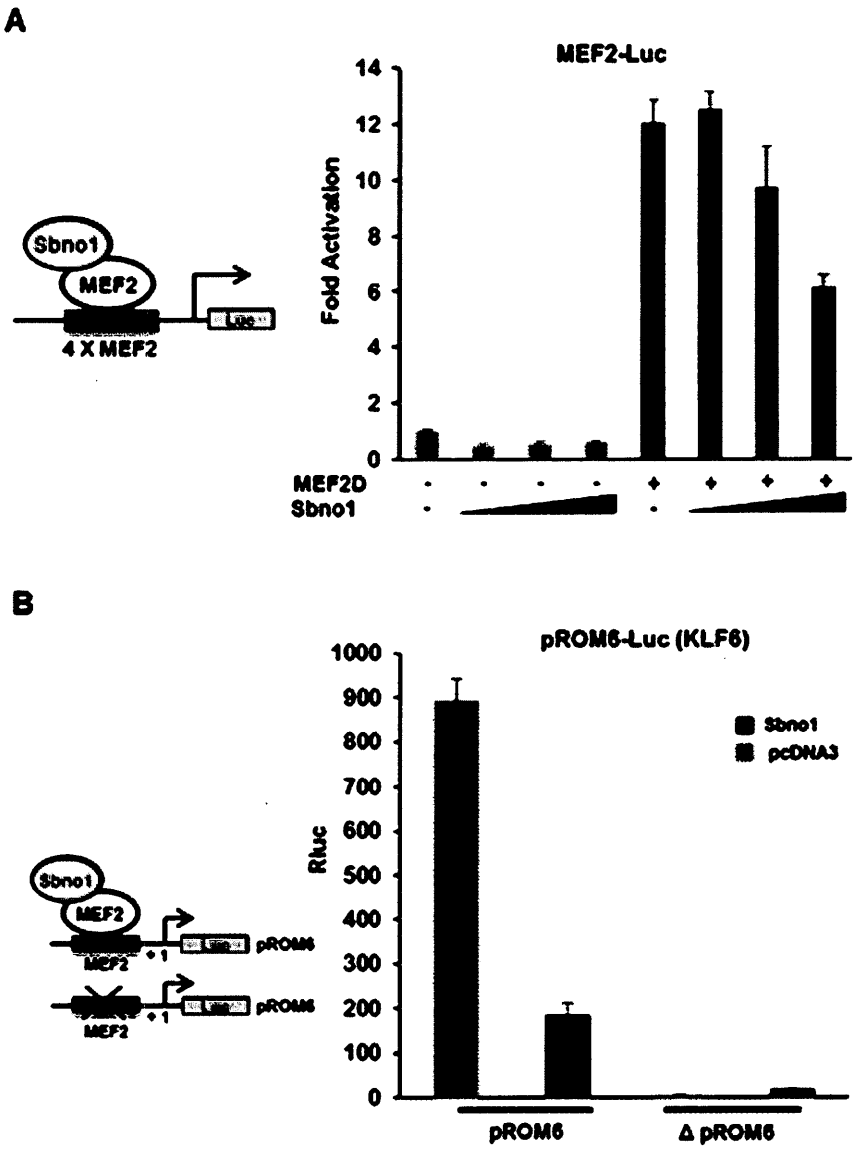

Figure 37. Effect of exogenous Sbno1 expression in C2C12 cells.
(A) Sbno1 suppresses MEF2 transcriptional activity in mammalian cells. C2C12 cells were transiently transfected with pGL3-4XMEF2-Luc reporter gene, pCMV-β-Galactosidase to normalize transfection efficiencies and increasing amount of Sbno1 by using calcium phosphate method. **(B)** Sbno1 suppresses KLF6 transcriptional activity through MEF2. Schematic illustrations of KLF6 promoter construct (pROM6) with intact MEF2 binding site or with MEF2 site mutated. C2C12 cells were co-transfected either with empty vector or Sbno1. MEF2

mediated transcriptional activity was determined by pGL3-pROM6-Luc or pGL3-ΔpROM6-Luc (KLF6 reporter gene) and pCMV-β-Galactosidase (to normalize transfection efficiencies). pGL3-basic empty vector was used as a control. Cell extracts were prepared for luciferase and β-gal assays as described in Material and Methods. (Data are the mean +/- S.E.M n=3).

Given that Sbno1 was identified as a potential co-repressor of MEF2, we then investigated whether Sbno1 can alter a known MEF2 downstream target gene Krüppel-like factor 6 (KLF6) through targeting MEF2. KLF6, a newly identified MEF2 target gene, is involved in neuronal survival (Salma & McDermott, 2012) and in muscle differentiation (unpublished data). To investigate the possible role of Sbno1 in the regulation of MEF2 target genes, C2C12 cells were transfected with the pROM6 (KLF6) reporter construct containing the intact or mutated MEF2 binding site in the presence and absence of Sbno1. Figure 37B demonstrated that the pROM6 reporter gene activity driven by MEF2 site was markedly reduced by Sbno1 (Figure 37B, 2nd bar) compared to control. Similar results were seen with or without Sbno1 when cells were transfected with pROM6 reporter construct which lacks the MEF2 binding site (Figure 37B, 3rd and 4th bar). Together, these results provide evidence for an important role of Sbno1 in the regulation of MEF2 activity. This association of Sbno1 with MEF2D suggests that Sbno1 may be acting as a co-repressor, inhibiting MEF2 activity in myogenic cells.

Exogenous expression of Sbno1 inhibits differentiation of C2C12 cells

Considering our finding that Sbno1 associates with MEF2D in myogenic cells and regulates MEF2 dependent gene expression. We proceeded to examine the effect of exogenous expression of Sbno1 on C2C12 skeletal muscle cell differentiation. The MEF2 factors have been shown to be essential for muscle differentiation (Black & Olson, 1998). MEF2 also cooperate with the basic helix-loop-helix transcription factors (bHLH) in the activation of the muscle specific gene expression and function within a regulatory network. Together, they regulate the differentiation of myoblasts into multinucleated myotubes by activating muscle-specific contractile genes (Molkentin et al. 1995). Therefore, we investigated the ability of Sbno1 in the regulation of muscle-specific transcription factors. C2C12 cells (myoblasts=MB) were transiently transfected with either Sbno1 or empty vector by using calcium phosphate precipitation method. Cells remain proliferative in the presence of growth medium (GM) containing 10% FBS until they were 80-90% confluent, but start to differentiate upon withdrawal of GM (high serum content) and induced to differentiate by culturing them in a differentiation medium (DM) containing 5% horse serum (as described in Materials and Methods) and were incubated additional 4 days. We used Western blot analysis to examine early differentiation markers MEF2 and myogenin and late differentiation marker skeletal myosin heavy chain (MyHC) protein expression in the presence of Sbno1. Total cellular extracts were isolated from MB in GM and 48hrs, 72hrs, and 120 hrs after induction in DM. Western blots indicate that ectopically expressed Sbno1

completely abolished expression of early and late differentiation markers myogenin, MyHC, and MCK respectfully.

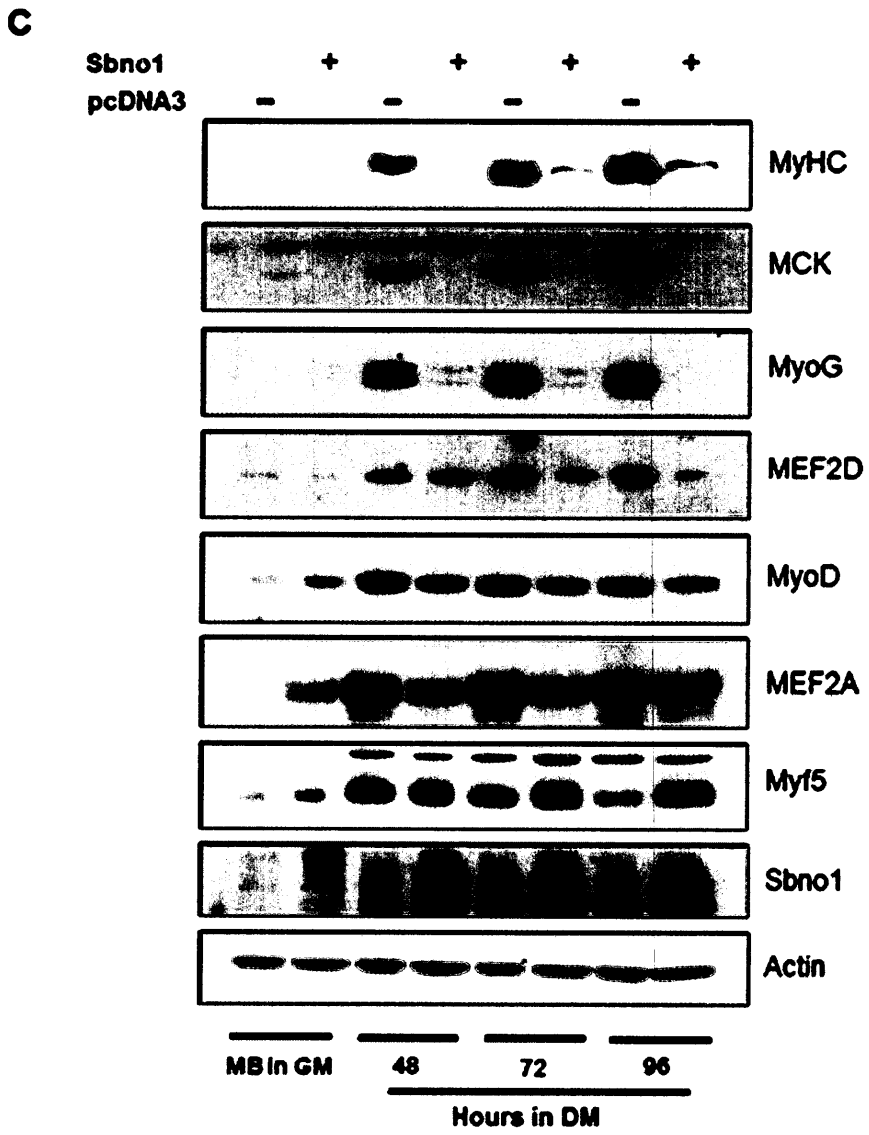

Figure 37. Effect of exogenous Sbno1 expression in C2C12 cells.
(C) Overexpression of Sbno1 inhibits myoblasts differentiation. C2C12 myoblasts were co-transfected either with empty vector and Sbno1. Transfected cells were allowed to recover and grow to 80% confluence in growth media (GM) (MB, 0 h) and then switched to differentiation media(DM) for a period of 48, 72, 96 hrs. Samples were harvested at the indicated time points and analyzed for early and late

differentiation markers (MyoD, Myogenin, MyHC, MCK) expression by Western blot analysis. MEF2 expression was gradually decreased in the presence of Sbno1 when cells grown in DM and monitor equal loading expression with Actin (polyclonal, 1:2000).

The expression of MEF2 factors, and MyoD were markedly decreased with Sbno1 relative to the pcDNA3 expressed cells. Interestingly, Sbno1 increased the Myf5 expression in the differentiating cells relative to the pcDNA3 expressed cells (Figure. 37C). Together these results show that overexpression of Sbno1 is involved in impaired expression of myogenic factors resulting in inhibition of myoblast differentiation and initiation of multi-nucleated myotubes formation by repressing MEF2 and muscle specific gene expression such as MyoD and myogenin. This might be occurred through MEF2 dependent regulation due to absolute requirement of MEF2 factors for myoblast differentiation and terminal myotubes formation.

Immunostaining analysis of Sbno1-MEF2D during C2C12 differentiation

Since we observed the potential role of Sbno1 in the regulation of MEF2 activity and in muscle differentiation, we initiated to further explore localization of Sbno1 and MEF2D during the process of skeletal muscle cell differentiation by examining double immunofluorescence analysis. In order to performed time course immunostaining, C2C12 cells were fixed at MB (GM), 48hrs, 96hrs and 120 hrs after induced to differentiate by culturing them in a DM. We found strong nuclear localization of Sbno1 (red) and MEF2D (green) in proliferating myoblasts and merged images clearly depict co-localization of Sbno1 and MEF2D in nuclear

region of proliferating myoblasts. Cell nuclei were counter-stained with DAPI (in blue) (Figure. 38, left panel 1).

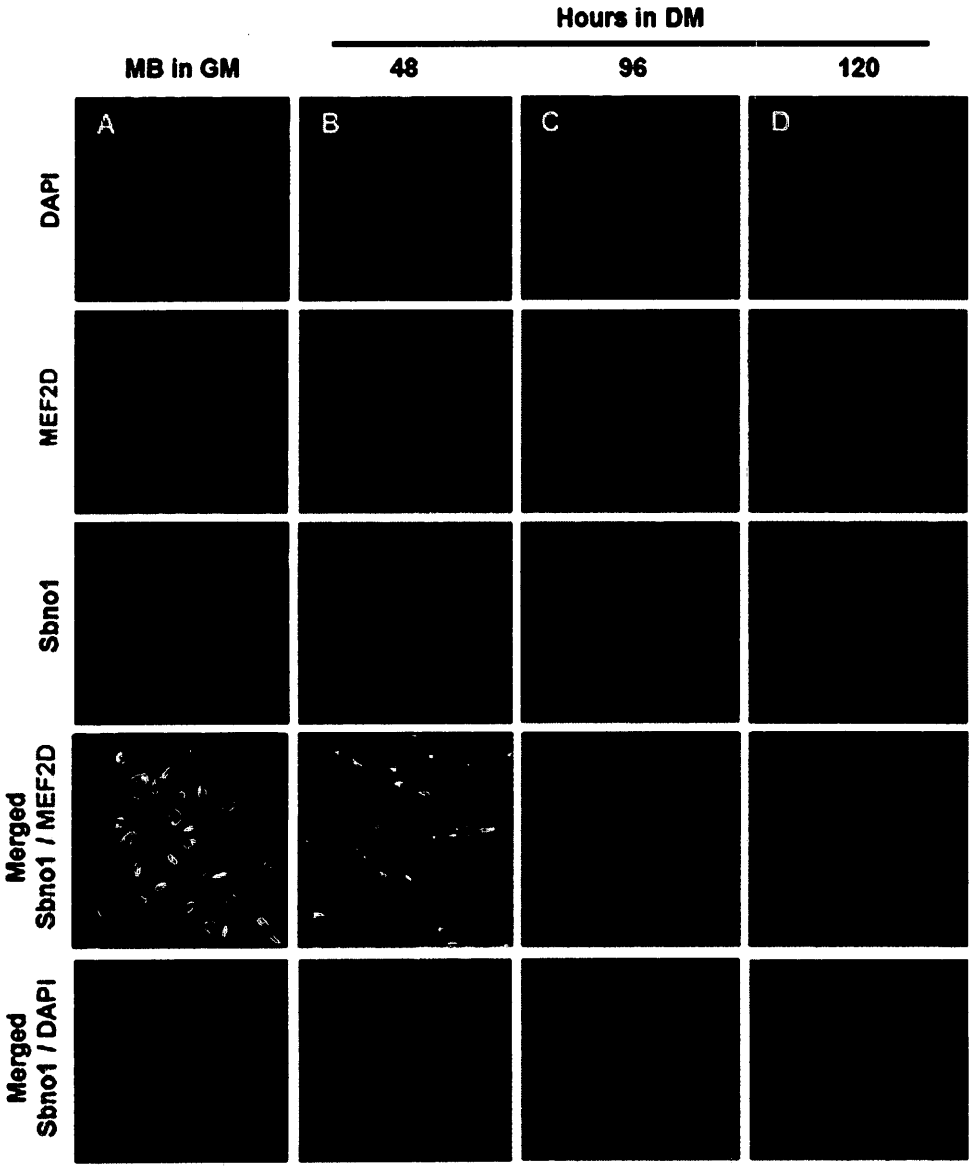

Figure 38. Cellular localization of Sbno1 & MEF2D during C2C12 differentiation. C2C12 proliferating myoblasts were grown to confluence in GM (0h, panel A) and then switched to DM for a period of 48, 96 and 120 hrs (panels B-D). Immunofluorescence analysis of C2C12 cells were performed at the indicated

time points. Cells were fixed in 4% paraformaldehyde (panels A-D) followed by double immunofluorescence analysis (as described in Materials and Methods). Cells were stained with primary antibodies to anti-Sbno1 (rabbit) shown in red were merged with anti-MEF2D (mouse) labeled with green. Co-localization of both proteins in nuclei (yellow) was revealed in the myoblasts (panel A). Nuclei were counterstained with DAPI (blue). All images were acquired from same field using a confocal laser scanning microscope.

The detailed analysis of immunostaining of Sbno1 during the progression of differentiation revealed a dramatic change in the localization of Sbno1 from mononucleated myoblasts, when cells remain proliferative in the presence of growth medium (GM), to formation of terminally differentiated myotubes (multinucleated). Interestingly we observed translocation of Sbno1 from nucleus to cytoplasm at 48hrs in C2C12 cells in DM. This localization was clearly seen when DAPI and Sbno1 images were merged (Figure. 38, left bottom panel 2). Both 96 and 120 hrs images demonstrated that Sbno1 was localized in cytoplasm of un-differentiated cells (reserve/satellite cells) whereas MEF2D showed strong nuclear localization in majority of cells in the early differentiating myoblasts (48hr) and in the late differentiated myotubes (96 & 120hrs) (Figure. 38, right panel 3 & 4). Taken together these observations indicate that Sbno1 highly localizes in nucleus of undifferentiated cells but gradually declines in differentiating cells suggesting that heterogenous cell types exists during myogenic differentiation. Sbno1 negative cells (myotubes) are surrounded by Sbno1 positive cells which maintain reserve cells population. Collectively, these results suggest that Sbno1 might be a crucial regulator to balance differentiated and undifferentiated cells.

Localization of Sbno1 during Myoblasts differentiation

The transition of cellular differentiation is associated with up/down-regulation of variety of regulators and co-regulators with direct association of myogenic factors such as MEF2 which requires for terminal process of differentiation. Normally MEF2 expression is induced upon activation of muscle differentiation signal which contributes to enhancement of muscle genes expression. Therefore, we intended to examine when Sbno1 and MEF2D interact to form physical complex in myogenic cells during the process of differentiation. C2C12 cell lysates, both from myoblasts and myotubes were immunoprecipitated with anti-Sbno1 antibody and immunobloted with anti-MEF2D monoclonal antibody. Detection of endogenous MEF2D was observed only when cell extracts from undifferentiated C2C12 myoblasts (cultured in GM) was immunoprecipitated with anti-Sbno1 antibody and immunobloted with anti-MEF2D antibody but not in differentiating myotubes in DM (data not shown). This revealed endogenous interaction between Sbno1 and MEF2D which occurred in a native cellular environment (Figure 39A, Co-IP). Present results demonstrated that these two proteins associate together in myoblasts and, not in myotubes.

In addition we performed immunocytochemistry on differentiated cell culture to observe the heterogenic cellular localization of Sbno1 and skeletal myosin heavy chain (MyHC) after 120hrs induction of muscle differentiation. Differentiated mature myotubes were positively identified by immunostaining with a monoclonal antibody directed against the MyHC, a terminal myogenic differentiation marker (in green) (Figure 39B).

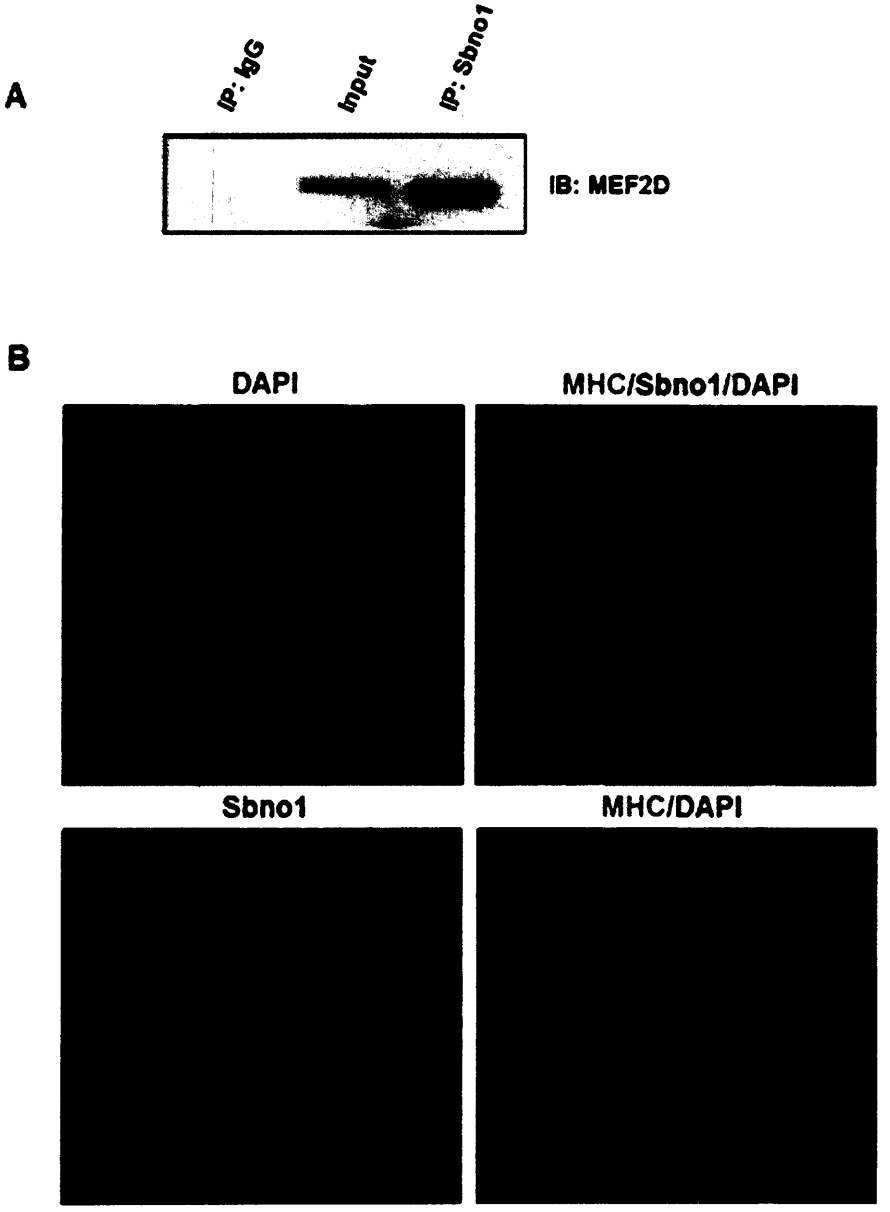

Figure 39. Physical Interaction between endogenous Sbno1 and MEF2.
(A) Endogenous co-immunoprecipitation analysis was performed using C2C12 cells at proliferative myoblast and differentiated myotubes stages (myoblasts were grown to confluence then switched to DM for a period of 120hrs). 500 μg total protein extracts was used for coimmunoprecipitations diluted with NP-40 lysis buffer. Protein complexes were immunoprecipitated (IP) with 2 μg anti-Sbno1 polyclonal antibody or 2 μg normal rabbit IgG (control), and 20 μl matrix beads

(50% slurry) ImmunoCruz (Santa Cruz) (as described in Materials and Methods). Protein complexes were resolved by SDS-PAGE and immunoblotted (IB) with MEF2D monoclonal (1:1000). **(B)** C2C12 cells were grown to confluence in GM and then switched to DM for a period of 120 hrs. Cells were fixed in 4% paraformaldehyde after 120 hrs in DM followed by double immunofluorescence analysis (as described in Materials and Methods). Localization of Sbno1 were observed in majority of mononucleated reserve cells stained with primary antibody anti-Sbno1 (rabbit) shown in red. Mature multinucleated myotubes were analyzed staining with anti-MyHC (mouse) shown in green as indicated. Nuclei were counterstained with DAPI (blue). All images were acquired from same field using a confocal laser scanning microscope.

Interestingly Sbno1 localization was mainly observed in the cytoplasm of undifferentiated cells (reserve cells) (in red) and absent in the nuclear compartment. Cells were immunostained for Sbno1 and MyHC, counterstained with DAPI allowing us to identify the nucleus of both differentiated and undifferentiated cells (Figure 39B). Together, these results suggesting that this Sbno1 may act to maintain the myogenic cells (reserve cells) in an undifferentiated proliferating state. Collectively present results further confirm that physical interaction between Sbno1-MEF2D occurs only in proliferating myoblast, not in differentiated myotubes. It is possible that Sbno1 inhibits progression of myoblasts differentiation to myotubes by inhibition of myogenic factors. As we know MEF2D is required for an efficient activation of muscle specific genes during myogenic differentiation but consequently inhibition of MEF2D by Sbno1 prevent myogenic events.

Delta1 expressing cells inhibits differentiation of C2C12 cells

Activation of Notch signaling inhibits muscle differentiation *in vitro* and *in vivo* (Wilson-Rawls et al. 1999; Conboy & Rando, 2002; Buas et al. 2009) and

previous studies suggested that Sbno1 is a downstream component of the Notch signaling (Coyle-Thompson & Banerjee, 1993; Majumdar et al. 1997) so it is possible that Sbno1 may be involved in muscle differentiation through regulation of MEF2. Therefore, we sought to address two questions: (1) whether Sbno1 constitute as an important downstream effector of Notch signaling in skeletal muscle, (2) the role of Notch-Sbno1in regulation of myoblast differentiation. Activation of Notch signaling is initiated by cell-cell contact, the Notch receptors and their ligands are both cell surface molecules and Notch ligands can modulate cell fate through activation of a Notch receptor. Therefore to address these questions, we utilized a well-characterized co-culture method to examine the effect of Notch signals delivered by OP9-Delta cells, a stable cell line expressing notch ligands (Delta1) (Lehar et al 2005), on C2C12 myoblasts cells (expressing endogenous Notch receptors) (Luo et al. 2005). This method allowed us to monitor C2C12 differentiation either by the appearance of multinucleated myotubes or by the expression of muscle-specific genes such as myogenin/MyHC. In order to analyze the intracellular events induced by Notch activation, we first examined the ability of OP9-Delta1 to transmit Notch signals to C2C12 and determine whether Notch activation prevents myogenic differentiation, C2C12 cells were co-cultured with the control OP9 cells and OP9-Delta1 cells initially in GM and induce to differentiate for up to 120 h in DM (see material and method).

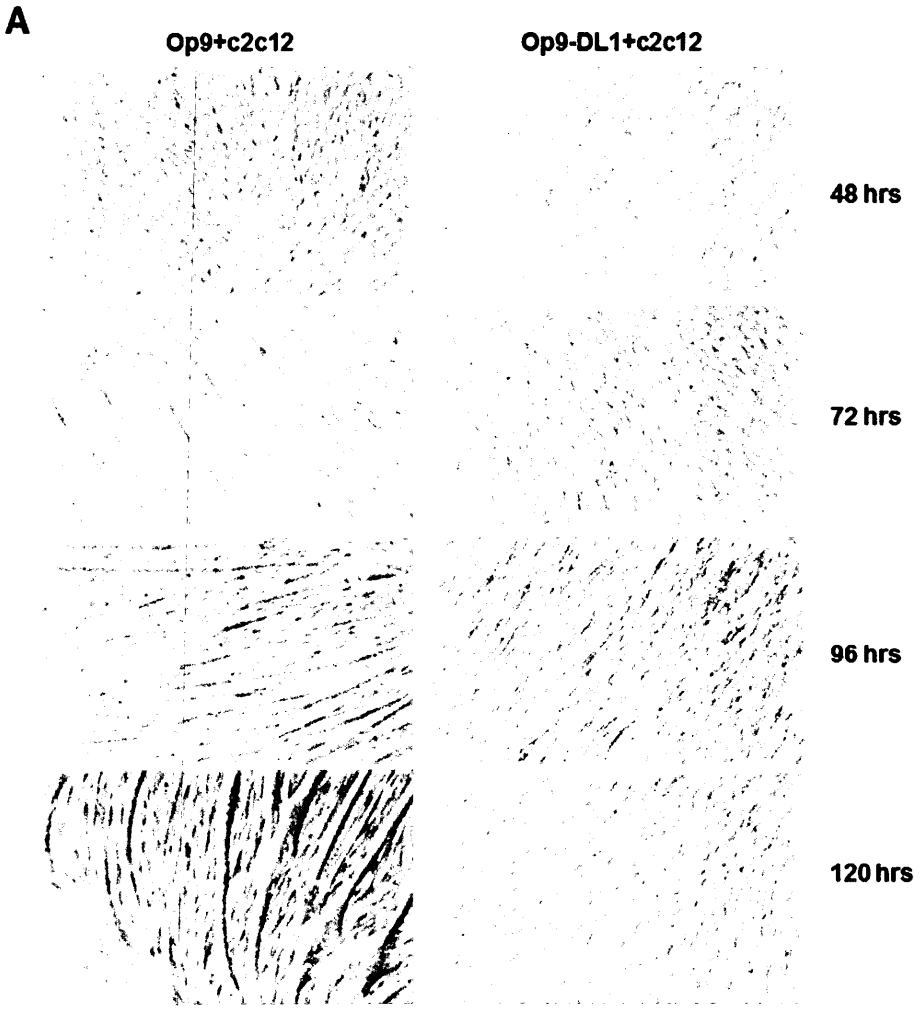

Figure 40. Delta1-expressing cells inhibit myoblasts differentiation.
Co-culture of C2C12 myoblast cells with notch ligand expressing (OP9-Delta1) cells blocks myotubes foramtion. C2C12 cells were co-cultured with the control OP9 cells and OP9-Delta1 cells initially in GM and induce to differentiate for up to 48, 72, 96, and 120 hrs in DM (as described in Materials and Methods). **(A)** Live cells images were obtained at the indicated time points using bright field phase-contrasts microscopy. C2C12 cells in the absence of notch ligand expressing (OP9-Delta1) cells differentiated normally and fused to form multinucleated mature myotubes. However in the presence of notch ligand expressing (OP9-Delta1) cells prevented myoblasts to differentiate into multinucleated myotubes.

When proliferating myoblast cells are induced to differentiate in DM they undergo myogenic conversion to form mature myotubes formation. Usually few myotubes started to appear 48 hours after the induction in DM but more were clearly seen at 72 to 120 hours indicating that myoblast cells progressed into differentiation normally and fused into multinucleated myotubes in the absence of delta-Notch signaling (Figure 40A left panel). As shown in Figure 40A right panel, C2C12 cells co-cultured with OP9-Delta1 cells prevent the myoblasts to differentiate into multinucleated myotubes, in contrast when C2C12 cells were cultured in the presence OP9 control cells. Together these results suggest that Delta1 transmit Notch signals to C2C12 myoblasts and play a role in inhibition of myoblast differentiation to become mature myotubes.

To further determine the molecular mechanism of inhibition of myoblast differentiation when C2C12 cells were co-cultured with OP9-Delta1 cells, we proceeded to examine expression of early differentiation markers MEF2 and myogenin and late differentiation marker skeletal myosin heavy chain (MyHC). Total cellular extracts were isolated from MB in GM and 48, 72, 96, and 120 hrs after induction in DM. Co-culture of C2C12 cells with Notch-ligand expressing cells completely blocked myoblast differentiation by inhibiting muscle differentiation markers. As shown in Figure 40B, expression of muscle specific differentiation markers such as myogenin, MyHC, and MCK was markedly decreased when C2C12 cells co-cultured with OP9-Delta1cells but not with control OP9 cells. The expression of Sbno1, both in C2C12 cells co-cultured with control

OP9 cells and with OP9-Delta1 cells, were considerably increased after 48 hrs of differentiation induction.

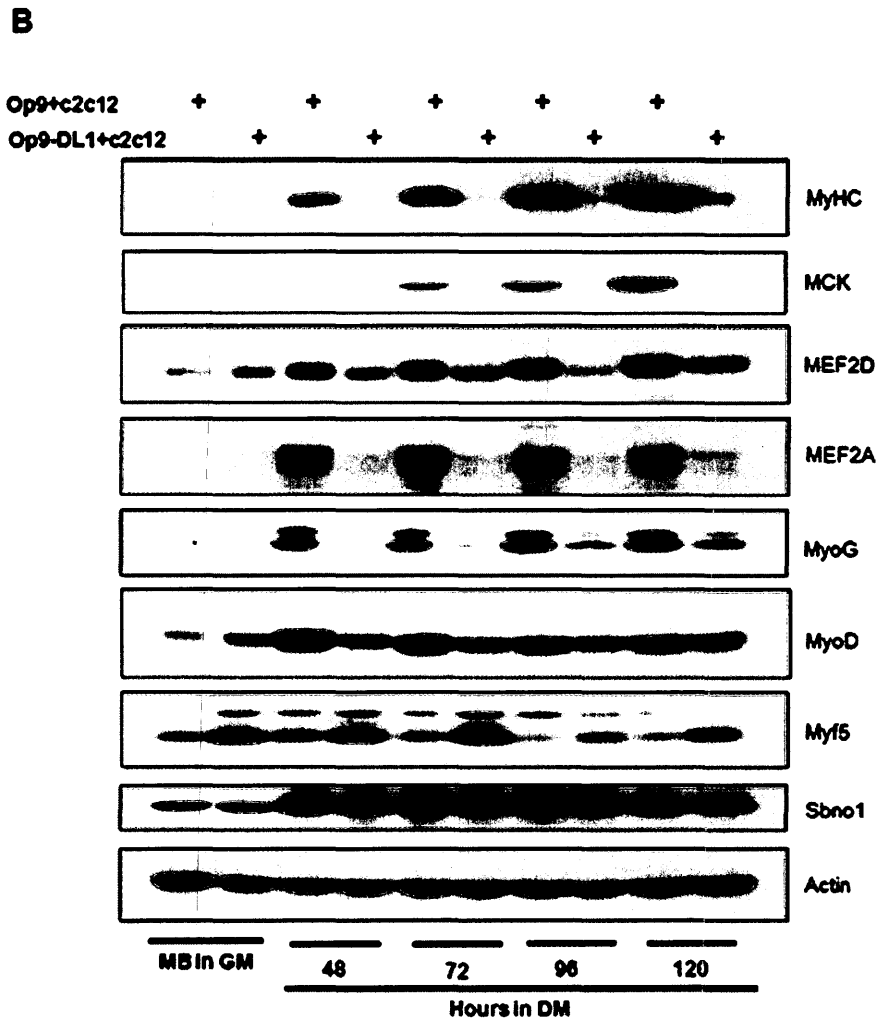

Figure 40. Delta1-expressing cells inhibit myoblasts differentiation.
(B) Total cellular extracts were isolated from MB in GM and after induction in DM at the indicated time points mentioned above. Equal amount of cell extracts were subject to Western blot analysis for detection of early and late differentiation markers. Actin was used as a control to monitor an equal loading.

Furthermore, stimulation of Notch signaling increases the expression of Myf5 before and 48 hrs after differentiation induction, consistent with previous reports, Myf5 progressively active in muscle progenitor cells/reserve cells (Figure 40B). Collectively, these results indicate that Notch signaling is activated by delta1 in C2C12 cells when co-cultured with OP9-Delta1 expressing cells. Delta1 acts as a functional ligand for Notch receptor in C2C12 cells and ligand-receptor association involves in inhibition of muscle specific genes and myogenic differentiation of C2C12 cells through activation of notch signaling and downstream effector Sbno1.

Pharmacological targeting of Notch rescue differentiation of C2C12 cells

Because Notch signaling plays an important role in muscle development, it is possible that targeting notch signaling may have opposite effects on muscle differentiation. During the cell-cell contact ligand and receptor undergoes sequential proteolytic cleavage by secretases. One of the approaches for pharmacologic targeting of the Notch signaling is γ-secretase inhibitor, which blocks the proteolytic cleavage and subsequent activation of the Notch receptor and release of intracellular domain of Notch (NICD)/active notch. Therefore, to explore whether OP9-Delta1expressing cells transmit the signal to the neighboring cells which directly involve in muscle specific differentiation genes suppression, we used γ-secretase inhibitor in co-culture experiment to monitor phenotypic effects of γ-secretase inhibitor and rescue of myotubes formations by immunostaining experiments that detect expression of myosin heavy-chain (MyHC), a terminal myogenic differentiation marker (Figure 41A).

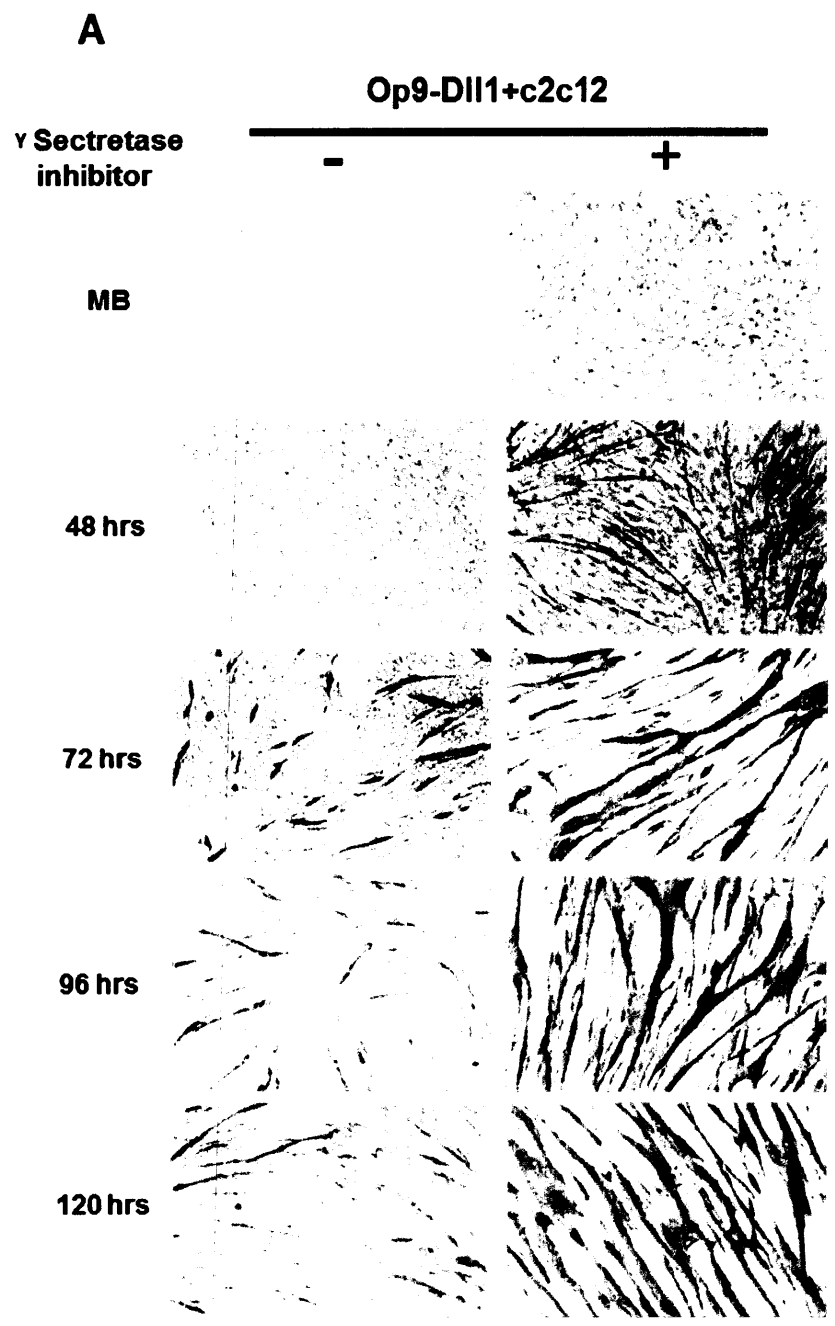

Figure 41. Inhibition of notch signaling triggers myoblasts differentiation.
(A) Inhibition of notch activity by treatment of γ-secretase inhibitor, enhanced myoblasts differentiation by preventing proteolytic cleavage of notch receptor and release of intracellular notch domain. C2C12 were co-cultured with notch ligand expressing cells (OP9-Delta1) either in the presence or absence of γ-secretase inhibitor. Cells were grown initially in GM and allowed to differentiate in DM for

up to 48, 72, 96, and 120 hrs. **(A)** Cells were fixed at the indicated time points followed by immunocytostaining using the anti-MyHC antibody (MF20) (see Materials and Methods for details). MyHC expressing myotubes (shown in brown staining) can be detected in co-cultures treated with γ-secretase inhibitor.

C2C12 cells were initially co-cultured with the control OP9 cells and OP9-Delta1 cells in GM and induce to differentiate for up to 120 h in DM (see material and method). We found that γ-secretase inhibitor blocked notch signaling and rescued myoblast differentiation that was observed by considerably increased multinucleated myotubes formation and accumulation of MyHC expression from 48 hrs to 120 hrs (Figure 41A, right panel) in contrast without γ-secretase inhibitor (Figure 41A, left panel). We next determined whether γ-secretase inhibitor could able to rescue endogenous expression of muscle specific differentiation markers, C2C12 myoblasts were induced to undergo myogenic differentiation in the presence and absence of γ-secretase inhibitor. Total cellular extracts were isolated from MB and 48hrs, 72hrs, 96hrs and 120 hrs after differentiation induction and subsequently analyzed by Western blotting. Expression of muscle specific differentiation markers was highly increased with γ-secretase inhibitor treatment (Figure 41B) in contrast without inhibitor. Whereas expression of Sbno1 decreases in the presence of γ-secretase inhibitor throughout myogenic differentiation suggesting that Sbno1 may act as downstream effector or Notch target gene in myogenic cells.

To obtain insight into whether the mechanism leading to myogenic inhibition was through Sbno1-Notch singling, transcriptional reporter gene assays were performed using a luciferase reporter driven by the MCK promoter (MCK-Luc) (Figure 41).

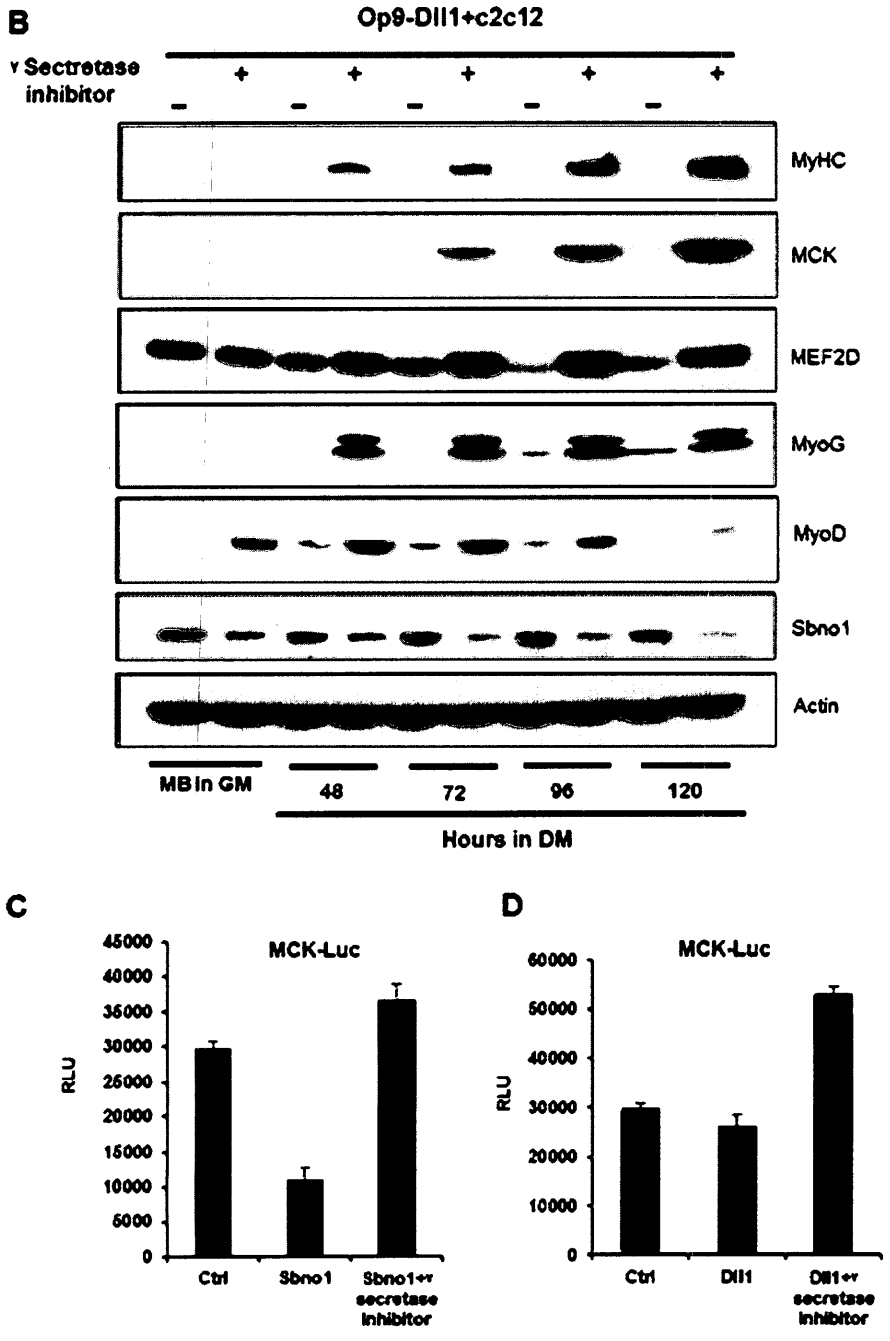

Figure 41. Inhibition of notch signaling triggers myoblasts differentiation.
(B) Western blot analysis of early and late differentiation markers from co-culture treated with or without γ-secretase inhibitor. Cells extract were prepared for the indicated time points under differentiation inducing conditions (see Materials and Methods for details). Equal amount of total protein were separated by 10%SDS-PAGE followed by detection of early and late differentiation markers as indicated

in figure 8B. Acitn was used as a loading control. **(C & D)** γ-secretase inhibitor rescued MCK promoter activity in myogenic cells. C2C12 cells were transiently co-transfected with pMCK-Luc reporter gene, pCMV-β-Galactosidase to normalize transfection efficiencies and with Sbno1/Dll1 or empty vector in combination with or without (1μM) γ-secretase inhibitor (as indicated). Luciferase and pCMV-β-galactosidase activities were measured 48 h after cells induce to differentiate in DM as described in Material and Methods (Data are the mean +/- S.E.M n=3).

C2C12 cells were co-transfected with Sbno1 and MCK-Luc reporter. Transfected cells were cultured with or without pharmacological inhibitor of Notch, γ-secretase inhibitor in differentiation medium for 48 h before harvesting and measuring luciferase activities. Reporter gene analysis demonstrated a reduction of MCK reporter activity in Sbno1 transfected cells which was reversed by γ-secretase inhibitor (Figure 41C). Similar transcriptional assay was performed co-transfected with Delta-1 (Dll1) using the MCK promoter luciferase reporter (MCK-luc). We found that γ-secretase inhibitor blocked Dll1 effects and rescued MCK reporter gene activity in C2C12 cells (Figure 41D). Taken together these results indicate that modulation of Notch signaling can alter myogenic program through activation of ligand-receptor and their association induces downstream effector, Sbno1. Inhibition of MEF2 through Sbno1 causes reduction in muscle gene activation, providing evidence that Sbno1-MEF2 association play a crucial role in muscle development. Together these results strongly suggest a potential role of Sbno1 as a negative regulator of myogenic differentiation.

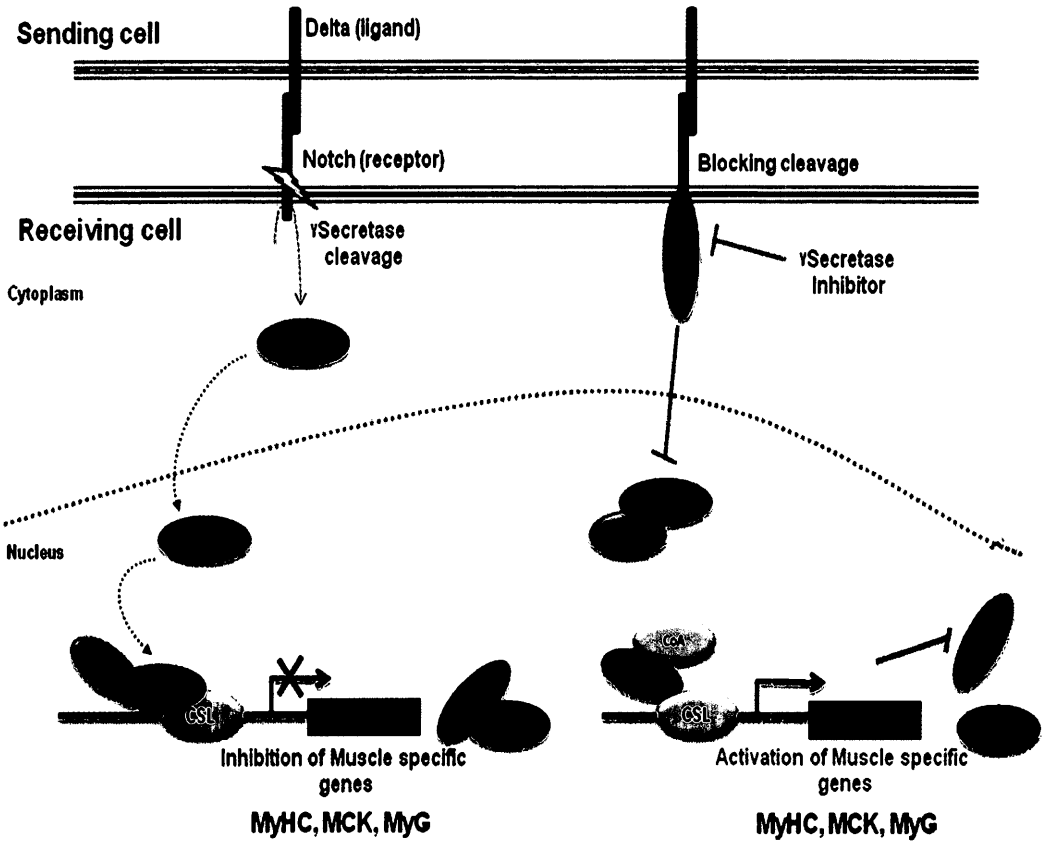

Figure 42. Proposed model for the Notch-Sbno1 signaling pathway.

Discussion

The Myocyte enhancer factor 2 (MEF2) family of transcription factor is potential target of multiple signaling pathways and number of cofactors that regulates specific gene expression in diverse developmental programs. MEF2 is emerged as a key factor in controlling the differentiation, proliferation, and survival in various cell types including muscle, and neurons (Perry et al. 2009; Salma & McDermott, 2012). In the present study, we revealed a novel interacting partner of MEF2 known as Strawberry notch 1 (Sbno1). We have provided evidence that Sbno1 acts as a negative regulator of MEF2 in myogenic cells. Ectopic expression of Sbno1 represses MEF2 mediated transcriptional activity and inhibits myogenic differentiation. Interestingly, a recent report found a synergism between MEF2 and Notch in *Drosophila* (Pallavi et al. 2012). Sbno1 was originally identified in Drosophila, encodes a conserved nuclear protein that functions downstream of Notch signaling and involve in wing development (Coyle-Thompson & Banerjee, 1993; Majumdar et al. 1997; Nagel et al. 2001). Currently, there is no evidence that Sbno1 is regulated by activation of notch signaling. However, previous studies reported that Notch signaling pathway is critical for satellite cell activation and myogenic precursor cell expansion in postnatal myogenesis (Conboy & Rando, 2002). The control of skeletal muscle differentiation is regulated by the muscle specific factors MEF2 and cofactors during transcription and the activation of the myogenic program. Previously, it was shown that notch signaling and notch mediated target genes inhibit myogenesis by targeting myogenic factors such as MEF2 and MyoD (Kopan et al. 1994; Wilson-Rawls et al. 1999). Here, our study

strongly suggests that terminal skeletal muscle differentiation is linked to MEF2-Sbno1 interaction which leads to inhibition of MEF2-dependent myogenic gene expression.

Differentiation of C2C12 myoblasts is a multistep process that requires a coordinated sequence of molecular events, involving an initial withdrawal of cells from the cell cycle, followed by expression of terminal differentiation genes and subsequent fusion of cells into multinuclear myotubes. It was shown previously that activation of Notch signaling inhibits myogenesis of cultured C2C12 cells and blocked terminal differentiation events (Kopan et al. 1994; Lindsell et al. 1995; Shawber et al.1996; Nofziger et al. 1999; Kuroda et al. 1999). Notch signaling is also able to block activation of postnatal myogenic differentiation in muscle specific stem cells (Luo et al. 2005). Ectopic expression of notch ligand *Dll1* in the limb bud of chick embryos inhibited muscle precursor cells differentiation (Delfini et al. 2000). These studies suggested that there may be multiple molecular mechanisms involve in regulation of muscle differentiation process. A previous study demonstrated that high levels of notch ligand *Dll1* suppress MyoD in cultured C2C12 myoblasts and NICD can directly bind the muscle differentiation factor MEF2C (Kuroda et al. 1999). Activation of Notch signaling blocks its DNA-binding site that lead to impaired transcriptional activity of MEF2C which might cause inhibition of myogenic process (Kopan et al. 1994; Shawber et al. 1999; Wilson-Rawls et al. 1999; Gagan et al. 2012). Notch-induced trans-activation of DNA-binding protein RBP-J directly regulates the transcription of *Hes1*, Notch target gene, which in turn blocked the expression of the muscle determining gene

MyoD (Kuroda et al. 1999). However, experiments with mutant Notch receptors lacking RBP-J binding site demonstrated that Notch signals are able to inhibit myogenic differentiation in the absence of activated RBP-J (Tamura et al. 1995; Delfini et al. 2000).

The process of myogenesis is essential not only for muscle development, but also for the regeneration of injured and aged muscle fibers characterized by the expression of muscle-specific genes (Karalaki et al. 2009; Taoa et al. 2010). Here, we reveal physical association between MEF2D and Sbno1 in mammalian cells. Sbno1 is highly localized in proliferating myoblasts, and diminished in mature myotubes during differentiation. Furthermore, overexpression of Sbno1 in C2C12 myoblasts inhibits muscle differentiation. MEF2 and myogenic regulatory factors (MRFs) including MyoD, Myf5, myogenin, and MRF cooperate together during the regulation of myogenic expression program (Black and Olson 1998; Berkes & Tapscott, 2005). Together, MEF2 and MRFs proteins regulate the differentiation of myoblasts into multinucleated myotubes by activating muscle-specific genes. Our study suggests that MEF2-Sbno1 interactions with respect to MEF2-MRFs cooperation inhibited by Sbno1 during myogenic differentiation. The role of Sbno1 may be linked with the notch signaling directly/indirectly in muscle differentiation inhibition (Buas et al. 2009 & 2010).

Skeletal muscle development and regeneration in vertebrates requires a balance between myogenic differentiation and the maintenance of progenitor cells (Conboy & Rando, 2002). The role of Notch signaling is to sustain the balance between differentiation and the maintenance of undifferentiated cells or reserve

cells (Conboy et al. 2003; Morgan & Partridge, 2003). Current studies also confirm above findings by establishing that there might be a connection between Sbno1 and notch signaling in sustaining the balance between myogenic differentiation and the maintenance of undifferentiated cell (reserve cells) population *in vitro*. A population of reserve cells is usually maintained aside and later these cells are the primary source of stem cells of post-natal skeletal muscle (Mourikis et al. 2012). These cells are essential for the growth and regeneration of muscles (Conboy & Rando, 2002; Rios et al. 2011; Gude & Sussman, 2012). Some mechanisms by which the Notch signalling pathway maintains activated reserve cells in an undifferentiated state have been documented previously. The NICD interacts with MyoD and Myf5 in the nucleus as an active repressor (Kopan et al. 1994). In the limb development, Delta-1 and Serrate-2 activate the Notch pathway and inhibit muscle differentiation through Pax3, Myf5, and MyoD (Delfini et al. 2000). Ectopic expression of NICD promoted proliferation of satellite cells and attenuated the myogenic differentiation, i.e., upregulated Pax3 and downregulated MyoD and desmin (Conboy & Rando, 2002). Conversely overexpression of Numb, suppressor of Notch signaling upregulated the expression of muscle differentiation genes and reduced proliferation of satellite cells ex vivo. Here we provide the evidence that Dll1, a Notch ligand, play a critical role in modulation of Notch signaling that leads to control myogenic events by maintenance of the reserve cells and differentiation. The induction of Sbno1 was observed in cell-cell contact due to ligand/receptor connection, when C2C12 cells were co-cultured with ligand expressing cells (OP9-Delta 1) then inhibition of myogenesis was observed. Pharmacologic targeting of

Notch pathway by blocking proteolytic cleavage of notch using γ-secretase inhibitor relieved repressive effects of Sbno1. In this study, we also showed that the ligand-induced Notch signaling activates the Sbno1, a notch downstream effector, leading to inhibition of MEF2 mediated myogenic differentiation by an increased expression of Sbno1. We also provided evidence that overexpression of Sbno1 in C2C12 myoblasts results in impaired expression of muscle specific genes such as myogenin, MyoD, and MyHC.

Our studies also suggest that Notch-Sbno1 signaling may play a dual role in maintaining muscle progenitors during skeletal muscle development by suppressing myogenic differentiation. The mechanistic links between Notch signaling and the proliferation and differentiation of skeletal muscle cells are apparently governed by more than one mechanism. Finally present data illustrated that Sbno1-Notch signaling may maintain the reserve cells population by promoting their self-renewal and inhibit their differentiation. The decreased proliferation and increased differentiation of C2C12 cells, upon treatment of gamma secretase inhibitor, may be due to downregulation of Sbno1, the Notch downstream effector. Therefore, it may be suggested that Sbno1-Notch signaling play a critical role in the proliferation and differentiation of C2C12. Over-expression of activated Sbno1 in myoblasts results in inhibition of differentiation into fusion-competent myoblasts. In addition, activation of the Notch-Sbno1 signaling inhibits the myogenic factors such as MEF2D, MyoD and myogenin. Furthermore, Sbno1 directly inhibits the transcriptional activity of MEF2.

Future work is still in progress to investigate the functional role of Sbno1 in cardiac and neurogenic cells. Beyond doubt this study may help to understand the role of Sbno1 in cardiac and neurogenic cells and also provide novel insight of Notch signaling pathway. Most importantly, a better understanding of MEF2 interacting partner that, possibly, involve in the development of mammalian tissues through MEF2 regulation.

Chapter V

Krüppel-like factor 6 (KLF6) promotes cell proliferation in skeletal myoblasts in response to TGFβ/Smad3 signaling

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Experimental design and drafting manuscript

Mathew G Dionyssiou, Jahan Salma and Dr. John C McDermott

Experiments conducted by

Dionyssiou, M.G (Figure 43C; Fig 45B & C; Fig 46A, B, C, & D)

Jahan Salma (Figure 43A & B; 45A)

Maria Bevzyuk (Figure 45B & C; Fig 46A, B, C)

Lucine Zakharyan (Figure 45B & C)

Stephanie Wales (Figure 44A & B)

Rationale:

Our previous study documented KLF6, as a novel MEF2D target gene, involved in hippocampal neuronal survival. MEF2D is a key transcriptional regulator of muscle differentiation. Furthermore, TGFβ is a potent inhibitor of myogenic differentiation by maintaining myoblasts in a proliferative state (undifferentiated myoblasts). Previous reports indicated that TGFβ and KLF6 regulate each other's expression in other cell types, we therefore sought to investigate the possible role of KLF6 in a myogenic context and assessed whether TGFβ activation regulated KLF6 protein expression and function in C2C12 myoblasts.

Krüppel-like factor 6 (KLF6) promotes cell proliferation in skeletal myoblasts in response to TGFβ/Smad3 signaling

Dionyssiou, M [1,2,3,4]. Salma, J [1,2,3,4], Bevzyuk, M [1]., Wales, S [1,3,4], Zakharyan, L [1], and John C. McDermott [*1,2,3,4]

Author affiliation:

[1]Department of Biology, York University, 4700 Keele Street, Toronto, Ontario Canada M3J 1P3

[2]Centre for Research in Mass Spectrometry (CRMS), York University, 4700 Keele Street, Toronto, Ontario Canada M3J 1P3

[3]Muscle Health Research Centre (MHRC), York University, 4700 Keele Street, Toronto, Ontario Canada M3J 1P3

[4]Centre for Research in Biomolecular Interactions (CRBI), York University, 4700 Keele Street, Toronto, Ontario Canada M3J 1P3

*Corresponding author:
J.C. McDermott, Department of Biology, 327 Farquharson, York University, 4700 Keele Street, Toronto, Ontario, Canada. M3J 1P3
E. mail: jmcderm@yorku.ca
Phone: (416)-736-2100 ext.30389, Fax: (416)-736-5698,

Key Words: Myoblasts; Krüppel-like factor 6; Transforming Growth Factor β; Cell proliferation

Abstract

Krüppel-like factor 6 (KLF6) is a key MEF2D target gene involved in neuronal cell survival. Since MEF2D also fulfills a crucial role in skeletal myogenesis, we wanted to identify whether KLF6 also functions in a myogenic context. MEF2D and KLF6 are co-localized in the nucleus of myogenic cells suggesting that KLF6 is a MEF2 target gene in these cells which was confirmed in reporter gene assays using the KLF6 promoter. In non-myogenic cells TGFβ and KLF6 have been shown to regulate each other's expression and, in view of TGFβ's potent effect on survival and proliferation of myoblasts, we assessed whether TGFβ activation regulated KLF6 protein expression and function in C2C12 myoblasts. Indeed, TGFβ potently enhanced KLF6 protein levels and this effect was repressed by pharmacological inhibition of Smad3. Mutation analysis revealed that activation of the KLF6 promoter by TGFβ was dependent on a MEF2 *cis* element. Interestingly, pharmacological inhibition of MEK/ERK(1/2) signaling resulted in re-activation of the differentiation program in myoblasts treated with TGFβ, which is ordinarily repressed by TGFβ treatment. Conversely, MEK/ERK (1/2) inhibition had no effect on TGFβ induced KLF6 expression whereas Smad3 inhibition negated this effect, together supporting the existence of two separable "arms" of TGFβ signaling in myogenic cells. Loss of function analysis using siRNA mediated KLF6 depletion resulted in enhanced myogenic differentiation whereas TGFβ induced myoblast proliferation was reduced in KLF6 depleted cells. Collectively these data implicate KLF6 in myoblast proliferation and survival in response to TGFβ with

consequences for our understanding of muscle development and a variety of muscle pathologies.

Background

KLF6 is a member of the Krüppel-like Factors (KLF) gene family which are a group of transcription factors that contain three highly conserved Cys_2-His_2 type zinc fingers invariably located in the C-terminus (Schuh et al. 1986; Kaczynski et al. 2003). Subsequently, these proteins regulate a vast range of target genes by preferentially binding to cognate GC-boxes or CACCC elements. KLF6 was originally identified due to its ability to regulate TATA-less gene promoters that can regulate glycoproteins in placental cells (Koritschoner et al, 1997) Since then, KLF6 has been found to be expressed in most tissues including neuronal, hindgut, heart and limb buds (Matsumoto et al. 2006) and is localized in the nucleus (Shields & Yang, 1997). Interestingly, homozygous null *KLF6* mice resulted in failure in the development of the liver and yolk sac vasculature, resulting in early lethality at (E) 12.5 (Matsumoto et al. 2006). To date, the most well established target gene of KLF6 is TGFβ and its receptors (Kojima et al. 2000) and subsequent studies have shown a positive feedback loop by which TGFβ activation enhances KLF6 *trans*activation properties through the formation of a Smad3-Sp1-KLF6 protein complex (Botella et al. 2009). TGFβ and KLF6 cooperatively regulate a wide range of cellular processes such as cell differentiation, proliferation and epithelial-to-mesenchymal transitions (EMT) (Haldar et al. 2000; Holian et al. 2008). Recently KLF6 was identified as a MEF2 target gene that is involved in neuronal cell

survival (Salma & McDermott, 2012). Since TGFβ and MEF2 are two key regulators of skeletal myogenesis and since KLF6 was identified in the myogenic transcriptome (Blais et al. 2005), we wanted to investigate the role of KLF6 in skeletal muscle cells.

Regulation of skeletal myogenesis is a complex process. Initially paracrine factors instigate the migration of designated myotome progenitor cells to the dermomyotome region of the somite. These proliferating cells grow and divide until cell contact triggers differential gene expression and activation of the myocyte enhancer factor 2 (MEF2) proteins and muscle regulatory factors (MRFs). This cascade of events causes morphological changes in the progenitor cells that allow them to align and fuse to form multinucleated myotubes that can eventually spontaneously contract as functional muscle fibers. Transforming growth factor β (TGFβ) antagonizes this process by preventing cells from exiting the cell cycle hence maintaining myoblasts in a proliferative state. TGFβ ligands bind to a type II receptor which becomes activated and autophosphorylated (Luo & Lodish,1997). The activated type II receptor can then phosphorylate and activate a type I receptor which in turn phosphorylates receptor mediated Smads(2/3) enabling them to dimerize with Smad4 and translocate into the nucleus where they can bind to other transcription factors and DNA to repress essential muscle genes and the expression of their downstream targets (Liu et al. 2001; Kollias & McDermott, 2008). In addition, TGFβ also regulates the mitogen-activated protein kinase (MAPK) pathway, which involves a cascade of protein kinases (MAPKKK, MAPKK, MAPK) which become activated in sequence by G-proteins in response to TGFβ

binding its receptors (Pelicci et al. 1992; Yue & Mulder, 2000; Derynck & Zhang, 2003). Upon TGFβ activation, MEK1/2 (MAPKK) can phosphorylate and activate ERK1/2 MAPK at conserved TEY sites, causing it to translocate into the nucleus to regulate gene expression. These two TGFβ regulated pathways converge to inhibit the function of MEF2 and hence muscle specific genes (Liu et al. 2004) and, ultimately result in cell proliferation (Liu et al. 2001; Jungert et al. 2006).

Not surprisingly inhibition of either or both of these pathways, (either pharmacologically or through ectopically expressed Smad7, which can antagonize the canonical Smad-pathway), enhances myotube formation (Kollias et al. 2006; Miyake et al. 2010). Cross-talk between these pathways is further supported by Smad7 antagonizing the repressive effects of MEK1 on MyoD (Perry et al. 2001 Miyake et al. 2010).

In this report, our goal was to assess the role of KLF6 in myogenic cells based on its regulation by both MEF2D and TGFβ. We report that TGFβ up-regulates KLF6 specifically through a Smad3-dependent pathway which enhances proliferation in myoblasts. In addition, we observed that (i) TGFβ enhanced KLF6 promoter activation in a MEF2 site dependent manner and, (ii) that TGFβ recruited MEF2 to the KLF6 promoter region. TGFβ induction coupled with pharmacological inhibition of Smad3 repressed KLF6 expression and cell proliferation but, importantly did not re-activate the differentiation program which is potently repressed by TGFβ signaling. Conversely, TGFβ treatment coupled with pharmacological inhibition of MEK1/2, enhanced myotube formation but had no effect on KLF6 expression and function. Loss of function assays using siRNA for

KLF6 revealed that KLF6 is required for cell proliferation. These experiments tease apart two independent functions of TGFβ signaling in myogenic cells. One is a repressive effect on differentiation which is mediated by ERK activation; the other being an enhancement of proliferation which is dependent on Smad3 and KLF6.

Methods

Plasmids

Expression plasmids for pcDNA3-MEF2D, pCMV β-galactosidase (Du et al. 2008; Perry et al. 2009) and, reporter gene constructs for 3TP-lux (Wrana et al. 1992) MCK-Luc (Donoviel et al. 1996) MEF2-Luc (Quinn et al. 2001) pROM6 ΔMEF2 (Salma & McDermott, 2012) have been previously described. KLF6 reporter constructs pRMO6 were generously provided by Dr. Nicolas P. Koritschoner (Faculty of Bioquimica y Ciencias Biologicas, Universidad Nacional del Litoral, Santa Fe, Argentina).

Antibodies

Anti-MEF2A rabbit polyclonal, anti-Myosin heavy chain mouse monoclonal and anti-Myogenin mouse monoclonal antibodies were produced with the assistance of the York University Animal Care Facility; anti-MEF2D (1:1000; BD Biosciences); Smad3, phospho-Smad3 and phospho-ERK1/2 (1:1000; Cell Signaling); Klf6, actin, ERK1/2 (1:1000; SantaCruz) were used for immunoblotting experiments. IgGs were also purchased from Santacruz Biotechnologies.

Cell culture, transfections and drug treatments

C2C12 cells were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone), 1% L-glutamine and 1% penicillin-streptomycin. Cells were maintained in a humidified, 37°C incubator with a 5% CO_2 atmosphere. For transfections, cells were seeded on pre-gelatin coated plates 1 day prior to transfection and transfected according to the standard calcium phosphate method previously described by Perry *et al.*, 2001. A mixture of 50µl 2.5M $CaCl_2$ per 25µg DNA with an equal volume of 2x HeBS (2.8M NaCl, 15mM Na_2HPO_4, 50mM HEPES, pH=7.15) was used and the cells were and incubated overnight followed by washing and addition of fresh media. Drugs treatments were used at the following concentrations: 2ng/ml TGFβ, 5µM Sis3 and 10µM U0126 as indicated.

siRNA gene silencing

Small interfering RNAs (siRNA) targeting Klf6, MEF2D and non-specific scramble RNA were purchased from Sigma. Transient transfections were performed using TurboFect Transfection Reagent (#R0531, Fermentas) according to the manufacturer's instructions. TurboFect (Fermentas): A 1:2 mixture ratio of DNA to turboFect reagent (including 4ng/ml siRNA) in 200µl serum-free DMEM was prepared for 19 hours incubation.

Immunocytochemistry

C2C12 cells were treated as previously described by Salma and McDermott, 2012 [14] and, incubated overnight with at 4°C with primary MEF2D and Klf6

antibodies (1:100) diluted in 1.5% goat serum. Cells were washed 3X with PBS for 10min and incubated with the appropriate TRITC/FITC-conjugated secondary antibodies (1:500) in 1.5% goat serum (PBS) for 2h at RT following DAPI (4',6-diaminidino-2-phenylindole) staining for 15min at RT. Cells were washed 3X with PBS and cover slips were mounted with DAKO mounting media (Dako) on glass slides. The fluorescence images were captured using Fluoview 300 (Olympus).

Protein extractions, immunoblotting and reporter gene assays

Cells were harvested using an NP-40 lysis buffer (0.5% NP-40, 50mM Tris-HCl [pH 8.0], 150mM NaCl, 10mM sodium pyrophosphate, 1mM EDTA [pH 8.0], 0.1M NaF) containing 10μg/ml leupetin and aprotinin, 5μg/ml pepstatin A, 0.2mM phenylmethylsulfonyl fluoride and 0.5mM sodium orthovanadate. Protein concentrations were determined using the Bradford method (Bio-Rad) with bovine serum albumin (BSA) as a standard. 20μg of total protein extracts were used for immunoblotting, diluted in sample buffer containing 5% β-mercaptoethanol and boiled. Transcriptional assays were done using luciferase reporter plasmids. The cells were harvested for these assays using 20mM Tris, (pH 7.4) and 0.1% Triton-X 100 and the values obtained were normalized to β-galactosidase activity expressed from a constitutive SV40 driven expression vector and represented as relative light units (RLU) or in some cases corrected Luciferase values for control, reporter alone transfections were arbitrarily set to 1.0, and fold activation values were calculated. Bars represent the mean (n=3) and error bars represent the standard error of the mean (n=3).

Coimmunoprecipitation assays

Protein extracts were prepared as described above. Immunoprecipitaion was performed using the ExactaCruz kit (SantaCruz Biotechnology), as per manufacturer's instructions. Precipitated proteins were separated by SDS PAGE and immunoblotting of proteins was performed as described above

Chromatin Immunoprecipitation (ChIP)

ChIP experiments followed the guidelines set by EZ ChIP™ (Upsate) with minor modifications. Approximately 1x 10^7 C2C12 cells were fixed with 1% formaldehyde (Sigma) for 15 minutes at 37°C. Fixing was quenched by Glycine (Bioshop) at a final concentration of 0.125M. Cells were collected in PBS containing PMSF (Sigma) and protease inhibitor cocktail (Roche). Cells were pelleted at 5000 rpm for 5 min at 4°C. Nuclei were treated with Wash Buffer I (10mM HEPES pH 6.5, 0.5M EGTA, 10mM EDTA, 0.25% Triton X-100, protease inhibitor cocktail, PMSF) for 5 minutes on ice. Nuclei were collected and resuspended in Wash Buffer II (10mM HEPES pH 6.5, 0.5 mM EGTA, 1 mM EDTA, 200 mM NaCl, protease inhibitor cocktail, PMSF) for 10 min on ice. Nuclei were again collected and then treated with lysis buffer (50mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS). Chromatin was sheared using Misonix X at 3 x 10s (power 8) to produce 500 bp fragments. Crosslinked sheared chromatin was collected following a 15 minute spin at maximum speed. 20% of total chromatin was set aside as input. Sheared crosslinked chromatin was diluted 1:10 with IP dilution buffer (0.01% SDS, 1.1% Triton-X 100, 1.2 mM EDTA, 16.7 mM Tris-HCL pH

8.1, 167 mM NaCl) and incubated with antibody overnight at 4°C with rocking. Protein G Dynabeads (Invitrogen) were blocked with 20 ug salmon sperm DNA in IP dilution buffer (15 ul beads + 135 ul IP dilution buffer + 20 ug salmon sperm DNA per IP) overnight at 4°C with rocking. 152 ul of pre-blocked beads were incubated with the IP reaction at 4°C for 1 hr. Dynabead-bound antibody:chromatin complexes were washed using IP Wash Buffer I (20 mM Tris pH 8.1, 2mM EDTA, 150 mM NaCl, 1% Triton-X 100, 0.1% SDS) and II (20 mM Tris pH 8.1, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS), each incubated for 10 minutes at 4°C, and followed with two washes in TE buffer at 4°C. Protein:DNA complexes were freed from Dynabeads through the addition of elution buffer (0.1 M NaHCO3, 1% SDS) for 30 minutes at room temperature. To separate protein from DNA samples were treated with 12 ul of 5 M NaCl (BioShop) at 65°C for 4 hours to overnight. Protein was further degraded by the addition of Proteinase K (Sigma), EDTA, Tris pH 6.5 for 1 hr at 45°C. Samples were then purified using a PCR clean up kit (Qiagen).

ChIP-qPCR

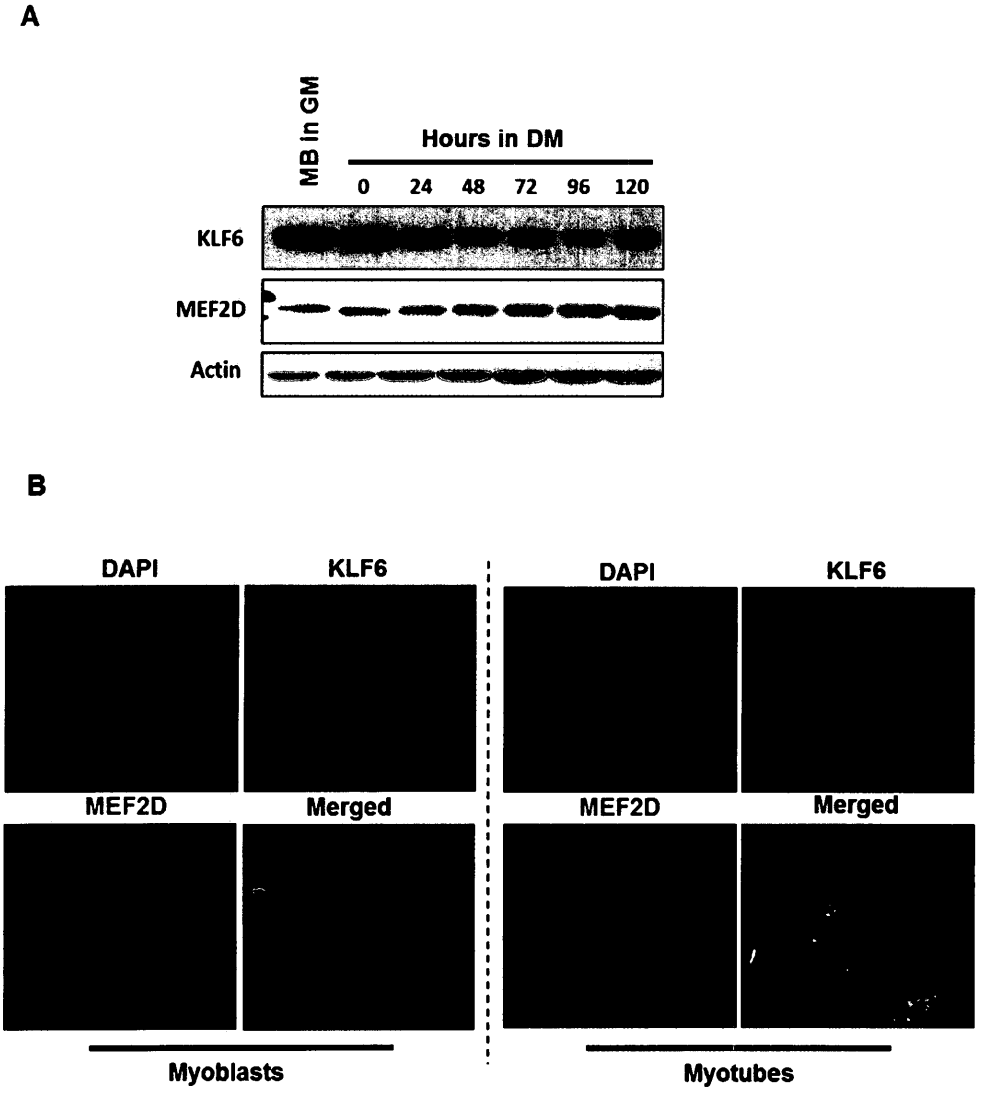
ChIP-qPCR analysis on the KLF6 promoter was done using BioRad Sybr Green as per the user manual with a final primer concentration of 0.5 uM. Antibodies used in ChIP: MEF2 (Santa Cruz, sc-313X, D1112; 5 ug), H3K9ac (Abcam, X; 2 ug). Primers flanking the ME2 site on the KLF6 promoter are: 5'-CTGCAACGTTGGGCTGTA-3' and 5'-TTGGAAAGACGTCTCACAGG-3'. Data were analyzed using percent input or fold enrichment.

Results and Discussion

MEF2D and KLF6 expression and co-localization in the nucleus, in skeletal myoblasts.

Since KLF6 was identified in the skeletal muscle transcriptome (Blais et al. 2005) and has since been shown to be a MEF2D target gene that is involved in the cell survival pathway in primary embryonal hippocampal neurons (Salma & McDermott, 2012) and, since MEF2D is also a crucial regulator of skeletal myogenesis, we wanted to investigate the role of KLF6 in skeletal myoblasts. We determined that KLF6 and MEF2D are indeed both co-expressed in C2C12 myoblasts and, co-localized in the nucleus using western blot analysis and immunocytochemistry respectively (Figures 43A and B). Endogenous expression of KLF6 is detected in C2C12 myoblasts in growth conditions and sustained upon serum withdrawal and throughout the course of myogenic differentiation up to 120h. Interestingly, we observed that KLF6 protein expression is down regulated at 48h, up regulated at 72h, down regulated at 96h and up-regulated again at 120h in a manner that is not easily explainable (Fig. 43A). Immunofluorescence labeling was conducted in order to observe the cellular localization of KLF6 with respect to MEF2D in proliferating myoblasts and then in differentiated myotubes. The data indicated strong nuclear localization of both KLF6 (red) and MEF2D (green) in conjunction with nuclear (blue) 4',6-diaminidino-2-phenylindole (DAPI) staining in myoblasts and, less so in differentiated myotubes (Fig. 43B). Since TGFβ has also been shown to regulate KLF6 expression, we tested the effect of TGFβ on previously characterized KLF6 promoter constructs (pROM6-Luc and pROM6-Luc

ΔMEF2). Serum was withdrawn 24h after transfection and treated with 2ng/ml TGFβ for 24h as indicated in the figure. The data indicates a 4-fold increase in transcriptional activity of pROM6-Luc in response to TGFβ treatment, but no effect on pROM6-Luc ΔMEF2, indicating that TGFβ regulates the KLF6 promoter through the MEF2 cis element (Figure. 43C).



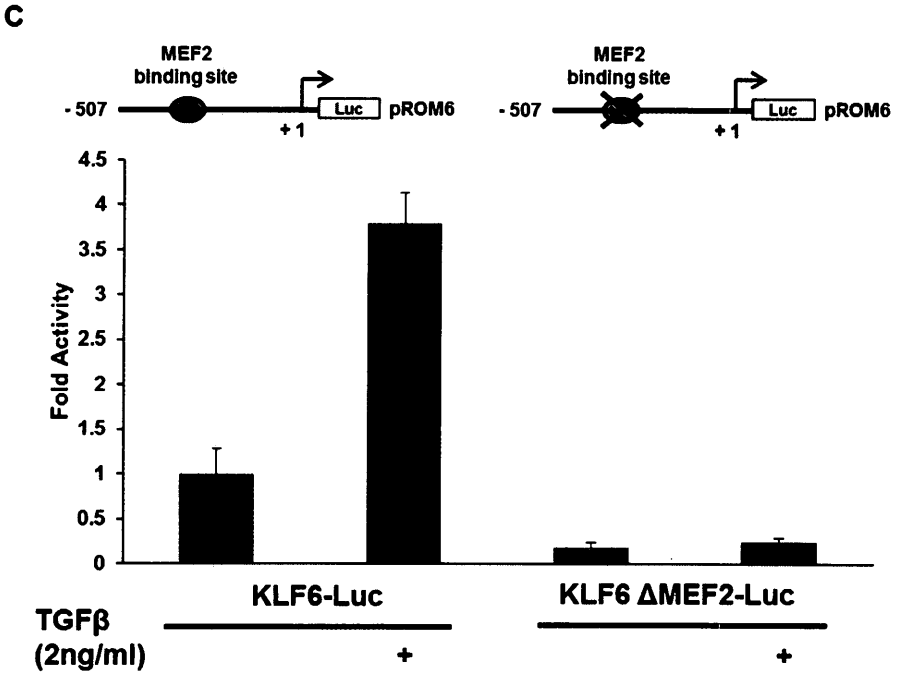


Figure 43: (A) Western blot analysis reveals that KLF6 and MEF2D are co-expressed in C2C12 myoblasts. Myoblasts were cultured in growth medium (10% serum), followed by serum withdrawal (2%) for 144h and harvested at 24h time intervals. Cells were then lysed and equal amounts of protein (20μg) were used for western blot analysis. The levels of the indicated proteins were assessed by a standard immunoblotting technique using specific primary antibodies for each. Actin was used as a loading control. **(B)** Immunocytochemistry reveals that KLF6 and MEF2D are co-localized in the nucleus at the myoblast stage but to a lesser extent in differentiated myotubes. C2C12 cells were treated as previously described by Salma and McDermott, 2012. DAPI staining was used for nuclear staining, green and red were used for MEF2D and KLF6 respectively and then merged. **(C)** TGFβ treatment potentiates KLF6 promoter region through MEF2. KLF6 promoter constructs (pROM6 Luc and pROM6 ΔMEF2 Luc) were used, and luciferase activities were analyzed upon serum withdrawal, with and without 2ng/ml TGFβ treatment as indicated

MEF2A/D expression is not required for KLF6 protein expression in skeletal myoblasts

Since we had already observed that TGFβ regulates the KLF6 promoter through MEF2 we wanted to assess the effect of MEF2A/D knock down using RNA silencing and observed an overall effect on KLF6 protein expression levels (Figure. 44A). Although siRNA2 for MEF2A appears to affect KLF6 expression slightly, this observation did not indicate a strong and consistent effect. On the other hand, siMEF2D appears to de-repress KLF6 expression. Since MEF2D is a potent HDAC4 co-factor, siMEF2D might be preventing the recruitment of HDAC4 to the promoter and hence de-repressing KLF6. Contrary to our initial hypothesis, these data indicate that MEF2 is not necessarily required for KLF6 expression, or that its requirement is only at the myoblast stage when the cells are responsive to TGFβ signaling. To further corroborate this observation, we looked at MEF2 recruitment on the KLF6 promoter with or without TGFβ treatment (Figure 44B). The data indicates that whilst MEF2 is indeed recruited to the KLF6 promoter in C2C12 myoblasts, there is no change in MEF2 recruitment upon TGFβ treatment with respect to the control, implicating a different mechanism for TGFβ activation of KLF6.

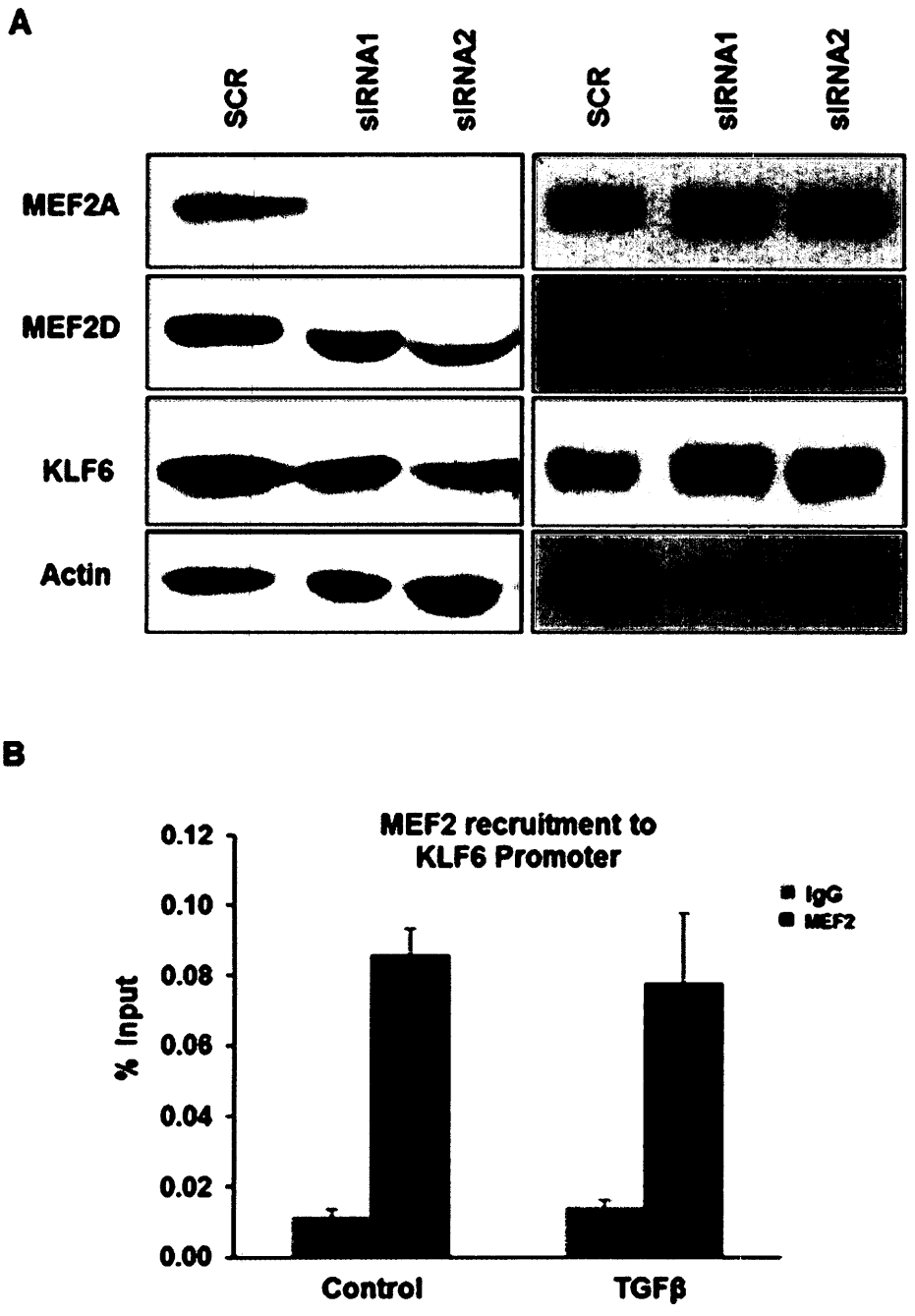


Figure 44: (A) MEF2A/D RNA silencing reveals that MEF2A/D expression is not required for endogenous KLF6 protein expression. In contrast siMEF2D appears to de-repress endogenous KLF6 protein levels. **(B)** Chromatin immunoprecipitation analysis of MEF2 recruitment onto the KLF6 promoter revealed no change upon TGFβ treatment.

TGFβ regulates KLF6 through a Smad3 specific pathway and inhibits skeletal myogenesis through MEK/ERK specific pathway

Since Smad3 is activated in proliferating myoblasts and is also regulated by TGFβ, we observed that Smad3, along with MEF2 and KLF6 are co-expressed in skeletal myoblasts (Figure. 45A). To further investigate the effect of TGFβ on KLF6 we used well documented pharmacological inhibitors of the Smad and ERK MAPK pathways. We tested the effect of TGFβ on KLF6 protein expression in C2C12 myoblasts in the presence and absence of a Smad3 inhibitor, Sis3 (Figure. 45B). The data in Fig. 45B reveal that indeed TGFβ treatment increases KLF6 protein levels and this corresponded with a decrease in myogenin as an indicator of myogenic differentiation. Interestingly, pharmacological inhibition of Smad3 with 5μM Sis3 reduced TGFβ induced KLF6 protein expression but had no effect on myogenin. This indicates that TGFβ regulates KLF6 and myogenin through two distinct pathways. Smad2/3 and phospho-Smad2/3 antibodies were used as positive controls for Sis3 treatment since Sis3 inhibits Smad3 phosphorylation and hence its translocation into the nucleus (Jinnin et al. 2006). Since TGFβ also regulates the MEK/ERK (1/2) MAPK pathway we wanted to test the effect of pharmacological inhibition of that pathway on KLF6 using 10μM U0126. The data summarized in Figure. 45C confirms that TGFβ induces KLF6 protein expression while inhibiting myotube formation (using sarcomeric myosin heavy chain expression as an indicator). In this experiment Smad3 inhibition repressed TGFβ induction of KLF6 but did not reverse the effects on MyHC (Figure. 45C). Strikingly, pharmacological inhibition of ERK1/2 had no effect on KLF6 levels but instead rescued myotube

formation and MyHC expression, hence supporting the idea that TGFβ regulates KLF6 and myogenic differentiation through Smad3 and ERK1/2 distinctively.

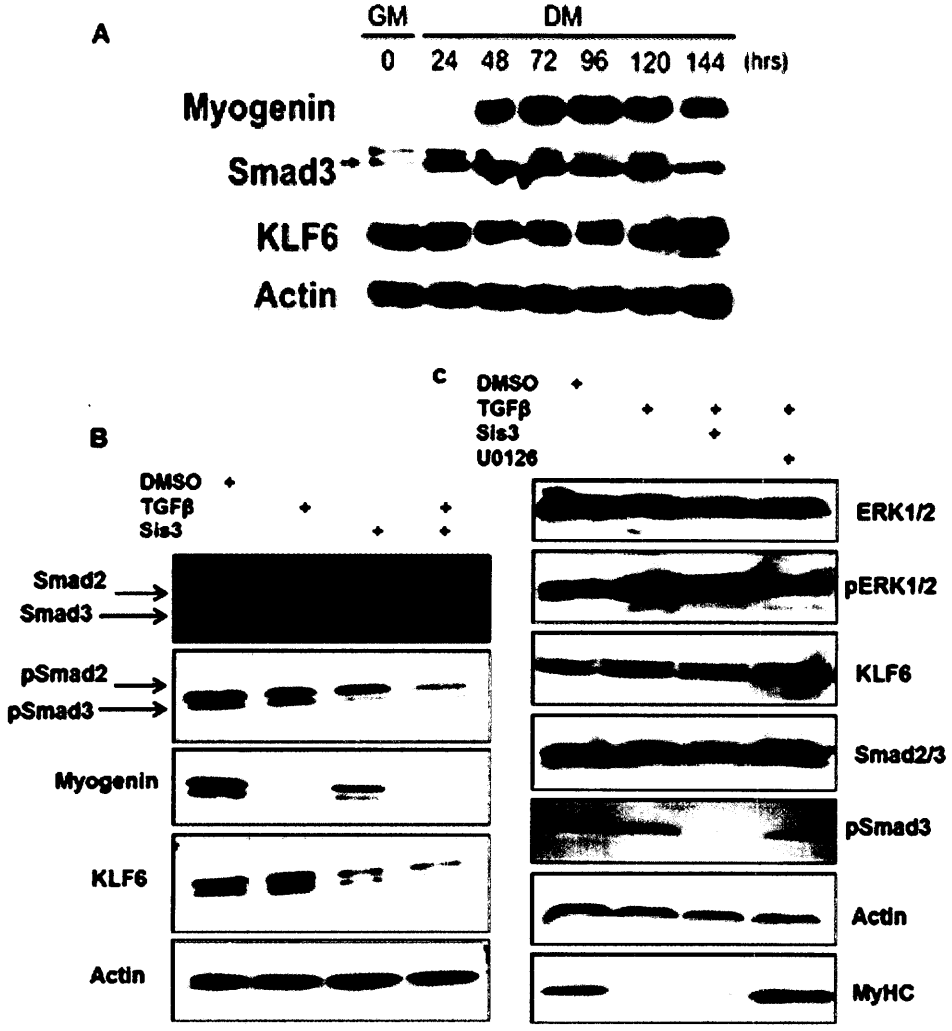


Figure 45: (A) Western blot analysis revealed that Smad3 and KLF6 are co-expressed in C2C12 myoblasts. Myogenin was used as a protein marker for differentiation and actin was used as a loading control. Pharmacological manipulation of TGFβ signaling pathway reveals that TGFβ regulates KLF6 protein expression through Smad3 but not MEK/ERK MAPK. **(B)** Western blot analysis indicates that 2ng/ml TGFβ treatment elevates KLF6 protein expression and that this effect is abrogated in the presence of 5μM specific inhibitor of Samd3, Sis3.

TGFβ treatment also inhibited myogenic differentiation marker, myogenin protein expression levels and, this effect was not abrogated by Sis3. **(C)** Western blot analysis revealed that TGFβ treatment enhances KLF6 expression through Smad3 but not ERK1/2 MAPK and that TGFβ treatment repressed myogenic differentiation through ERK1/2 MAPK but not Smad3. 10μM U0126 was used as an inhibitor of the MEK/ERK MAPK pathway, 5μM Sis3 was used for Smad3 inhibition and 2ng/ml TGFβ were all used as indicated. Actin was used as a loading control.

TGFβ induces cell proliferation in C2C12 myoblasts through KLF6

Since TGFβ represses skeletal myogenesis by retaining cells in a proliferative state, we wanted to test the effect of KLF6 mRNA silencing using siRNA mediated gene silencing. siRNA3 was chosen as the most efficient in knocking down KLF6 expression based on Fig. 46 A. Subsequent KLF6 silencing resulted in increased MyoD and myogenin protein expression (Figure. 46B; upper panel) and this corresponded with a 2.5 fold increase in muscle creatine kinase (MCK) promoter activity indicating, as predicted, that KLF6 is anti-myogenic (Figure. 46B; lower panel). Furthermore, an MTT cell proliferation assay was performed, and the data showed that a 24h, 2ng/ml TGFβ treatment doubles the number of proliferating cells (Figure. 46C). This effect is negated upon KLF6 gene silencing thus implicating KLF6 in the proliferative response to TGFβ signaling. In support of this, siKLF6 on its own reduced the number of proliferating cells indicating a functional role in proliferation of skeletal myoblasts (Figure. 46C).

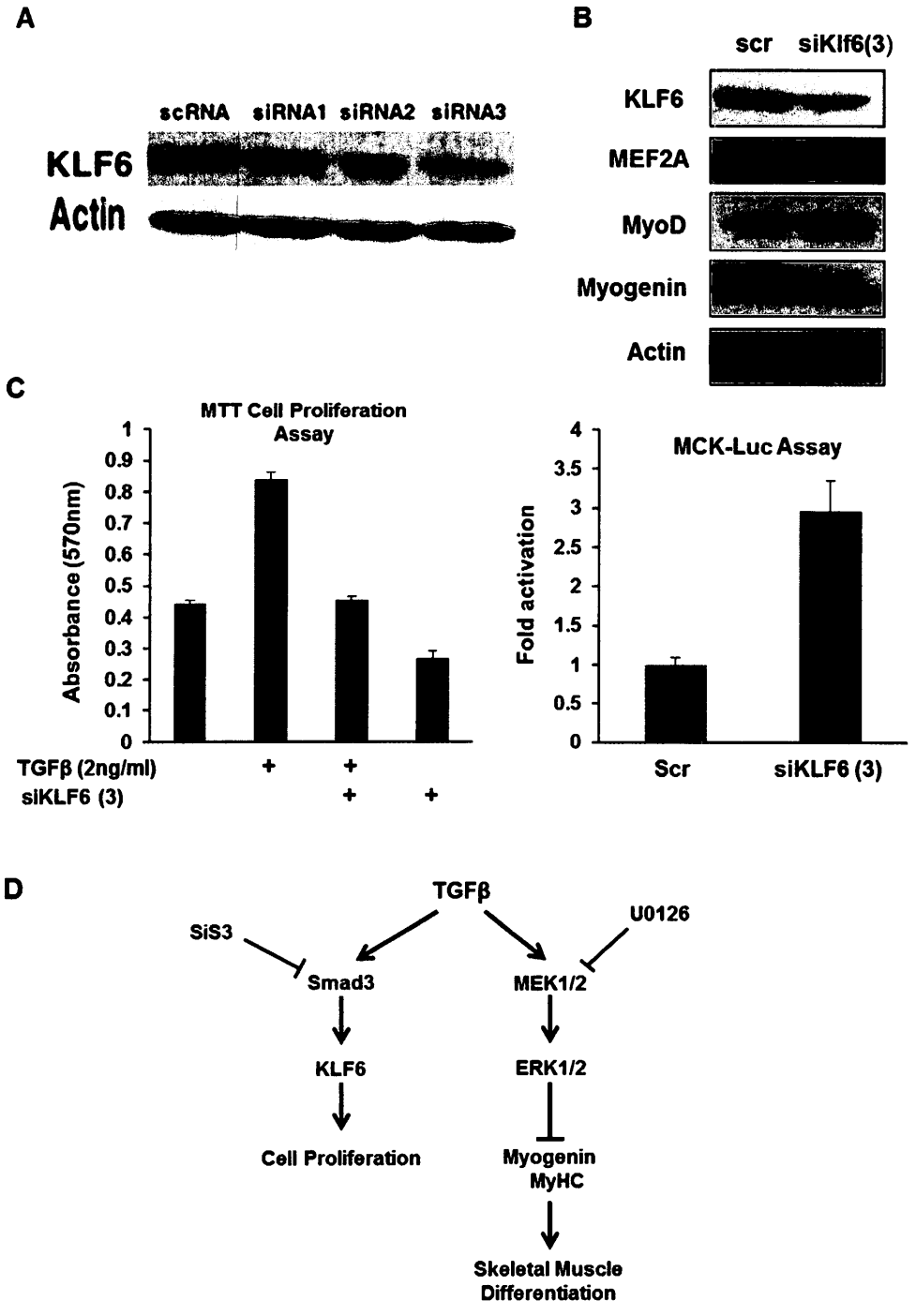


Figure 46: KLF6 RNA silencing reveals that **(A)** KLF6 protein expression was successfully repressed, particularly by siRNA3 which was used in subsequent experiments. **(B)** KLF6 RNA silencing resulted in (i) increased MyoD and myogenin protein levels, (ii) enhanced MCK Luciferase activity and, (iii) reduced

TGFβ induced cell proliferation. **(C)** Cell proliferation was measured using the MTT cell proliferation assay kit. The number of proliferating cells is directly proportional to the absorbance at 570nm. TGFβ treatment doubled the number of proliferating cells and this effect was repressed with KLF6 silencing. **(D)** A schematic summary of the data presented, in which TGFβ/ERK signaling represses myogenic differentiation while TGFβ/Smad signaling regulates KLF6 gene expression and myoblast proliferation.

Conclusions

In this study we report a novel role for KLF6 in skeletal myoblasts. Based on our data we propose that KLF6 is a downstream effector of the TGFβ/Smad3 pathway that regulates cell proliferation in skeletal myoblasts. We identify Smad3 as a key regulator of KLF6 expression, through TGFβ. In addition we were able to functionally distinguish between the TGFβ/Smad and TGFβ/MAPK pathways in that TGFβ inhibits skeletal myogenesis through the MEK/ERK (1/2) MAPK pathway and concomitantly enhances cell proliferation through Smad3 mediated induction of KLF6 expression. Our findings are summarized in figure 46 D. Many myopathies and muscle loss disorders have been linked with increased TGFβ signaling (Burks & Cohn, 2011) and hence, our findings identify KLF6 as a potential therapeutic target for such pathological conditions as well as for cancers such as embryonal rhabdomyosarcoma where TGFβ promotes cell proliferation (Bouché et al. 2000).

Chapter VI: Summary

Collectively, the goal of these studies was to further define the role of MEF2 in myogenic and neurogenic cells. MEF2s are tightly regulated via posttranslational modifications, directly targeted by phosphorylation or through association with particular co-activators/repressors. Dissecting components of key posttranslational modifications is an essential step in further understanding the complex role of MEF2 in developmental processes and phenotypes. Here our investigations highlight the complex and redundant mechanisms that regulate MEF2 proteins in myogenic and neurogenic cells, as presented in chapter III, IV, and V.

MEF2 family members (MEF2 A, C, and D) are highly expressed in neurons and exhibit distinct patterns of expression in different regions of the brain. MEF2 expression level is highest in the cerebral cortex, cerebellum and hippocampus and is required to regulate neuronal development and synaptic plasticity. In the mammalian nervous system, regulation of MEF2 is a crucial determinant of neuronal cell survival, and death. Phosphorylation by kinases is an important process through which the activity of MEF2 is up or down-regulated in various cell types. Several kinases have been linked to muscle and neuronal development in part due to their modulation of MEF2 function. In Chapter III, we demonstrated that MEF2D is localized in the nuclear compartment of primary hippocampal neurons. Previous studies from our lab and others reported that MEF2D is directly targeted by PKA (Belfield et al. 2006; Du et al. 2008). Here, we assessed whether cAMP signaling converges on the pro-survival role of MEF2D in hippocampal neurons.

We observed that experimental induction of cAMP/PKA signaling promotes apoptosis in primary hippocampal neurons as indicated by TUNEL and FACS analysis. MEF2 protects neurons from apoptotic cell death, which contrasts with its pro-apoptotic function in other cell types such as T-cells. The ability of MEF2 to regulate neuronal specific transcriptional programs occurs through activation of MEF2 survival genes. In this study we identified that cAMP-dependent protein kinase (cAMP/PKA) signaling negatively regulates MEF2D function in neuronal cells suggesting the importance of MEF2D in neuronal gene expression. Luciferase reporter gene assays revealed that PKA potently represses MEF2D *trans*-activation properties in neurons. This effect was largely reversed by engineered neutralizing mutations of PKA phospho-acceptor sites on MEF2D (S121/190A). For the first time we characterised a MEF2D target gene named Krüppel-like factor 6 (KLF6) as a critical survival gene. siRNA mediated of suppression KLF6 expression promotes neuronal cell death and antagonizes the pro-survival role of MEF2D in primary hippocampal neurons. In addition, we observed nucleo-cytoplasmic shuttling of HDAC4 in the presence of activated protein kinase A and PKA signaling prevents HDAC4 export from the nucleus resulting in suppression of MEF2 mediated gene transcription. In this study, we found that PKA signaling promotes the formation of HDAC4/MEF2 repressor complexes by increasing the levels of HDAC4 in the nuclear compartment leading to downstream repression of key MEF2 target genes involved in neuronal survival such as KLF6. Based on our current observations and experimentations, we suggest that there is a bi-partite mechanism regulating MEF2 mediated gene expression: by direct phosphorylation; and increased physical

association with HDAC4 which together contribute to PKA-mediated inhibition of MEF2 activity and numerous biological processes. Overall, this chapter demonstrated that cAMP/PKA signaling events modulate gene expression by post-translationally modifying MEF2 to control neuronal development in hippocampal neurons suggesting an important role for these proteins in the mammalian nervous system.

In chapter IV, we tested ideas concerning how MEF2 controls diverse cellular processes in muscle development in the presence or absence of cofactors. Therefore, we initiated experiments to identify MEF2 interacting proteins by using Tandem affinity purification combined with mass spectrometry analysis. This approach has proven to be a useful proteomics tool to identify novel co-factor interactions of physiological relevance. These studies identified Strawberry notch 1(Sbno1) as a novel interacting partner of MEF2D which is a previously documented downstream effecter of Notch signaling. Notch signaling regulates the balance between differentiation and maintenance of progenitor cells by inhibition of differentiation. Notch signaling is involved in blocking the expression and activity of the myogenic factors such as MEF2. Here we utilized C2C12 myoblasts which provide a useful *in vitro* model to study skeletal muscle differentiation to understand the role played by the Sbno1-MEF2 interaction. Western blot analysis indicated that exogenous Sbno1 expression inhibits expression of myogenic markers. Sbno1 also represses MEF2 trans-activation properties suggesting Sbno1 plays a role in inhibition of skeletal muscle differentiation through targeting MEF2D. Since Sbno1 is known as downstream effector of Notch signaling, we

focused on the detailed mechanism of myogenic inhibition by Notch-Sbno1 signaling. Here we report ectopic expression of Sbno1 in C2C12 myoblasts blocks terminal differentiation in myogenic cells and decreases expression of myogenic factors such as myogenin and Myosine heavy chain. Immunocytochemistry analysis revealed that Notch-Sbno1 might be involved in maintaining "reserve" population in a differentiated muscle culture. Our data indicate that protein-protein interactions between Sbno1 and MEF2D results in interference with the function of myogenic factors that repress skeletal muscle differentiation. Thus, we propose that Sbno1 serves as an important MEF2 cofactor, functioning to maintain a balance between committed myogenic differentiated cells and "reserve" progenitor population.

Lastly, Chapter V documents a role of MEF2D target gene, KLF6, in skeletal muscle development. In the previous study (chapter III) we documented that KLF6 is involved in hippocampal neuronal survival and is a MEF2 target gene. TGFβ, a potent inhibitor of myogenic differentiation, maintains myoblasts in a proliferative undifferentiated state. Previous studies documented that together TGFβ and KLF6 regulate each other's expression in other cell types. Therefore we sought to investigate the possible role of KLF6 in a myogenic context and assessed whether TGFβ activation regulates KLF6 protein expression and function through MEF2 in C2C12 myoblasts. We observed that TGFβ enhanced KLF6 protein expression levels in myogenic cells and this effect was repressed by pharmacological inhibition of Smad3. Collectively these results illustrated that TGFβ enhances skeletal muscle cell proliferation and survival through KLF6 in response to TGF β.

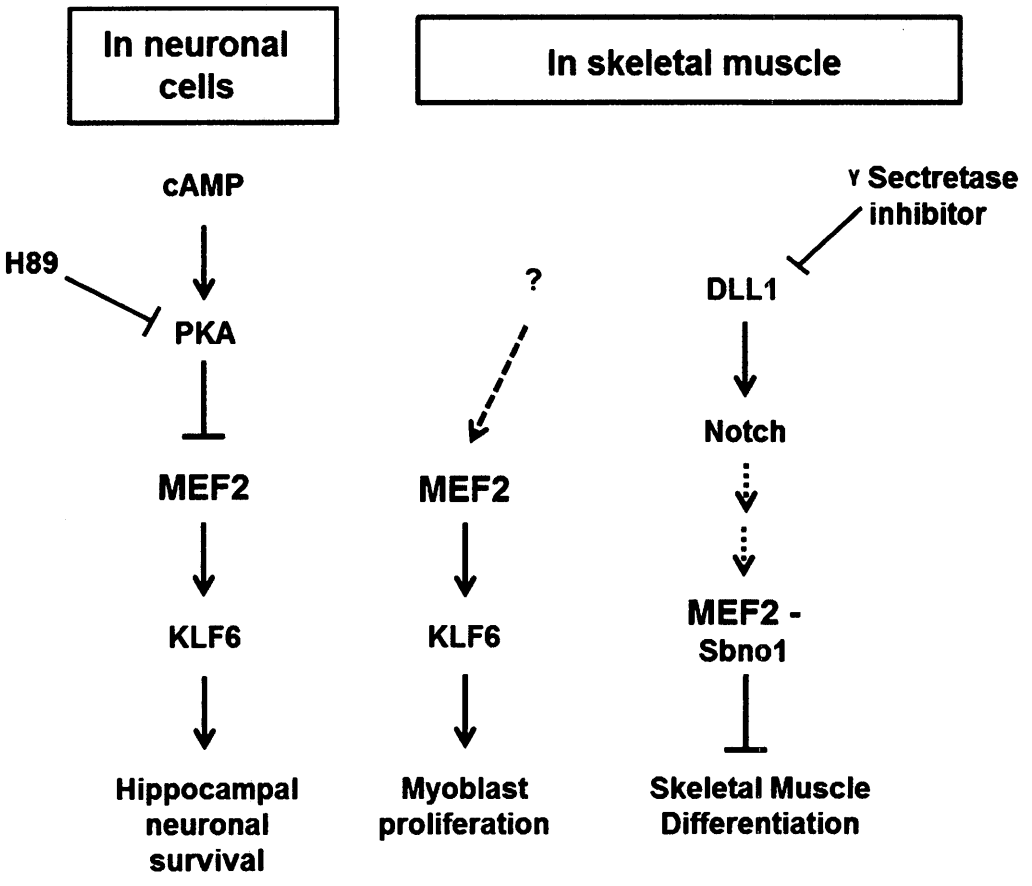


Figure 47. A schematic summary of regulation of MEF2 in Myogenic and neurogenic cells.

In conclusion, we report that differential activation of signaling cascades and co-factors regulate the MEF2 transcriptional complex and subsequently MEF2 dependent gene expression in myogenic and neurogenic cells (Figure 47). It is established now that cAMP/PKA signaling pathway regulates diverse cellular functions and biological processes including muscle differentiation and neuronal

survival. This pathway may also represent a mechanism by which cAMP/PKA signaling modulates human diseases such as cardiac hypertrophy. Since KLF6 is identified as a pro-survival MEF2 target gene in neurons, future work will focus on characterizing whether KLF6 plays a corresponding role in development of skeletal or cardiac muscle. Here, we speculate that cAMP/PKA could be involved in regulation of the MEF2 mediated gene expression in cardiac tissues. Thus, understanding the cAMP/PKA signaling and its role in MEF2 function in cardiac cell survival and death will be useful to the field of cardiac biology. It will also be of interest to examine the role of Notch-Sbno1 signaling in other cell types such as neurons and cardiac cells. Notch signalling pathway plays an important role in the development and maintenance of the nervous system at many different levels. Notch is required for normal neurites morphology, synaptic plasticity, and memory processing. Further analysis of the exact role that Notch-Sbno1 plays in neural development will not only provide insights into neurobiology and underlying mechanisms regulating these cells but also provide potential pharmacological targets with implications for neurodegenerative diseases.

Appendix: Materials and Methods

Cell Culture

The following cell lines were utilized in the aforementioned studies, C2C12, Cos7 and rat hippocampal neuronal primary cultures. What follows are general cell culture guidelines, recommendations by ATCC were also adhered to.

Reagents:

1. 1X Dulbecco's PBS (without Ca^{2+})

NaCl	8 g
KCl	0.2 g
Na_2HPO_4 $7H_20$	1.44 g
KH_2PO_4	0.24g
Add 800 mL ddH_2O	
pH to 7.4 with HCl	
take to volume 1 L	

2. Versene

EDTA	0.2 g
1X Dulbecco's PBS (without Ca^{2+})	1 L

3. 0.125% Trypsin-EDTA

4. 1% penicillin/streptomycin

5. 10% FBS (GM= Growth Medium)
   Fetal bovine serum (FBS), heat inactivated at 56° for 30 minutes.
   DMEM
   Penicillin/streptomycin

6. 5% HS (DM=Differentiation Medium)
   Horse serum (HS), heat inactivated at 56° for 30 minutes.
   DMEM
   Penicillin/streptomycin

Freezing medium:

Growth media (10%FBS) in which the cells are normally cultured, supplemented with 10% DMSO; sterilize the freezing medium by passing through a 0.2um filter.

Thawing Frozen Cells

Materials

1. Vial of frozen cell stock
2. 10% FBS (Growth Medium)

Method

1. Remove the vial from -80°C freezer/ liquid-nitrogen and thaw in 37 °C
2. Dissociate clumps of cells using a sterile glass pasteur pipette.
3. Transfer the cell suspension to a centrifuge tube containing 5 ml of 10% FBS medium.
4. Centrifuge for 10 minutes at 1500 x g.
5. Aspirate the supernatant and agitate tube vigorously to remove cell clumps
6. Resuspend cells in 10ml of 10% FBS (Growth medium).
7. Count cells in haemocytometer
8. Seed cells at appropriate dilution (10^6 cells/100 mm dish in 10 ml of growth medium).

Passaging of Adherent Cells

1. Remove medium from established cell stock.
2. Rinse the cell monolayer with 5ml Versene to remove any traces of serum.
3. Add 1.0 ml of 0.125% Trypsin-EDTA to 100 mm dish.
4. Incubate at 37°C for 1-4 minutes.
5. Inactivate the trypsin by adding 9 mL of 10% FBS (GM) and triturate cells several times (pipette the cells up and down) to ensure complete removal of the cells from the dish and to dissociate clumps of cells.
6. Count cells in a haemocytometer and seed the cells at the appropriate dilution in GM.

Freezing Cells

Materials

1. dimethylsulfoxide (DMSO)
2. freezing vials
3. freezing chamber- polystyrene foam box
4. freezing medium

Method

1. Prepare a cell suspension and pellet the cells by centrifugation at 1500 x g.

2. Resuspend the cells in freezing medium at a concentration of $1x10^6$ - $8x10^6$ cells/ml
3. Dispense 1 ml of cell suspension into each freezing vial.
4. Place the vials in the polystyrene box.
5. Place the box into a -80°C freezer and freeze overnight.
6. Store vials in -80°C freezer for short term (1-3 months)
7. Store vials under liquid nitrogen for long term cell stock storage.

Preparation of primary hippocampal neuron culture

Materials

1. Pregnant female rats (Sprague-Dawley) at gestational day 18 (E18)
2. 95% ethanol
3. HBSS (Hanks balance salt solution)
4. Neurobasal Medium
5. B-27(2ml/100 ml)
6. L-Glutamine (1ml/100 ml)
7. High-glucose MEM/10% (v/v) FBS
8. Trypsin solution
9. Poly-D-lysine coated coverslips
10. Poly-D-lysine coated 6 well dishes/10cm cell culture plates
11. 0.04% (w/v) trypan blue
12. Hemacytometer for cell counting
13. Dissecting tools, sterile:
14. Stainless steel scissors
15. Curved forceps (2 pairs)
16. Dumont forceps,
17. Dissecting microscope
18. Anesthetizing chamber connected to a CO2 tank

Embryo Isolation Protocol

(Approved by Animal Care and Use Committee, York University)

1. Anesthetize the pregnant rat in an anesthetizing chamber filled with CO_2.
2. Sacrifice a pregnant female rat (E18) by cervical dislocation.
3. Place rat, ventral side up and sterilize the abdomen with 95% ethanol.
4. Rinse forceps and scissor in 95% ethanol.
5. Make an incision down the midsection to expose uterine horns.
6. Gently grasp uterine horns at one of the constrictions and remove horns.
7. Pull embryos and uterine horns away from amniotic sac.
8. Place embryos in a 100-mm Petri dish filled with HBSS.
9. Working under the dissecting microscope, gently isolate hippocampi.
10. Add trypsin solution, shake gently and incubate for 5 min at 37°C.

11. Inactivate the trypsin by adding 5 mL HBSS and triturate several times
12. Gently shake and allow the cells to pellet at the bottom of the tube.
13. Determine the total cell number and viability with a haemocytometer and an inverted phase contrast microscope.
14. Trypan blue is used to distinguish viable from dead cells. Viable cells exclude trypan blue while dead or damaged cells are stained (dark blue).
15. Plate and incubate hipppocampal neurons culture in neurobasal medium at 37°C humidified incubator with a 5% CO_2 in air.
16. After 24 h replenish media.

Transfection of Mammalian Cells with DNA

Reagents:

2X HEBS *(2.8 M NaCl, 15mM Na_2HPO_4, 50mM HEPES)*

8.18 g NaCl
5.95 g HEPES
0.1065 g Na_2HPO_4 (MW=142) or 0.201 g Na_2HPO_4-$7H_2O$
Add 400 ml ddH_2O, pH to 7.15, bring volume up to 500 ml, filter sterilize, store at -20 °C.

2.5 M $CaCl_2$

2.78 g $CaCl_2$ (MW=111)
Add ddH_2O up to 10 ml, filter sterilize, store at -20 °C.

Transient transfection of adherent cells with Calcium-phosphate

Methods

1. Seed cells 24 h prior to transfection (30-50% confluent at time of transfection).
2. Refeed cells 2-3 hrs (C2C12) prior transfection with growth media.
3. Calcium-phosphate-DNA precipitate (for 100 mm plate):
   label 15 mL sterile polystyrene tubes and add 500 µl 2 x HEBS to each tube.
4. Prepare DNA-$CaCl_2$ solution containing 50 µl 2.5 M $CaCl_2$ and 20-30 µg total DNA in label sterile eppendorf (Total volume 500 µl), mix gently.
5. While vortexing 2x HEBS at low speed add DNA-$CaCl_2$ solution drop by drop.
6. Incubate for 25-30 minutes at room temperature.
7. Triturate the DNA precipitate and add DNA mix drop by drop to cell cultures.
8. Incubate cells with DNA precipitate for up to 16 hours.

9. Wash cells 2X with PBS and re-feed with 10% FBS (growth media)
10. Incubate cells in growth media for at least 8 h before experimentally treating cells.
11. For differentiation: remove GM and wash cells 2X with PBS and re-feed with 5% HS (DM) (~80% confluent cells at time of change media to DM).
12. Harvest Cells according to experimental time points.

Transfection with Lipofectamine

1. Seed cells at 80% confluence in 10 cm plates.
2. Dilute 8 μg of DNA in 800 μl serum- and antibiotic-free media.
3. Mix 20 μl of Lipofectamine reagent in 800 μl serum- and antibiotic-free media.
4. Combine above, mix and incubate for 15 minutes (up to 45 minutes).
5. Add 1.6 ml of serum- and antibiotic-free media to the mix.
6. Re-feed cells in 3.2 ml of serum- and antibiotic-free media.
7. Add the DNA/Lipo mix and gently rock.
8. Incubate of 2 hours.
9. Wash 2X in PBS and re-feed in growth media.

Luciferase assay.

***Reagents*:**

Luciferase assay Lysis buffer (*20 mM Tris, pH 7.4, 0.1% Triton-X 100*), Luciferase substrate (Promega).

Methods

1. Wash adherent cells 3X with ice-cold 1XPBS.
2. Add 300 μl of lysis buffer per well/dish (35mm).
3. Incubate 15 minutes at 4°C while rocking.
4. Scrape cells with rubber policeman and collect into labelled eppendrof tubes.
5. Spin-down cell debris at 15000rmp for 10min.
6. Transfer cell lysate into new tubes.
7. Transfer 50/100 μl lysate to Luciferase assay tube.
8. Use the Berthold luminometer to detect light units

β-Galactosidase Assay

Reagents:

ONPG *(4 mg/ml in ddH_2O)*

1 M Na_2CO_3

Z buffer

16.1 g Na2HPO4-7H2O (60 mM)
5.5 g NaH2PO4-H2O (40 mM
0.75 g KCl (10 mM)
0.246 g MgSO4-7H2O (1 mM)
Add 800 ml ddH_2O, pH to 7, bring volume up to 1 L, filter sterilize, store at RT

Reaction mix

500 µl Z buffer/sample
100 µl ONPG/sample
2.74 µl β-mercaptoethanol/sample
Prepare fresh and mix well
Prepare volume which is sufficient for all samples and blank to be read

Methods

1. Prepare reaction mixture (per sample (500 µl Z buffer, 100 µl ONPG, 2.74 µl β- mercaptoethanol)).
2. Mix 600 µl of reaction mix with 100 µl of cell lysate
2. Incubate tubes at 37 °C until a color change is apparent (yellow).
3. Add 300 µl of 1M Na_2CO_3 to each tube to stop reaction.
4. Measure absorbance of samples at 420 nm using spectrophotometer.

Protein Extracts

Reagents:

Lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM Sodium vanadate, 1 mM PMSF (add fresh)
Protease inhibitor cocktail (add fresh, Sigma, P-8340),
ice-cold 1XPBS,
2X SDS sample buffer (BioRad) (supplemented with β-mercaptoethanol)

Methods:
(Keep protein samples on ice at all times).

1. Remove media and wash cells twice with ice-cold 1XPBS.
2. Add 700 µl PBS and gently scrape cells and transfer to a new tube.
3. Centrifuge at 1500 xg for 5min.
4. Remove PBS, and re-suspend the pellet with five times (vol/vol) lysis buffer.
5. Vortex cells briefly every 10 min for 30 min on ice.
6. Centrifuge cell lysate at 10,000 xg for 15 min, and transfer supernatant to new tube.
7. Determine protein concentration by Bradford assay
8. Dilute protein samples to equal concentration (0.5 µg/µl – 2.0 µg/µl), with equal amounts 2 X SDS sample buffer added.
9. Boil samples for 3-5 min, chill on ice for five minutes, store at - 80 °C.

SDS-PAGE

Reagents:

10% Resolving gel (15ml)
(5.9 ml ddH_20; 3.8 ml 1.5M Tris pH 8.8; 5 ml 30% acrylamide; 0.15 ml 10% SDS; 0.15 ml 10% APS; 0.006 ml TEMED)

Stacking gel (4ml)
(2.7 ml ddH20; 0.5 ml 1.0 M Tris pH 6.8; 0.67 ml 30% acrylamide; 0.04 ml 10% SDS; 0.04 ml 10% APS; 0.004 ml TEMED)

10X Laemmli (1L)
ddH_20 800 ml
Tris 30.3g
Glycine 144.2 g
SDS 10g
pH to 8.3; bring volume up to 1L with ddH_2O

1XPBS

Methods:

1. Prepare resolving gel and then top with stacking gel in Hoefer gel apparatus
2. Fill bottom and centre well of mini-gel apparatus with 1X Laemmli buffer.
3. Load wells with equal amount of protein samples on a gel.
4. Run a gel at 100 V through stacking and 120 V through running gel.

Western blotting

Reagents:

Transfer buffer *(100ml)* Methanol 20ml; 1X Laemmli 80ml
(Prepare blocking buffer, washing solutions, ECL, and antibody diluent as per manufacturer's instruction).

1. Soak Whatman paper and Immobilon-P (Millipore) membrane in transfer buffer for 5-15 min.
2. After SDS PAGE, transfer protein from gel to Immobilon-P membrane by wet- transfer at 20 V for 16-18 hrs.
3. Block membrane with 5 % (w/v) skim milk powder in 1XPBS/TBS (blocking solution) for 1 hours at room temperature (RT)
4. Incubate membrane with primary antibody (1:1000 or 1:10 000) in blocking solution for 1-16 hrs at 4 °C.
5. Wash membrane with 1XPBS/TBST (3X for 5 min each).
6. Incubate membrane with secondary antibody (1:2000 or 1:100 000) in blocking solution for 1-2 hrs at RT.
7. Wash membrane with PBS/TBST (3 X 5 min each).
8. Use chemiluminescence reagent (Amersham), and expose blot to film and develop.

Co-Immunoprecipitation

1. Prepare cell lysates as described in protein extracts section.
2. Prepare IP antibody-IP matrix complex: add 40-50 µl of suspended (25% v/v) IP matrix (ImmunoCruz™ IP/WB kit), 1-5 µg of primary antibody (IP antibody) and 500 µl of PBS.
3. Incubate the complex at 4 °C for 1 h with gently agitation.
4. Pellet immuno-complex by centrifugation at 1000x g for 30 sec and discard supernatant.
5. Wash pelleted matrix 2X with 500 µl of PBS.
6. After the final wash, add lysis buffer and protein sample (250-500 µg of total cellular protein) to the palleted matrix and incubate at 4 °C on a rotator for O/N.
7. After incubation, microcentrifuge at 1000x g for 30 seconds at 4° C to pellet IP matrix, aspirate and discard supernatant.
8. Repeat wahing step 2-4X with either RIPA buffer/PBS.
9. Re-suspend pellet in 40 µl of 2 X SDS sample buffer and boil for 3 min.
10. Sample analyze by westernblot analysis.

Immunofluorescence

1. Wash cells 3X with cold PBS.
2. Fix cells with 4 % paraformaldehyde in PBS for 10 min at RT.
3. Wash cells 3X with PBS.
4. Permeabilize cells with 0.3 % Triton-X in PBS.
5. Block cells with 10 % goat serum in PBS at 37 °C for 30 min
6. Incubate cells with primary antibody (1:100 – 1:500) at 4 °C for O/N.
7. Wash cells 3X with PBS.
8. Incubate cells with appropriate TRITC/FITC-conjugated secondary antibody (1:500) directed against IgG from species the primary antibody was raised in, for 2 hours at RT.
9. DAPI (4, 6-diamidino-2-phenylindole) staining for 15 min at RT.
10. Wash cells 3X with PBS, add a drop of appropriate mounting media (DAKO), and cover slip. The fluorescence images are captured using a Fluoview 300 (Olympus)

TAP Protocol for Mammalian Cells

Reagents:

Lysis buffer

10 mM Tris-HCl, pH 8.0
150 mM NaCl
Protease inhibitor cocktail (Sigma)
1 mM PMSF

IPP150

10 mM Tris-HCl, pH 8.0
150 mM NaCl
0.1% NP40

TEV cleavage buffer

10 mM Tris-HCl, pH 8.0
150 mM NaCl
0.1% NP40
0.5 mM EDTA
1mM DTT

IPP150 Calmodulin binding buffer

69.4 μL / 100 ml β-mercaptoethanol (add fresh)
10 mM Tris-HCl, pH 8.0
150 mM NaCl
1mM Mg acetate

1mM imidazole
2mM $CaCl_2$
0.1% NP40

IPP150 Calmodulin elution buffer

69.4 µL / 100 ml β-mercaptoethanol
10 mM Tris-HCl, pH 8.0
150 mM NaCl
1mM Mg acetate
1mM imidazole
2mM EGTA
0.1% NP40

IgG beads from Sigma (A2909): Washed with IPP150 buffer

Calmodulin Beads from Stratagene (#214303): Washed with Calmodulin binding buffer (without β-mercaptoethanol)

Methods:

1. Transfect cells with TAP-protein expression vector. C2C12 (15= 100 mm plates)
2. Harvest cells as directed in Preparing protein extracts.
3. Add 40 µl of 50% IgG beads for every 1 ml of lysate (5 plates), rotate at 4 °C for 2 h.
4. Remove supernatant and wash beads 1 ml of IPP150 (all spins involving beads should be at 2000 g). Repeat.
5. Wash beads in 1ml of TEV buffer.
6. Add 100 µl of TEV buffer +2 µl (10 U) TEV protease (Gibco).
7. Incubate at 16°C for 1.5 hours, mix occasionally.
8. Briefly spin and transfer supernatant to new tube.
9. Add 200 µl of TEV buffer to beads, spin, and combine this supernatant with the supernatant from the previous step.
10. Add 900 µl of Calmodulin binding buffer +1 µl $CaCl_2$ (2M) to supernatant.
11. Add 20-40µl of 50% Calmodulin beads, rotate for 1hour at 4 °C.
12. Briefly spin and wash beads with 1 ml of CBB. Repeat twice.
13. Add 25 µl of 2x SDS sample buffer to beads to recover protein complex.
14. In-solution digestion of proteins for Mass spectrometry analysis

Coomassie Stain:

1. wash 3 x 5 min H_2O.
2. stain 5-60 min in GelCode Blue (Pierce).
3. destain 1-24 hours in H2O.

RNA Isolation

1. Add 1 ml of Trizol to 100 – 35 mm dish, agitate for 5 min and then transfer solution to microfuge tube.
2. Add 200 µl chloroform to cell suspension, vortex for 15 sec, and leave at RT for 2-3 min.
3. Centrifuge samples at 12 000x g for 15 min at 4 °C.
4. Transfer the aqueous phase to a fresh tube.
5. Add 500µl of isopropanol to the aqueous phase and incubate at RT for 10 min.
6. Centrifuge samples at 12 000x g for 10 min at 4 °C.
7. Following centrifugation, remove the supernatant and leave pellet.
8. Wash RNA pellet with 70% ethanol.
9. Centrifuge samples at 7500x g for 5 min at 4 °C.
10. Remove supernatant and air dry for 5-10 min.
11. Dissolve the pellet in 25-50 µl of DEPC-treated water by heating at 70°C for 5 minutes

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