Regulation of Myocyte Enhancer Factor 2 (MEF2) by Glycogen Synthase Kinase 3 β (GSK3β)

Nathaniel B. Nowacki

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

The myocyte enhancer factor 2 (MEF2) family of transcription factors plays a central role in cardiac and skeletal muscle development, neuronal survival and T-cell selection. Due to their disparate biological roles, the activity of MEF2 factors is precisely regulated at the post-translational level through protein:protein interactions and post-translational modifications. MEF2 has been shown to interact with a plethora of proteins which include the chromatin remodeling HATs and HDACs and various tissue-restricted transcription factors. Furthermore, congruent with its role as a signal sensor, MEF2 is acutely sensitive to several signal transduction cascades and has been demonstrated to be a direct target of several kinases.

Glycogen synthase kinase 3 (GSK3) has been implicated in multiple metabolic and signaling pathways and has recently emerged as a negative regulator of skeletal muscle hypertrophy and skeletal muscle differentiation; its exact mechanism of action however, has not been clearly defined. In this report, we document a novel convergence of GSK3β signaling on the MEF2A transcriptional regulator during skeletal myogenesis. GSK3β inhibition enhances MEF2A transcriptional activity, activates p38 MAPK and results in a post-translational modification of MEF2A. Taken together, these data suggest that p38 targets MEF2A in the absence of GSK3β signaling.

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To my dear sister Laureen, thank you for being an anchoring presence during my time here in Canada. I will always cherish the memories of our time together. Thank you for all of those succulent meals. You are undoubtedly the cordon bleu of the family. Keep the passion alive!

Finally, I wish to thank my grandmother (Aline) and my uncle and aunt (Alain and Annick) in France for their kindness, generosity and unrelenting prayers.

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Abbreviations

APC	adenomatous polyposis coli (protein)
APS	ammonium persulphate
AR	AR-A014418
ATF	activating transcription factor
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
bp	base pair
BSA	bovine serum albumin
bZIP	basic leucine-zipper
C	carboxyl terminus
cAMP	cyclic adenosine monophosphate
CArG	$CC(A/T)_6GG$
CBP	calmodulin binding peptide
CDK	cyclin dependent kinase
CEB	calmodulin elution buffer
CID	collision-induced dissociation
CK2	protein kinase CK2 (aka casein kinase-II)
CMV	cytomegalovirus
d	day(s)
Da	dalton
DBD	DNA binding domain
DM	differentiation medium
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpc	days post coitum
DTT	dithiothreitol
E	embryonic day
EDTA	ethylenediaminetetraacetic acid tetrasodium salt dihydrate
eGFP	enhanced green fluorescent protein
eIF2B	eukaryotic initiation factor 2B
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal regulated protein kinase
FBS	fetal bovine serum
β-Gal	β-galactosidase (LacZ)
GFP	green fluorescent protein
GM	growth medium
GSK3	glycogen synthase kinase 3
GST	glutathione S-transferase
h	hour(s)
HAT	histone acetyl transferase
HDAC	histone deacetylase

HEBS	hepes buffered saline
HRP	horse radish peroxidase
HS	horse serum
IGF	insulin-like growth factor
IgG	immunoglobulin G
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranoside
JNK	Jun N-terminal kinase (SAPK)
kDa	kilodalton
LB	Luria-Bertani
LEF	lymphoid-enhancer factor
LiCl	lithium chloride
MADS	MCM1, agamous, deficiens, serum-response factor
MALDI	matrix-assisted laser desorption/ionization
MAPK	mitogen-activated protein kinase
MAP3K	mitogen-activated protein kinase kinase kinase
MCK	muscle creatine kinase
MEF2	myocyte enhancer factor 2
MRF	myogenic regulatory factor
MRF4	muscle regulator factor 4 (aka myogenic factor 6 (herculin))
mRNA	messenger RNA
MS	mass spectrometry
MyBP	myelin basic protein
Myf5	myogenic determination factor 5
MyHC	myosin heavy chain
MyoD	myogenic determination factor
N	amino terminus
N	any nucleotide
NFAT	nuclear factor of activated T-cells
NLS	nuclear localization signal
NP-40	nonidet-P40
P	phosphorylated protein
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PEG	polyethylene glycol
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKD	protein kinase D
PMSF	phenyl methyl sulfonyl fluoride
PP1	protein phosphatase 1
PP2B	protein phosphatase 2B
PTM	post-translational modification

RLU	relative luciferase units
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcriptase PCR
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
siRNA	silencer RNA
SRF	serum response factor
SUMO	small ubiquitin-like modifier
t	time
TAD	transcriptional activation domain
TAP	tandem affinity purification
TBS	tris buffered saline
TCF	T-cell factor
TE	Tris-EDTA buffer
TEMED	N,N,N',N' - tetramethylethylene diamine
TEV	tobacco etch virus
TnT	troponin T
TOF	time-of-flight
Tween 20	polyoxyethelenesorbitan monolaurate
WNT	wingless-int
WT	wild type
X	any amino acid

Chapter 1: LITERATURE REVIEW

Myogenesis, or the process of skeletal muscle development, has become a paradigm for the study of cell lineage establishment and cellular differentiation (Ludolph and Konieczny, 1995). During ontogeny, proliferating skeletal muscle progenitor cells (myoblasts), migrate to pre-determined positions upon receipt of appropriate signals, withdraw from the cell cycle, fuse and terminally differentiate into multinucleated myotubes. The transition from myoblasts to myotubes is accompanied by striking morphological changes that can successfully be replicated in cell culture. The C2C12 myoblast differentiation system for example, reproduces many of the important stages of muscle development and has proven to be a highly tractable model for biologists studying myogenesis and its molecular underpinnings.

A considerable volume of research acquired over the past few decades, both in cell culture models and *in vivo*, has identified two families of transcription factors, the myogenic regulatory factors (MRFs) and the myocyte enhancer factor 2 (MEF2) family, which are vital to the process of muscle differentiation. In the first part of this review, we will briefly survey some of the characteristics of the MRFs and outline their reliance on MEF2 factors for optimal activation of the myogenic program. We will then introduce the MEF2 family of proteins and explore some of their general features as well as their individual roles during development. Finally, we will delve into the intricate regulatory mechanisms that act in concert to govern MEF2 function.

I. The Myogenic Regulatory Factors (MRFs)

The MRF family is composed of: MyoD, Myf5, MRF4 and myogenin which share a basic helix loop helix (bHLH) motif responsible for DNA binding and dimerization (Olson, 1990). Members of the MRF family form transcriptionally active heterodimers with the E-proteins and bind to the consensus DNA binding element CA*NN*TG, referred to as an E-box (Olson, 1990). The E-box is found in the control region of most, but not all muscle specific genes (Olson, 1992). MRFs are regulated by protein:protein interactions and post-translational modifications. Their primary role is to transactivate muscle-specific genes and to induce terminal cell cycle arrest during skeletal muscle differentiation by increasing the expression of the cell cycle regulator p21 (Halevy et al., 1995).

Interestingly, ectopic expression of any MRF family member in some non-muscle cell lines, such as C3H10T1/2 fibroblasts, results in their stable conversion to the myogenic lineage (Olson, 1990). This property suggests that the MRFs are key regulators of myogenesis.

Despite their fundamental importance in myogenesis, the MRFs cannot efficiently activate the myogenic program without interaction with another class of transcriptional regulators termed the myocyte enhancer factor 2 or MEF2 family. As previously mentioned, the MRFs bind to an E-box *cis* regulatory element, located in the control regions of most muscle specific genes. While E-boxes are needed for transactivation of many skeletal muscle genes, they are not sufficient and depend on the adjacent binding

sites of additional factors to activate gene transcription. MEF2 binding sites are often found in proximity to E-boxes in muscle gene regulatory regions, and have been shown to be required for transcriptional activation of those genes in skeletal muscle (Olson et al., 1995). MEF2 was initially identified as a DNA-binding activity that recognized an A/T-rich element in the E-box-containing muscle creatine kinase (MCK) enhancer (Gossett et al., 1989). Full enhancer activity required MEF2 binding. Since the discovery of MEF2, A/T-rich sequences have been found in the promoters and enhancers of the majority of skeletal and cardiac muscle structural genes (Olson et al., 1995).

Unlike members of the MRF family, MEF2 proteins cannot convert C3H10T1/2 fibroblasts to the myogenic lineage. Nonetheless, studies have demonstrated that MEF2 factors are essential for myogenic differentiation. Indeed, when the regions of MyoD that interact with MEF2 are mutated, MyoD is no longer able to fully activate the myogenic program (Davis and Weintraub, 1992). Furthermore, overexpression of the bHLH region of myogenin (without the transcriptional activation domain (TAD)) with MEF2C is capable of efficiently converting C3H10T1/2 fibroblasts to myotubes (Molkentin et al., 1995). As further proof, microinjection of a dominant negative form of MEF2A, lacking the TAD, into L6E9 or C2C12 myoblasts inhibits myotube formation (Ornatsky et al., 1997). Additionally, dominant negative MEF2A impairs the myogenic conversion of C3H10T1/2 fibroblasts by MyoD (Ornatsky et al., 1997). These findings highlight the importance of MEF2 proteins in the muscle differentiation program.

II. The MEF2 Family of Transcription Factors

MEF2 proteins belongs to the MADS (MCM1, agamous, deficiens, serum-response factor) superfamily of transcription factors. Vertebrates possess four *Mef2* genes: *Mef2a*, *b*, *c* and *d*, whereas *Drosophila*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae* have a single *Mef2* gene (Potthoff and Olson, 2007). When the single *Drosophila Mef2* gene (*D-mef2*) is deleted, embryos produce myoblasts that are normally specified and positioned, but they do not differentiate into functional myotubes (Lilly et al., 1995). In vertebrates, loss-of-function analyses have been complicated by the fact that there are four MEF2 members with overlapping expression patterns and some degree of functional redundancy.

1. MEF2 Structure

The amino-termini of MEF2 proteins contain a highly conserved, 57 amino acid, MADS-box domain and an immediately adjacent, 26 amino acid, MEF2 domain (Black and Olson, 1998). These two domains collectively mediate DNA-binding, dimerization and co-factor interactions (Black and Olson, 1998). MEF2 factors bind as homo- or heterodimers to the consensus DNA sequence (C/T)TA(A/T)$_4$TA(G/A) found in the control regions of most muscle specific genes (Pollock and Treisman, 1991). The MEF2 transcriptional activation domain is located in the carboxy- terminus of the protein. Additionally, MEF2A has been shown to contain a nuclear localization sequence (NLS) at its extreme carboxy-terminus, which is conserved in MEF2C and MEF2D (Yu, 1996). When the NLS is deleted, MEF2A fails to localize to the nucleus (Yu, 1996). The C-

terminus of MEF2 is highly divergent amongst the MEF2 members, as revealed by sequence analysis. It is subject to alternative splicing and multiple post-translational modifications in the form of phosphorylation, acetylation and sumoylation.

2. MEF2 Expression

The vertebrate *Mef2* genes are expressed in distinct but overlapping temporospatial patterns during embryogenesis, with highest levels occurring in the brain and striated muscles (Potthoff and Olson, 2007). During mouse development, MEF2C expression is first detected at 7.5 dpc in cells of the cardiac mesoderm that give rise to the primitive heart tube (Edmondson et al., 1994). By 8.5 dpc, MEF2A, MEF2C and MEF2D mRNAs are all detected in the myocardium. At 9.0 dpc, MEF2C is expressed in rostral myotomes, where its expression lags that of Myf5 by about a day and myogenin by several hours (Edmondson et al., 1994). MEF2A and MEF2D are expressed at a lower level than MEF2C in the myotome at 9.5 dpc. They are however detected in more embryonic tissues than MEF2C. After 12.5 dpc, MEF2 transcripts are detected at high levels in specific regions of the brain and ultimately in a wide range of tissues (Edmondson et al., 1994).

In cultured skeletal muscle cells, the expression pattern of *Mef2* genes follows a different course. MEF2D is expressed in proliferating myoblasts prior to the onset of differentiation and MEF2A appears as cells enter the differentiation pathway (Black and Olson, 1998). MEF2C appears at a later timepoint in the differentiation program (Black

and Olson, 1998). Interestingly, although MEF2D is present in myoblasts, it cannot initiate muscle specific gene expression in such a context (Martin et al., 1994).

Unlike the MRF family members, MEF2 expression is not confined to skeletal muscle. In fact, several reports have provided evidence that MEF2 mRNAs are ubiquitous (Breitbart et al., 1993; Martin et al., 1994). Despite the widespread distribution of MEF2 transcripts, MEF2 activity is tissue-specific. For example, EMSAs using the MCK MEF2 DNA binding site as a probe showed that HeLa, Schneider, and L6E9 muscle cells contain levels of MEF2 binding activity comparable to those in C2C12 muscle cells (Ornatsky and McDermott, 1996). However, only C2C12 cells show MEF2-dependent activation of a muscle-specific gene reporter (Ornatsky and McDermott, 1996). These observations are also supported by *in vivo* data where transgenic mice harboring a lacZ reporter gene, controlled by three copies of the MEF2 site, show MEF2-dependent expression in developing myogenic cell lines of the embryo but not in other cell types that also contain MEF2 (Naya et al., 1999). It therefore appears that the transcriptional activity of MEF2 proteins can be regulated independently of DNA binding in various cell types (Black and Olson, 1998).

3. MEF2 Function

Assessment of the role of the vertebrate *Mef2* genes has been challenging due to their overlapping expression patterns during development. In mice, null *Mef2c* embryos die at approximately 9.5 dpc due to cardiovascular defects. The heart tube fails to undergo looping morphogenesis, the future right ventricle does not form, and a subset of

cardiac muscle genes is not expressed (Lin et al., 1997). *Mef2b*-null mice are viable and do not display any obvious phenotypic defects (Black and Olson, 1998). *Mef2a*-null mice are also viable but are subject to sudden death within the first week of life. They exhibit pronounced dilation of the right ventricle, myofibrillar and mitochondrial disorganization and activation of a fetal cardiac gene program (Naya et al., 2002). The few *Mef2a*-null mice that do survive to adulthood show a marked reduction in size and number of cardiac mitochondria and susceptibility to sudden death (Naya et al., 2002). *Mef2d*-null mice are viable and show no apparent abnormalities (Arnold et al., 2007).

Despite MEF2's demonstrated importance in myogenesis, none of the aforementioned genetic deletions have shed light on the role of MEF2 factors in vertebrate skeletal muscle *in vivo*. It is noteworthy however, that the early lethality caused by the *Mef2c* gene knockout has precluded analysis of its function in skeletal muscle at later developmental stages (Potthoff et al., 2007). In this regard, tissue-specific gene deletion has proven to be a valuable method to further ascertain the role of MEF2 proteins. Recently, a study showed that myofibers from mice with skeletal muscle deficient in *Mef2c* differentiate and form normally during embryogenesis, but rapidly deteriorate after birth, due to disorganized sarcomeres and loss of integrity of the M line (Potthoff et al., 2007). In the heart, mice with a *Mef2d* loss-of-function mutation are viable but display an impaired response to stress signals that normally lead to MEF2 activation, cardiac hypertrophy, fibrosis and fetal gene activation (Kim et al., 2008). MEF2D is thus thought to play a crucial role in mediating stress-dependent gene expression in the adult heart (Kim et al., 2008).

Beyond its function in skeletal muscle, MEF2 has emerged as an essential component of the transcriptional circuits that control differentiation and development in disparate biological contexts. MEF2 is critically involved in vertebrate heart development, neural crest development, bone development, the maintenance of vascular integrity, neuronal differentiation and survival, and T-cell apoptosis (Potthoff and Olson, 2007). How MEF2 orchestrates such contrasting programs of gene expression is a penetrating question that is sure to stimulate new lines of inquiry in the area of MEF2 research. One plausible answer may lie in the elucidation of the intricate regulatory mechanisms that combine to govern MEF2 function.

4. MEF2 Regulation

MEF2 activity is precisely controlled by multiple regulatory mechanisms such as, alternative splicing, protein:protein interactions and post-translational modifications.

A. Alternative Splicing

The vertebrate *Mef2* genes are capable of generating multiple isoforms through a complex pattern of alternative splicing which includes use of alternative exons and splice versus no-splice options (Zhu et al., 2005). In total there are four alternatively spliced exons/domains, termed α1, α2, β, and γ, which give rise to numerous tissue-restricted MEF2 isoforms. MEF2A containing the acidic β exon (SEEEELEL) for example, is restricted to sarcomeric muscle and brain (Yu et al., 1992). Additionally, the inclusion or exclusion of specific domains alters MEF2 transcriptional activity (Zhu et al., 2005).

B. Protein:Protein Interactions

Another level of control of MEF2 activity involves the combinatorial association of MEF2 factors with a number of partner proteins which serve either as functional co-activators or co-repressors. MEF2 is known to interact with other MEF2 members, MRFs, histone acetyltransferases (HATs) and histone deacetylases (HDACs), various tissue-restricted transcriptional regulators and the phosphatase, PP1α.

i. *MEF2:MEF2 interactions*

MEF2 factors homo- and heterodimerize to activate gene expression. The composition of the MEF2 dimers is of functional consequence. For instance, in non-muscle cells, MEF2A:MEF2D heterodimers are formed and are commonly found to be transcriptionally inactive. On the other hand, in muscle cells, MEF2A:MEF2A homodimers are formed which can potently activate muscle-specific genes (Ornatsky and McDermott, 1996).

ii. *Interaction with HATs and HDACs*

MEF2 activity is tightly regulated by two families of chromatin-remodeling enzymes, HATs and HDACs. HATs and HDACs catalyze the acetylation/deacetylation of histones and thereby act as transcriptional activators and repressors, respectively. 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).

Antagonizing the action of the HATs are the class IIa HDACs (HDAC4, -5, -7, -9) which also associate with the MADS domain of MEF2. Class IIa HDACs are distinguished from other classes of HDACs by the presence of an 18-amino acid motif at their N-termini that mediates binding to MEF2 factors (McKinsey et al., 2002). Association of class IIa HDACs and other co-repressors with MEF2 promotes the formation of multiprotein repressive complexes on MEF2-dependent genes such as myogenin (Potthoff and Olson, 2007). It is thought that inhibition of MEF2 transcriptional activity, through interaction with HDACs, prevents the premature expression of myogenic genes prior to cells receiving appropriate differentiation cues. The fact that MEF2 is present but transcriptionally silent in proliferating myoblasts, lends credence to this idea. When cells are stimulated to differentiate, several calcium-regulated protein kinases, such as PKD and CaMKs, phosphorylate class II HDACs on a series of conserved serine residues, which creates a docking site for the intracellular chaperone protein 14-3-3 (McKinsey et al., 2001; Vega et al., 2004). Binding of 14-3-3 to phospho-HDACs results in dissociation of HDACs from MEF2, and subsequently export of the 14-3-3:HDAC complexes from the nucleus to the cytoplasm (McKinsey et al., 2001). MEF2 can then engage HATs and other positive regulators of differentiation and activate myogenic genes.

iii. *Interaction with NFAT*

MEF2 factors act as integrators of calcium signals (McKinsey et al., 2002). Variations in intracellular calcium concentration can alter MEF2's phosphorylation status

as well as the proteins that it interacts with. The serine/threonine phosphatase PP2B or calcineurin responds to sustained, low amplitude calcium transients and has been shown to activate MEF2 by recruiting members of the nuclear factor of activated T-cells (NFAT) family of transcription factors (McKinsey et al., 2002). Upon dephosphorylation by calcineurin, NFAT translocates to the nucleus where it directly associates with both MEF2A and MEF2D (Blaeser et al., 2000). NFAT stimulates MEF2-dependent transactivation by facilitating recruitment of p300 to MEF2 response elements (Youn et al., 2000).

iv. ***Interaction with a Phosphatase***

In a recent development, a novel physical interaction has been reported between the catalytic subunit of the phosphatase PP1α and MEF2. Binding of PP1α to MEF2 occurs within the nucleus and potently represses MEF2-dependent transcription (Perry et al., 2009). Interestingly, PP1α phosphatase activity is not required for MEF2 repression. Moreover, a MEF2-PP1α regulatory complex leads to nuclear retention and recruitment of HDAC4 to MEF2 transcription complexes (Perry et al., 2009). Two key results suggest that PP1α exerts a dominant level of control over MEF2 function. First of all, PP1α overrides the positive influence of calcineurin signaling on MEF2 and secondly, PP1α-mediated repression of MEF2 function interferes with the pro-survival effect of MEF2 in primary hippocampal neurons (Perry et al., 2009).

Taken together, these findings are the first demonstration of MEF2-mediated transcriptional repression due to a direct interaction with a phosphatase (Perry et al.,

2009). The association between PP1α and MEF2 therefore sets an important precedent in the literature and unveils a novel aspect of MEF2 regulation.

C. Post-translational Modifications

MEF2 proteins are subject to extensive post-translational modifications in the form of acetylation, sumoylation and phosphorylation. Phosphorylation in particular, plays an important role in controlling MEF2 activity (Cox et al., 2003).

i. *MEF2 Acetylation*

Protein lysine acetylation/deacetylation has emerged alongside phosphorylation as an important post-translational modification governed by the dueling actions of HATs and HDACs (Gregoire et al., 2007). In addition to histones, proteins known to be subject to lysine acetylation include over 50 transcription factors, and various other proteins (Gregoire et al., 2007). A study has shown that p300 acetylates six lysine residues in the transactivation domain of MEF2C, both *in vivo* and *in vitro* (Ma et al., 2005). Mutation of these lysines affects MEF2 DNA binding activity and transcriptional activity and inhibits myogenic differentiation (Ma et al., 2005).

Opposing the action of HATs are the HDACs which can deacetylate their substrates. For example HDAC3, a class I HDAC, deacetylates MEF2 *in vivo*, represses MEF2 transcriptional activity and inhibits myogenesis (Gregoire et al., 2007). The class IIa HDACs, HDAC4 and HDAC5, repress MEF2-dependent transcription however, they cannot directly deacetylate MEF2 (Gregoire et al., 2007).

ii. *MEF2 Sumoylation*

Sumoylation is a novel post-translational mechanism where small ubiquitin-like modifier (SUMO) proteins are covalently attached to the lysine residues of target proteins (Hay, 2005). Sumoylation regulates several critical cellular processes including cell cycle progression, maintenance of genome integrity, subcellular transport and protein transcriptional activity (Hay, 2005).

A series of reports suggest that sumoylation could play a pivotal role in controlling MEF2 transcriptional activity (Gregoire et al., 2006; Gregoire and Yang, 2005; Riquelme et al., 2006; Zhao et al., 2005). MEF2D and MEF2C are sumoylated on a single lysine residue located at a consensus sumoylation motif conserved among MEF2 proteins (Gregoire and Yang, 2005). This modification inhibits transcriptional and myogenic activities. MEF2A also undergoes sumoylation primarily at a single lysine residue (K395) both *in vitro* and *in vivo* (Riquelme et al., 2006). Mutation of K395 to arginine abolishes MEF2A sumoylation and enhances MEF2 transcriptional activity (Riquelme et al., 2006).

iii. *MEF2 Phosphorylation*

MEF2 is exquisitely tuned to a diversity of extracellular cues by virtue of its strategic position at the endpoint of various signaling cascades. Amino acid sequence analyses and multiple sequence alignment of MEF2 proteins reveal several, highly conserved, putative phosphorylation sites for kinases such as, CK2, MAP kinases, PKC,

PKA, GSK3 and other signaling molecules. Presently, MEF2 factors are documented targets of CK2, ERK5, p38 MAPK, CDK5, PKC and PKA (Fig. 1).

a. *CK2 and MEF2*

Protein kinase CK2 or casein kinase-II, phosphorylates MEF2C at a conserved serine residue (S59) located in the MADS-box domain (Molkentin et al., 1996). Phosphorylation of this site enhances the DNA binding and transcriptional activity of MEF2C. Intriguingly, MEF2A has been shown to be phosphorylated at a consensus CK2 site, S289, in response to p38 MAPK signaling (Cox et al., 2003). This region of MEF2A (and the equivalent region of MEF2C) is alternately spliced in different tissues, suggesting a possible tissue-specific functional role for this region. It also suggests a link between p38 MAPK activity and the phosphorylation of MEF2A by CK2 (Cox et al., 2003).

b. *ERK5 and MEF2*

Extracellular signal regulated protein kinase 5 (ERK5) was initially found to interact with the N-terminus of MEF2D in a yeast-two-hybrid screen (Yang et al., 1998). Co-immunoprecipitation experiments and GST pull-down assays have confirmed that MEF2A and MEF2C can also interact with ERK5 (Yang et al., 1998). ERK5 dramatically enhances the transcriptional activity of MEF2C by phosphorylating S387, which is located in the transactivation domain of the protein (Kato et al., 1997).

c. *p38 MAPK and MEF2*

p38 MAP kinase is recognized as a key regulator of skeletal muscle differentiation and has also been shown to play a crucial role in somitic myogenesis. Indeed, a critical interaction between p38 and MEF2 occurs in the somite myotome during development (de Angelis et al., 2005). Abrogation of p38 MAPK signaling blocks MEF2 activation in somite cultures and in embryos *in vivo*, and concomitantly inhibits myogenic differentiation (de Angelis et al., 2005).

Originally, a kinase inactive form of p38 was found to interact with MEF2C in a yeast-two-hybrid screen (Han et al., 1997). Later studies revealed p38 can also phosphorylate MEF2A, but not MEF2B, and dramatically enhance MEF2 transcriptional activity (Zhao et al., 1999). More recently, MEF2D has been documented to be directly phosphorylated by p38 *in vitro* as well as in fibroblasts and C2C12 cells (Penn et al., 2004; Rampalli et al., 2007).

p38 targets MEF2C *in vitro* at three residues, T293, T300 and S387. Mutagenesis of any of these phosphoacceptor sites to alanine impairs MEF2C transcriptional ability (Han et al., 1997). Similarly, MEF2A is phosphorylated by p38 *in vitro* at T312, T319, S453 and S479 (Zhao et al., 1999). However, only T312 and T319 appear to be required for increased transcriptional activation (Zhao et al., 1999). Phosphorylation of MEF2A by p38 *in vivo* shows a much more complicated pattern (Cox et al., 2003). In addition to the aforementioned sites, there is evidence that suggests that MEF2 is targeted by p38 at S98, S192 or S223, S408 and S494 (Cox et al., 2003).

The specificity of p38 MAPK phosphorylation is achieved by a docking site located in the transactivation domains of MEF2A and MEF2C (Yang et al., 1999). This domain is not phosphorylated by p38 but is responsible for the interaction that allows phosphorylation of MEF2 to occur. Deletion of the docking site prevents MEF2 from being targeted by p38 (Yang et al., 1999). Moreover, transferal of the docking domain from MEF2A to proteins which are not p38 substrates confers p38-responsiveness (Yang et al., 1999).

d. *CDK5 and MEF2*

Regulation of neuronal apoptosis plays a central role both during development of the central nervous system and in the adult brain (Tang et al., 2005). MEF2 plays a critical role in neuronal survival and cyclin dependent kinase 5 (CDK5) mediates neurotoxic effects by targeting and inhibiting MEF2 (Tang et al., 2005). CDK5 phosphorylates a conserved serine residue in the transactivation domain of MEF2 (S408 in MEF2A and S444 in MEF2D), in primary cortical neurons (Gong et al., 2003). Mutation of the CDK5 site restores MEF2 activity and protects neurons from CDK5 in neurotoxin-induced apoptosis (Gong et al., 2003). Studies in primary cerebellar granule neurons have shown that phosphorylation by CDK5 reduces MEF2 transcriptional activity by promoting degradation of MEF2A and MEF2D by caspase-3 (Tang et al., 2005).

Experiments performed in non-neuronal cells, show that CDK5 acts in concert with HDAC4 to stimulate MEF2D sumoylation at K439 by phosphorylating S444

(Gregoire et al., 2006). Opposing the action of CDK5, calcineurin dephosphorylates S444 and prevents sumoylation of K439, thus acting as an "editor" of MEF2's phosphorylation status, selectively removing inhibitory phosphates (Gregoire et al., 2006). The interplay of S444 phosphorylation with K439 sumoylation therefore regulates MEF2 activity in certain contexts.

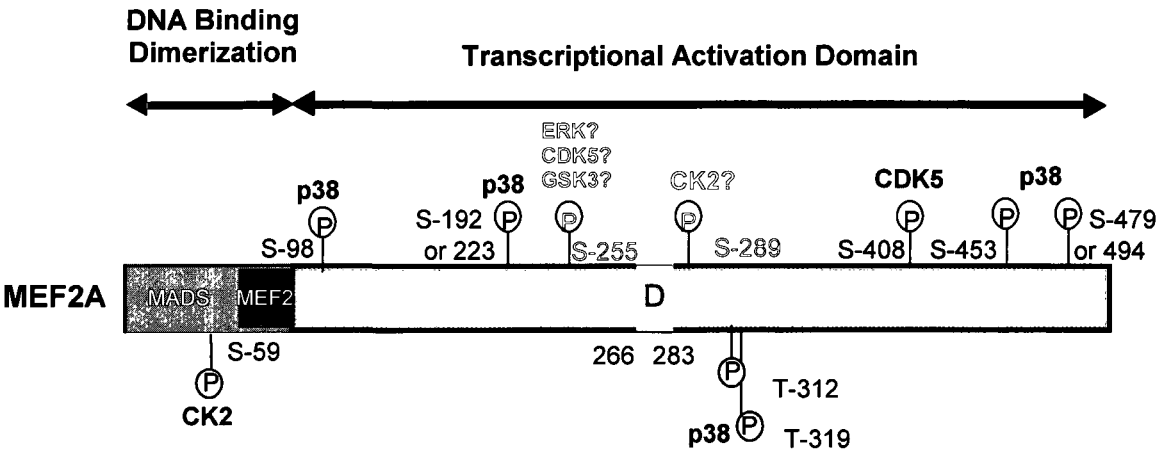

Figure 1: Kinase target sites in MEF2A. Schematic representation of the MEF2A transcription factor depicting important features such as the MADS/MEF2 domain mediating DNA binding and dimerization and the transcriptional activation domain. The p38 docking site (D) is also indicated. Shown in black are MEF2 phosphoacceptor sites known to be targeted by the indicated kinases. Shown in red are MEF2 sites that are phosphorylated by kinases whose identity is currently unknown. (Adapted from Cox et al., 2003)

e. *PKC and MEF2*

The PKC serine-threonine kinases participate in vital cellular processes such as growth and differentiation, acting as components of several signal transduction cascades initiated by ligand stimulation of transmembrane tyrosine kinase receptors (Ornatsky et al., 1999). There are multiple PKC isotypes that are classified as conventional cPKCs (α,

β and γ), novel nPKCs (η, ε, δ, and θ) and atypical aPKCs (ι and ζ) (Mellor and Parker, 1998). The nPKCs δ and ε have been shown to phosphorylate the TAD of MEF2A in HeLa cells and enhance its transcriptional activity (Ornatsky et al., 1999). The residues targeted by PKC however, are unknown.

f. *PKA and MEF2*

Activation of PKA by elevation of intracellular cyclic AMP (cAMP) level inhibits myogenesis by antagonizing the activity of the MRFs (Li et al., 1992). Repression by PKA however, does not require direct phosphorylation of these transcription factors, which implies an indirect mechanism of inhibition. It has recently been shown that PKA targets MEF2D at S121 and S190 and potently represses its transactivation properties (Du et al., 2008). PKA activation also results in an enhanced nuclear accumulation of HDAC4 and a subsequent increase in a MEF2D:HDAC4 repressor complex (Du et al., 2008). Neutralizing mutations of S121 and S190 confers PKA-resistance to MEF2D and efficiently rescues myogenesis from PKA-mediated repression (Du et al., 2008).

g. *GSK3*

As its name implies, glycogen synthase kinase 3 or GSK3, was originally identified as one of several kinases that could phosphorylate glycogen synthase, the enzyme that catalyzes the last step in glycogen synthesis (Cohen and Frame, 2001). Several decades of research have helped to release GSK3 from its traditional association with glycogen metabolism. Indeed, the name glycogen synthase kinase fails to adequately

describe the plethora of functions and substrates that are attributed to GSK3. It turns out that GSK3 has a key role in the regulation of many cellular functions such as signaling by insulin, nutrients and growth factors, cell fate specification during embryonic development, cell proliferation, apoptosis and microtubule function (Cohen and Frame, 2001).

- GSK3 Characteristics:

The GSK3 family of serine/threonine kinases consists of two isoforms, α and β, which are 98% identical within their kinase domains but differ substantially in their N- and C-termini (Force and Woodgett, 2009). GSK3 is ubiquitously expressed with high levels of expression occurring in the brain (Rayasam et al., 2009). Several unique features distinguish GSK3 from other protein kinases. First of all, it is constitutively active in unstimulated cells and is inhibited in response to cellular signals such as growth factors (Force and Woodgett, 2009). Secondly, phosphorylation of its substrates generally leads to their inactivation (Cohen and Frame, 2001). Furthermore, GSK3 preferentially targets substrates that are first phosphorylated by another protein kinase at a serine/threonine residue, termed the "priming phosphate", located four amino acids C-terminal to the GSK3 phosphorylation site (Cohen and Frame, 2001). The consensus recognition sequence is (S/T)XXX*(S/T)*P (priming phosphoacceptor residue shown in italics) (Doble and Woodgett, 2003).

- GSK3 Regulation:

GSK3 is a critical component of several distinct physiological pathways. Regulation of its activity is therefore essential to ensure proper function. GSK3 undergoes multiple phosphorylation events which, depending on the amino acids being modified, impact its activity (Rayasam et al., 2009). For example, phosphorylation of the N-terminal S21 in GSK3α, or S9 in GSK3β, turns the amino terminus into a pseudosubstrate that suppresses the activity of these kinases (Cohen and Frame, 2001). Conversely, phosphorylation of Y279 and Y216 in GSK3α and -β respectively, activates the enzymes (Rayasam et al., 2009). Of note, Y216 in GSK3β is constitutively phosphorylated in resting mammalian cells (Doble and Woodgett, 2003) but the kinase(s) responsible for the modification is not well characterized (Rayasam et al., 2009).

- Signaling to GSK3:

The role of GSK3 in insulin signaling and WNT signaling has been extensively studied. Binding of insulin to its receptor leads to activation of phosphatidylinositol 3-kinase (PI3K) and Akt /PKB, which phosphorylates GSK3 at its N-terminus and inhibits it. GSK3 inactivation results in dephosphorylation of glycogen synthase and eIF2B which, in turn, stimulate glycogen and protein synthesis respectively (Rayasam et al., 2009).

A proportion of GSK3 in cells is present in a multiprotein complex comprising axin, the adenomatous polyposis coli (APC) protein and β-catenin (Cohen and Frame, 2001). In the absence of WNT proteins, GSK3 in this complex is active and

phosphorylates β-catenin targeting it for proteosome-mediated degradation. WNT signaling disrupts the multiprotein complex and ultimately leads to dephosphorylation and stabilization of β-catenin. Stabilized β-catenin translocates to the nucleus, associates with members of the LEF/TCF family of transcription factors and enhances gene transcription (Cohen and Frame, 2001).

- GSK3 and Skeletal Muscle:

In recent years, GSK3β has been implicated in the negative regulation of skeletal muscle hypertrophy and skeletal muscle differentiation. The studies that led to the identification of GSK3's negative regulatory role were initially focused on understanding the hypertrophic effects of insulin-like growth factor-1 (IGF-1) on skeletal muscle cells. IGF-1 is a recognized inducer of both muscle differentiation and myotube hypertrophy, however its mechanism of action has not been fully clarified (Rommel et al., 2001). It is possible that IGF-1 mediates its hypertrophic effects in part through inhibition of GSK3β. In this regard, it has been demonstrated that IGF-1 treatment of C2C12 cells causes phosphorylation of GSK3 via a mechanism that is blocked by the PI3K inhibitor LY294002 (Rommel et al., 2001). The IGF/PI3K/Akt axis may therefore be crucial in regulating GSK3 and muscle hypertrophy. Since GSK3 normally acts to inhibit the translation initiation factor eIF2B, inactivation of GSK3 by Akt/PKB might promote translation initiation and protein synthesis and contribute to hypertrophy (Rommel et al., 2001). This hypothesis has been validated by the fact that expression of a dominant negative form of GSK3β in myotubes causes profound myotube hypertrophy (Rommel et

al., 2001). Furthermore, treatment of C2C12 cells with LiCl, a well characterized GSK3 inhibitor, enhances myotube hypertrophy (Vyas et al., 2002).

Studies point to a negative regulatory role of GSK3β during myogenic differentiation. Experiments performed in C2C12 cells show that treatment of cells with LiCl enhances differentiation (van der Velden et al., 2006). Similarly, GSK3β knockdown via RNA interference, in C2C12 myoblasts, stimulates differentiation (van der Velden et al., 2008). Furthermore, investigation of IGF-1-induced skeletal muscle differentiation suggests that the pro-myogenic effects of IGF-1 require GSK3β inactivation (van der Velden et al., 2006). In support of this, blockade of GSK3β inhibition abrogates IGF-1 but not LiCl-dependent stimulation of myogenic mRNA accumulation (van der Velden et al., 2006).

The mechanism detailing how GSK3β negatively regulates myogenesis is not well understood. One report proposes that GSK3β phosphorylates the transcription factor NFATc3 and suppresses its nuclear localization thus inhibiting NFAT-dependent muscle-specific gene expression during myogenic differentiation (van der Velden et al., 2008). Importantly however, NFATc3 silencing does not completely block muscle gene expression, during differentiation, following GSK3β inhibition (van der Velden et al., 2008). Other factors that are negatively regulated by GSK3β may therefore contribute to enhanced differentiation in the absence of GSK3β activity (van der Velden et al., 2008).

Interestingly, *in silico* analysis of MEF2 proteins reveals several highly conserved GSK3 consensus sequences. Since MEF2 is involved in the various aspects of muscle development, it represents a possible target for GSK3β-mediated myogenic repression.

Chapter 2: STATEMENT OF PURPOSE

Previous work in our laboratory focused on systematically documenting MEF2 phosphorylation patterns in mammalian cells using mass spectrometry tools (Cox et al., 2003). These efforts resulted in the discovery of a crucial phosphoacceptor site, S255 that regulates the stability and function of MEF2A. Phosphorylation of S255 is detected in several cell types (C2C12, COS, and HeLa) and is mediated by endogenous kinases. S255 is part of a consensus sequence that is recognized by several kinases such as MAPK members, cdc2-like kinases and GSK3. Interestingly, S255 phosphorylation represses MEF2A transcriptional activity and targets MEF2A for degradation.

The ubiquitous phosphorylation of S255, coupled with its role in the degradation of MEF2, points towards a role for GSK3. This ubiquitous kinase is involved in numerous cellular signaling functions and is known to phosphorylate several proteins, which are subsequently targeted for degradation. Recent studies have identified GSK3β as a negative regulator of skeletal muscle hypertrophy and skeletal muscle differentiation. Since MEF2 is critically involved in differentiation, we hypothesized that GSK3β phosphorylates MEF2 and inhibits its activity and thus impedes myogenesis.

To address our hypothesis we conducted a series of preliminary experiments aimed at: (i) determining if MEF2A is a GSK3β substrate, (ii) assessing the effects of GSK3 on MEF2A function and, (iii) examining the effects of GSK3 on MEF2A expression.

Chapter 3: MATERIALS and METHODS

Plasmids and Antibodies

MEF2A, Gal4-MEF2A and pCMV-β-Gal expression plasmids and the 5xGal4-Luciferase reporter construct were previously described (Du et al., 2008; Ornatsky et al., 1999; Perry et al., 2009). pGL4-3xMEF2-Luc was made by J. Gordon from pGL3-MEF2-Luc with 2 additional copies of the MEF2 site inserted. The MCK-Luciferase enhancer construct was cloned into pcDNA3 (Invitrogen) by T. Miyake. The multiple point mutations of the MCK enhancer were generated by S. Pustylnik using the QuickChange kit (Stratagene) according to the manufacturer's instructions.

Antibodies used include the following: α-MEF2A rabbit polyclonal antibodies, produced with the assistance of the York University Animal Care Facility; α-Actin (Santa Cruz Biotechnology); α-α/β-Tubulin (Cell Signaling Technology); α-β-catenin (Cell Signaling Technology), α-P-β-catenin (Cell Signaling Technology), α-p38 (Cell Signaling Technology), α-P-p38 (Cell Signaling Technology), α-ATF-2 (Active Motif), α-P-ATF-2 (New England Biolabs (NEB)).

Cell Culture and Transfection

Mouse C2C12 myoblasts were cultured in growth medium (GM) consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (HyClone), 1% penicillin-streptomycin (Gibco), sodium pyruvate and L-glutamine. Cells were maintained in a humidified 37°C incubator with a 5% CO_2 atmosphere. Cells were passaged every 48h and monitored daily for appropriate morphology.

Transient transfections were performed using the calcium-phosphate precipitation method (see Appendix). Cells were harvested 48h after transfection. Drug treatments were performed 16-19h prior to harvesting.

Protein Extraction and Immunoblotting

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 sodium fluoride) supplemented with a protease inhibitor cocktail (Sigma, P-8340)), 0.2mM PMSF and 0.5mM sodium orthovanadate. Protein concentrations were determined by the Bradford assay (BioRad) using BSA as a standard.

Equivalent amounts of protein (15-20μg) were electrophoretically resolved by SDS-PAGE on 6-10% gels, followed by electrophoretic transfer to an Immobilon-P membrane (Millipore) as directed by manufacturer. Blots were processed and incubated with primary antibodies, α-Actin (1:2000), α-α/β-Tubulin (1:1000), α-MEF2A (1:200, 1:250) α-β-catenin (1:1000), α-P-β-catenin (1:1000), α-p38 (1:1000), α-P-p38 (1:1000), α-ATF-2 (1:1000), α-P-ATF-2 (1:1000), in 5% milk in PBS or TBST or 5% BSA in TBST as directed by manufacturer. Appropriate HRP-conjugated secondary antibody was diluted (1:2000) in 5% milk in PBS. Enhanced chemiluminescence reagent (Amersham) was used to detect secondary antibody bound to the membrane by exposure to Biomax film (Kodak).

The experiments in figures 5 and 6 (with the exception of the ATF-2 panel) were performed multiple times (at least twice) showing near-identical results as the data presented.

Transcriptional Response Assay

Transient transfections of C2C12 myoblasts were performed using the calcium-phosphate precipitation method (see Appendix). pCMV-β-galactosidase was used as an internal control for transfection efficiency. Reporter gene constructs and expression plasmids were transfected as indicated in figures. Briefly, 16-18h following transfection, cells were washed with PBS and allowed to recover in GM. Cells were incubated for indicated times and conditions before cellular extracts were prepared to determine luciferase activity using Luciferase substrate (Promega) as directed by manufacturer and a luminometer (Berthold Lumat, 9501).

All experiments using luciferase reporter plasmids were performed in triplicate with data presented as means ± standard errors of the means. The experiments in figures 2 and 3 were carried out multiple times showing near-identical results as the data presented.

***In vitro* Kinase Assay**

Briefly, 2.5μg of purified recombinant GST-MEF2A 1-507 was incubated with or without 0.5μg purified recombinant GST-GSK3β 1-433 (Cell Signaling Technology) and with [γ-^{32}P]ATP or unlabeled ATP (for MS analysis) for 30min, and denatured by heating for 5min at 95-100°C in SDS sample buffer. Protein samples were separated by 8-10%

SDS-PAGE, then either exposed to X-ray film (Kodak X-Omat) for 1-74h to detect ^{32}P incorporation or stained with Coomassie Blue to visualize proteins for MS analysis.

In-Gel Trypsin Digestion

Coomassie blue-stained protein bands were excised, cut into small pieces, washed with 50% acetonitrile-25 mM ammonium bicarbonate, and placed on a shaker for 15min (three times). Gel pieces were reduced by incubation with 10mM dithiothreitol in 50mM ammonium bicarbonate (freshly made) for 30min at 50°C and then washed twice with acetonitrile, followed by incubation with 55mM iodoacetamide (Sigma) in 50mM ammonium bicarbonate (freshly made) for 20min in the dark at room temperature (RT). The gel pieces were then washed twice with acetonitrile, air dried, rehydrated with 12.5 ng/μl trypsin (sequencing grade; Roche) in 50 mM ammonium bicarbonate at 4°C for 1h, and then incubated at 37°C overnight. After overnight incubation, peptides were further extracted using 3% formic acid at 70°C for 2min, followed by 15min of shaking. The samples were then concentrated by centrifugation at 13200rpm for 1min.

Matrix-assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

For MALDI-MS sample preparation, C_{18} μZipTips (Millipore) were used to desalt and concentrate tryptic peptides from the tryptic digests per the manufacturer's protocol (see Appendix). The sorbed peptides were then eluted from the ZipTips and spotted onto a MALDI target steel plate, using 1μl of matrix solution (10mg/ml α-cyano-4-hydroxycinnamic acid in 65% acetonitrile and 0.3% trifluoroacetic acid).

Peptide mapping and CID were performed on a hybrid quadrupole-TOF tandem mass spectrometer (Sciex Qstar XL; Applied Biosystems/MDS), using the positive-ion reflector mode. The hybrid quadrupole-TOF instrument was optimized and calibrated daily. CID resulted in MS/MS spectra from which sequencing and identification of phosphorylation sites were possible by searching against a nonredundant protein database (NCBI), using the search program MASCOT (Matrix Science).

Chapter 4: RESULTS

GSK3β directly phosphorylates MEF2A *in vitro*.

Studies have shown that GSK3β functions as a negative regulator of both cardiac and skeletal muscle hypertrophy as well as skeletal muscle differentiation (van der velden 2008). Since MEF2A is critically involved in muscle development, we postulated that the repressive effect of GSK3β on myogenic differentiation was mediated in part by direct phosphorylation of MEF2A. To investigate this possibility, we performed an *in vitro* kinase assay with GST-MEF2A 1-507 and purified GST-GSK3β. Results of the *in vitro* kinase assay showed that MEF2A was a probable, albeit weak substrate for GSK3β (Fig. 2).

GSK3 typically phosphorylates proteins at the consensus site (S/T)XXX*(S/T)*P (Doble and Woodgett, 2003). The first serine/threonine in the consensus is phosphorylated by GSK3. Although not absolutely required, GSK3 preferentially targets substrates that are first phosphorylated by another protein kinase at a serine/threonine residue, termed the priming phosphate, located four amino acids C-terminal to the GSK3 phosphorylation site (Cohen and Frame, 2001). An *in silico* analysis of MEF2 protein isotypes revealed several highly conserved GSK3 consensus sequences in the C-termini of MEF2A, -C and -D.

Based on this observation, and having determined that MEF2A is a direct target for GSK3β, we next sought to characterize the GSK3β phosphoacceptor sites in the MEF2A protein. In vitro kinase assays were performed and combined with mass spectrometry (MS) analysis. Briefly, MEF2A protein bands were excised and digested in-gel with trypsin. Proteins were identified by either tryptic mass fingerprinting or

collision-induced dissociation (CID) fragmentation. The MALDI-TOF spectrum for the tryptic digest of MEF2A contained a number of peaks whose masses matched the expected mass of peptides from MEF2A, thus identifying the protein as MEF2A. Further confirmation was obtained by CID fragmentation of selected peptides. The fragmentation products revealed short sequences and characteristic ions that could only be derived from specific MEF2A peptides. The analysis however failed to identify phosphopeptides.

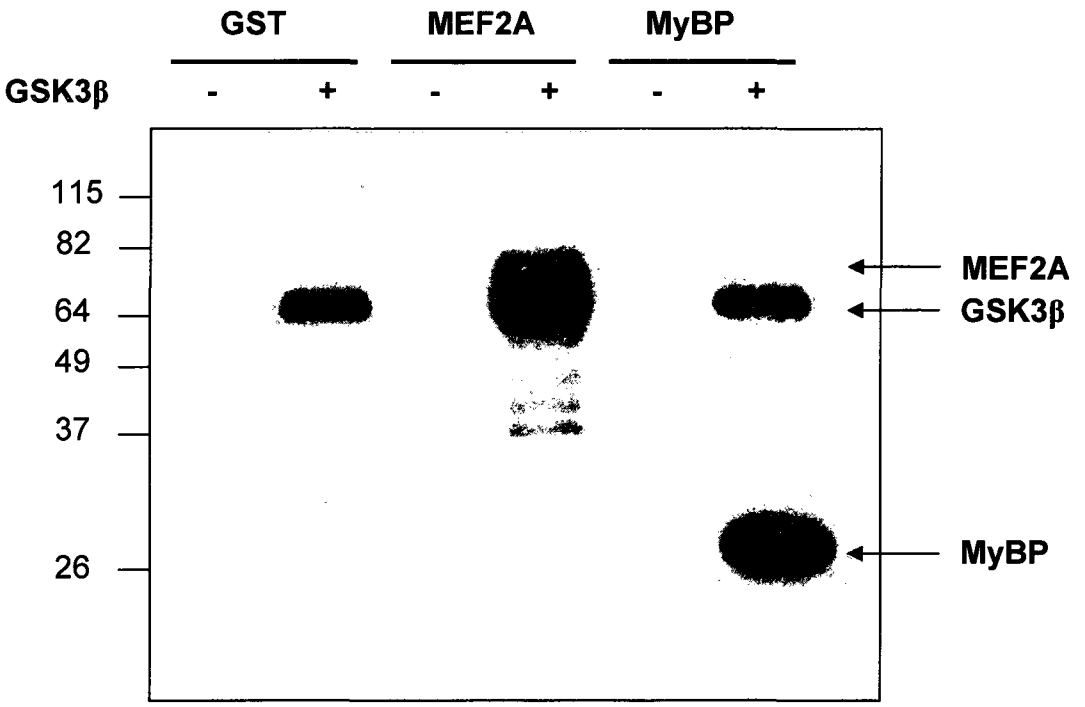

Figure 2: GSK3β phosphorylates MEF2A directly *in vitro*. Purified GST-MEF2A 1-507 was incubated *in vitro* with or without purified GST-GSK3β 1-433 and [γ-^{32}P]ATP. GST and myelin basic protein (MyBP) proteins were used as negative and positive controls respectively. Bands were resolved using SDS-PAGE and visualized by Coomassie Blue staining. Gels were dried and exposed to X-ray film for 21h after the assay. MEF2A, autophosphorylated GSK3β and MyBP are indicated by an arrow.

GSK3β inhibition enhances MEF2A transcriptional activity.

While attempting to map the MEF2A sites targeted by GSK3β, a series of experiments were performed in parallel to assess the role of GSK3β in relation to MEF2 function in myogenic cells. Given that GSK3β suppresses myogenic differentiation and that phosphorylation by GSK3β is generally inhibitory, we hypothesized that inhibition of GSK3β would enhance MEF2A transcriptional activity. To test our hypothesis, we utilized AR-A014418 (AR), a potent, highly-specific GSK3 inhibitor (Bhat et al., 2003). C2C12 cells transfected with MEF2A were treated with increasing concentrations of AR (1-50μM). MEF2 activity was assessed using a 3xMEF2-Luciferase construct, a MEF2-dependent reporter gene containing three copies of a consensus MEF2 enhancer element. Results showed that MEF2A transcriptional activity increases in a dose-dependent manner in response to GSK3β inhibition (Fig. 3). It was also observed that GSK3β inhibition enhanced MEF2-Luc activity when no MEF2A was transfected, an effect probably due to de-repression of endogenous MEF2 in these cells.

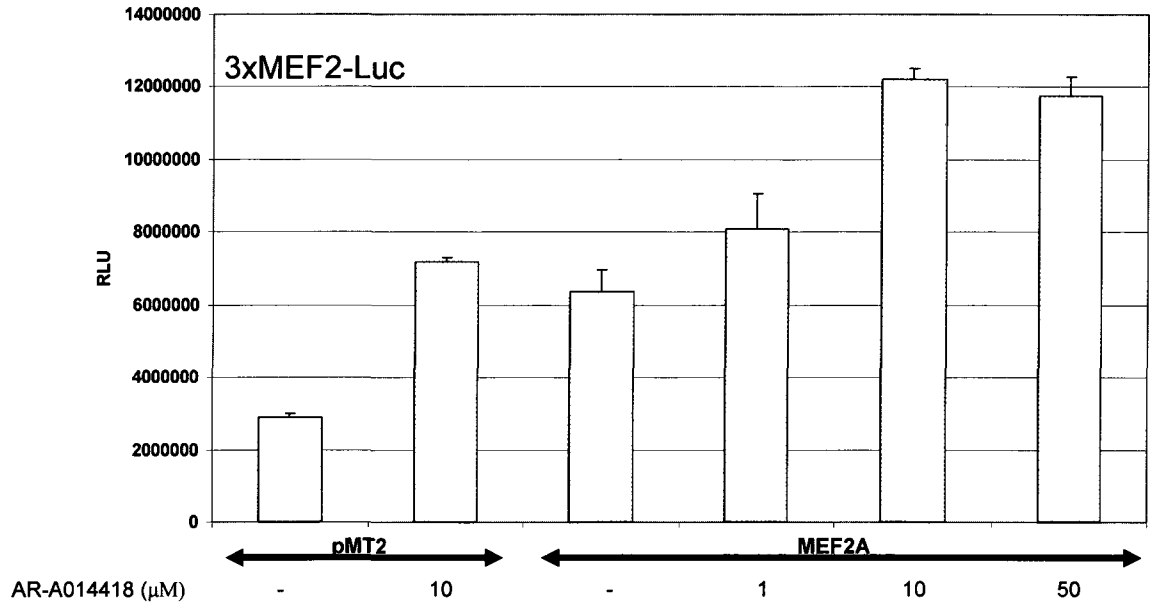

Figure 3: GSK3β inhibition enhances MEF2A transcriptional activity. C2C12 cells, maintained in growth medium (GM), were transiently transfected with either empty vector (pMT2) or pMT2-MEF2A and the pGL4-3xMEF2-Luc reporter gene. pCMV-β-gal was used as an internal control for transfection efficiency. Cells were treated with AR-A014418 (1-50μM) or solvent (DMSO) for 19h prior to harvesting. Luciferase activity was normalized to β-galactosidase activity and expressed as Relative Light Units (RLU). Data are the mean ± S.D. (n=3).

To further corroborate these findings, the effect of GSK3β inhibition on the muscle creatine kinase (MCK) enhancer, a physiological MEF2 target, was analyzed. Activation of the MCK enhancer is a useful index of muscle specific gene expression since it is highly dependent on MEF2, CArG box and E-box *cis* elements for its activation in myogenic cells. C2C12 cells were co-transfected with MEF2A and the MCK-Luciferase construct, or an alternate version of the construct containing mutated MEF2 binding sites (MCK-Luc ΔMEF2), and treated with AR. In the presence of the inhibitor, MCK-Luc activity was enhanced. However, when the MEF2 sites on the

enhancer were mutated, preventing MEF2 binding, its activity decreased (Fig. 4) demonstrating the specificity of the effect of GSK3β inhibition on MEF2 transcriptional activity.

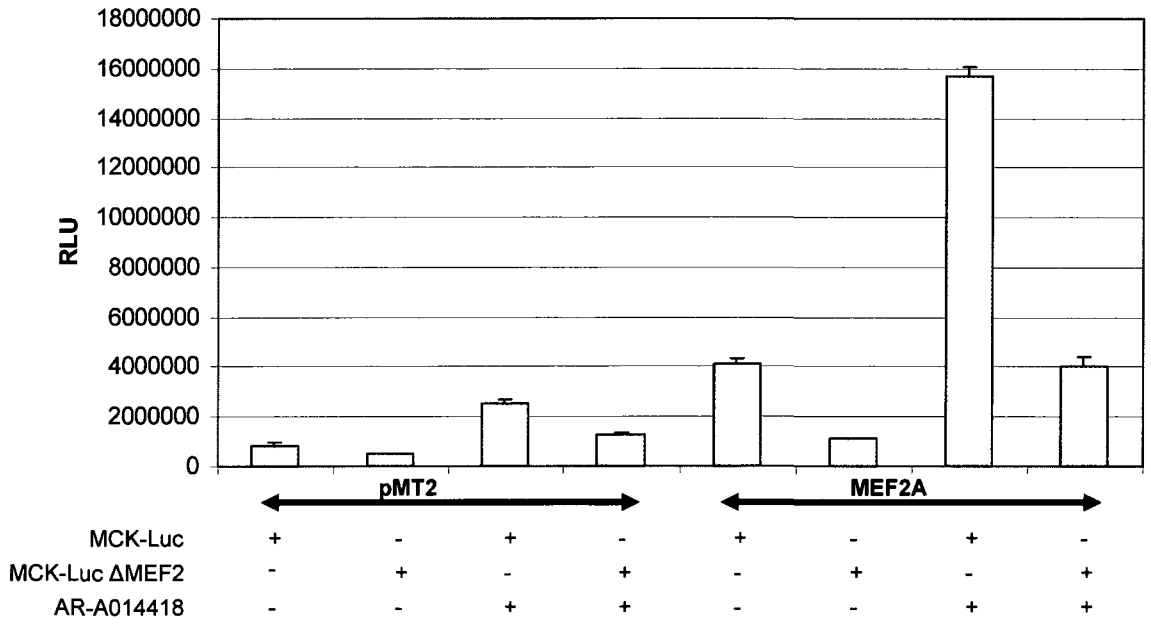

Figure 4: Inhibition of GSK3β enhances MEF2A-mediated transactivation of the MCK enhancer. C2C12 cells, maintained in GM, were transiently transfected with either empty vector (pMT2) or pMT2-MEF2A. Luciferase activity was assessed using either the intact MCK-Luc construct or an alternate version of the construct containing mutated MEF2 binding sites. Luciferase values were normalized to β-galactosidase activity. Cells were treated with AR-A014418 (10μM) or solvent (DMSO) for 19h prior to harvesting. Data are the mean ± S.D. (n=3).

We next attempted to determine if the effects of GSK3β inhibition that we observed on the wild type MEF2A protein were mediated by the MEF2A transactivation domain. To this end, we used a GAL4-based system. The N-terminus of MEF2A (amino

acids 1-92) containing the DNA-binding and dimerization domains was substituted by the DNA-binding domain of the yeast transcription factor GAL4, yielding a chimeric fusion protein: GAL4-MEF2A 92-507. Such a molecule would allow us to study the effects of the kinase on the activation domain of MEF2A without complication from the effects of other endogenous interacting proteins or changes in DNA-binding affinity. C2C12 cells were co-transfected with GAL4-MEF2A 92-507 and the 5xGal-Luciferase construct containing five copies of the GAL4 binding site. Results showed that suppression of GSK3β in cells treated with AR potentiates GAL4-MEF2A transcriptional activity when compared to GAL4-MEF2A activity in untreated cells (Fig. 5). The transactivation domain of MEF2A is therefore potentially regulated by GSK3β.

Taken together, data from these functional studies identified MEF2A as a putative downstream target of GSK3β signaling.

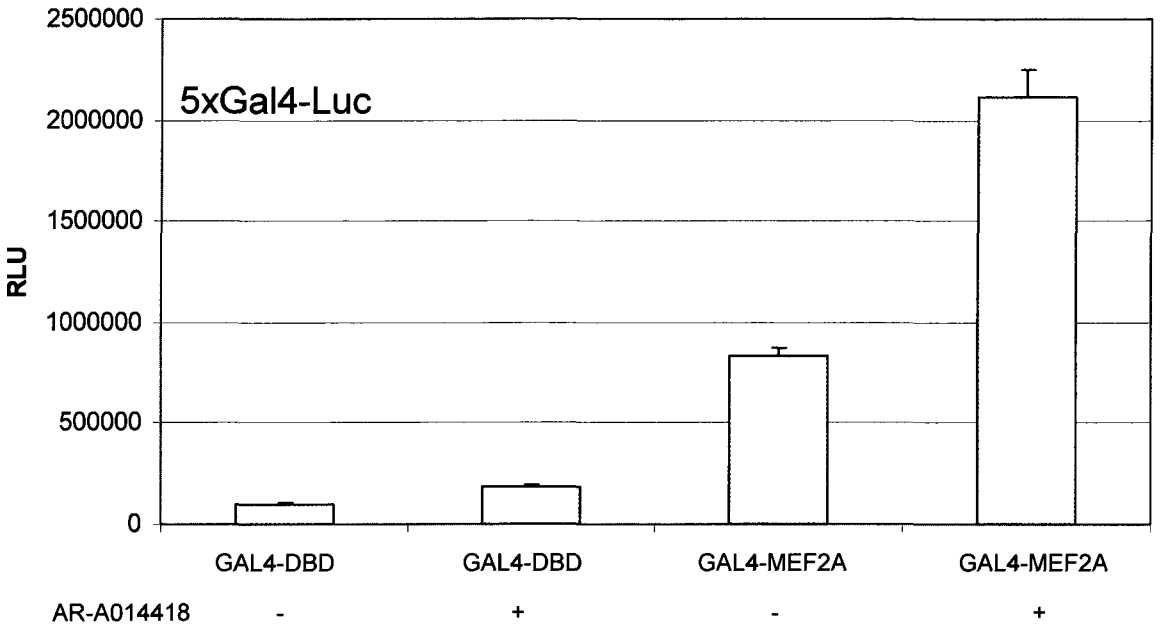

Figure 5: GSK3β inhibition enhances GAL4-MEF2A transcriptional activity. C2C12 cells, maintained in GM, were transiently transfected with either Gal4-DBD or Gal4-MEF2A (amino acids 92-507). Luciferase activity was assessed using a 5xGal4-Luc reporter gene and normalized to β-galactosidase activity. Cells were treated with AR-A014418 (10μM) or solvent (DMSO) for 19h prior to harvesting. Data are the mean ± S.D. (n=3).

GSK3β inhibition leads to a post-translational modification of MEF2A.

To further characterize the effects of GSK3β on MEF2A, we examined MEF2A expression levels when GSK3β activity is inhibited. C2C12 myoblasts cultured in growth medium were treated with varying concentrations (0.1-50μM) of the GSK3-specific inhibitor AR-A014418. Following treatment, endogenous protein expression levels were analyzed via Western immunoblotting. We confirmed the ability of AR to suppress GSK3β signaling by looking at the expression of β-catenin/phosho-β-catenin, a known GSK3β substrate targeted for degradation by phosphorylation at Ser33, 37 and Thr41

(Yost et al., 1996). At 50μM AR, phosho-β-catenin levels were substantially reduced compared to control indicating that GSK3β activity was repressed (Fig. 6). Interestingly, there was also a noticeable alteration in MEF2A's gel migration pattern at this concentration. Previous studies investigating the post-translational control of MEF2A have detected at least two forms of the protein by Western immunoblotting (Ornatsky et al., 1999); a high mobility (faster migrating), low molecular weight form and a low mobility (slower migrating), high molecular weight form that is post-translationally modified. At 50μM AR, the faster migrating band shifted and merged with the slower migrating band forming a single high molecular weight band which strongly suggested that MEF2A was post-translationally modified (Cox et al., 2003; Ornatsky et al., 1999) (Fig. 6). In addition to this, it is noteworthy that MEF2A expression was enhanced at 1 and 10μM AR.

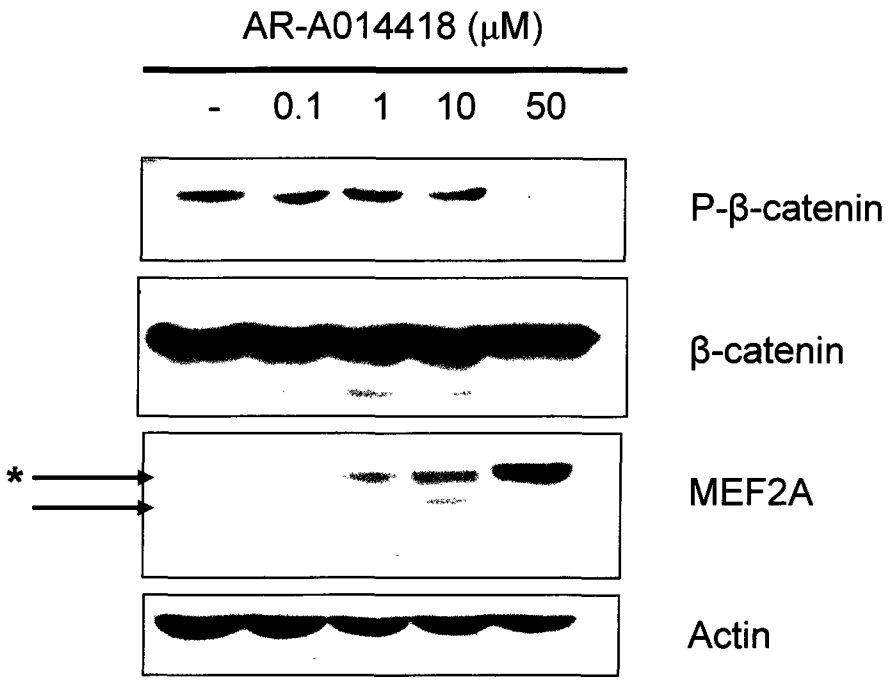

Figure 6: GSK3β inhibition leads to a post-translational modification of MEF2A. C2C12 cells were maintained in GM for 48h and subsequently treated for 4h with AR-A014418 (0.1-50μM) or solvent (DMSO). Following treatment, cells were lysed and total protein was extracted from cells. Equal amounts of protein (20μg) were used for Western blotting analysis. The levels of the indicated proteins were assessed by a standard immunoblotting technique with a specific primary antibody. Actin was used as a loading control. The arrows indicate MEF2A protein. The arrow with an asterisk points to a low mobility, high molecular weight form of MEF2A that is post-translationally modified.

GSK3β inhibition activates p38 MAP Kinase

The alteration in MEF2A's gel-mobility pattern upon GSK3β inhibition indicated that the protein was post-translationally modified. Previous reports have demonstrated that MEF2 is targeted by phosphorylation by multiple kinase signaling cascades. We thus postulated, based on our earlier examination of the interaction between MEF2A and the p38 MAP kinase (Cox et al., 2003), that MEF2A was being phosphorylated by p38 upon

GSK3β inhibition. We performed a time course analysis in which C2C12 cells, cultured in growth medium, were treated for various time intervals with AR. Following treatment, cells were harvested and endogenous protein levels were analyzed via Western immnunoblotting. β-catenin/phospho-β-catenin expression was used as a positive control to verify GSK3β inhibition. As shown in Fig. 7, p38 is activated when GSK3β is suppressed. Phospho-p38 levels rose after 1/2hr treatment with AR and remained above baseline after 2, 4, 8 and 24h treatment. Furthermore ATF-2, a known downstream target of p38 phosphorylated at Thr69 and Thr71, was also activated after 1/2h treatment. Phospho-ATF-2 levels paralleled phospho-p38 levels up until the 8hr time period, providing further evidence of p38 activity in cells treated with the inhibitor. When we examined MEF2A expression, we noticed an upward shift and enrichment of the upper band of the MEF2A doublet after the 1/2h mark (Fig. 7). This change in gel-mobility was more pronounced after 4h, consistent with our previous result. The concurrent activation of p38 and post-translational modification of MEF2A following AR treatment lend support to our hypothesis that p38 is the kinase that targets MEF2A when GSK3β is inhibited.

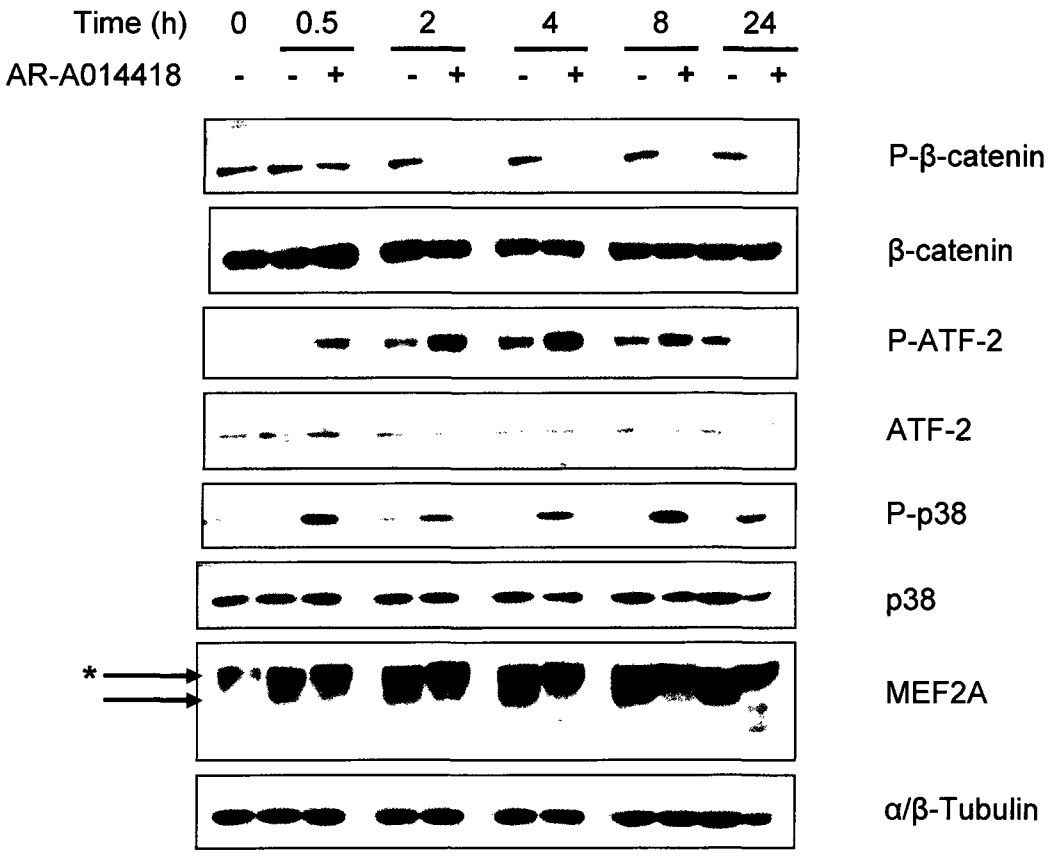

Figure 7: GSK3β inhibition activates p38 MAPK. C2C12 cells were maintained in GM for 48 hours and subsequently treated for 1/2-24h with 50μM AR-A014418 or solvent (DMSO). Following treatment, cells were lysed and total protein was extracted from cells. 15-20μg of protein was used for Western blotting analysis. The levels of the indicated proteins were assessed by a standard immunoblotting technique with a specific primary antibody. α/β-Tubulin was used as a loading control. The arrows indicate MEF2A protein. The arrow with an asterisk points to a low mobility, high molecular weight form of MEF2A that is post-translationally modified.

Chapter 5: DISCUSSION

It is well established that GSK3β signaling suppresses skeletal muscle differentiation, although the exact molecular mechanism has not yet been precisely defined. In this report, we document a novel convergence of GSK3β signaling on the MEF2A transcriptional regulator during skeletal myogenesis. These studies characterize an inhibitory effect of GSK3β on the transactivation properties of MEF2A leading to repression of MEF2-dependent gene transcription.

Results from our *in vitro* kinase assay showed that GSK3β directly phosphorylates MEF2A. However, phosphate incorporation was weak and mass spectrometry analysis failed to detect phosphopeptides. A possible reason for this result is that a priming phosphorylation event may be necessary to stimulate GSK3β activity. In our assay, MEF2A was bacterially expressed and subsequently GST-purified and therefore did not contain any prior physiological modification that could be crucial for GSK3β action. Although not strictly required, priming phosphorylation greatly increases the efficiency of substrate phosphorylation of most GSK3 substrates by 100-1000-fold (Doble and Woodgett, 2003). For example, glycogen synthase, requires priming phosphorylation by CK2 and then undergoes multiple rounds of phosphorylation by GSK3 (Doble and Woodgett, 2003).

Functionally, it appears that GSK3β represses MEF2 transcriptional activity. Reporter assays performed with the 3xMEF2-Luc construct and with the MCK-Luc construct demonstrate that MEF2A-mediated transactivation is enhanced when C2C12 cells are treated with AR-A014418, a GSK3-specific inhibitor (Fig. 3, 4). Furthermore,

GAL4-MEF2A 92-507 activity is enhanced in the presence of AR (Fig. 5) which suggests that the inhibitory effect of GSK3β on MEF2A is mediated by the MEF2A transactivation domain. Since the majority of predicted GSK3β phosphoacceptor sites are situated in the C-terminus of MEF2A, between amino acids 92 and 507, we hypothesize that GSK3β targets a site in this region and suppresses MEF2A activity. Additional experiments will be needed to confirm this. Alternatively, GSK3β could mediate its inhibitory effect on the MEF2A TAD through another kinase or through a mechanism that does not require phosphorylation but rather is dependent on disruption of a crucial protein:protein interaction. For example, upon dephosphorylation by calcineurin, NFAT translocates to the nucleus where it directly associates with both MEF2A and MEF2D (Blaeser et al., 2000). NFAT stimulates MEF2 transactivation by facilitating recruitment of p300 to MEF2-response elements (Youn et al., 2000). GSK3β phosphorylates NFAT and promotes its translocation to the cytoplasm which could potentially abrogate the stimulatory effect of NFAT on MEF2 transcriptional activity.

Treatment of C2C12 cells with low concentrations of AR (1-10μM) stabilizes MEF2A expression (Fig. 6) which is another possible explanation for the increase in MEF2A activity observed upon GSK3 inhibition. Intriguingly, treatment of cells with a higher concentration of AR (50μM) causes MEF2A to be post-translationally modified (Fig. 6). We strongly suspect this modification to be phosphorylation based on the characteristic banding pattern exhibited by MEF2A (Cox et al., 2003; Ornatsky et al., 1999). Data from our time course analysis (Fig. 7) further reinforces the prospect that MEF2A is phosphorylated. Treatment of cells for 1/2h with AR causes an upward shift

and enrichment of the upper band of the MEF2A doublet. Kinetically, such a rapid response rules out effects of AR on gene transcription and translation and points to a PTM. Together with our findings that GSK3β inhibition enhances MEF2A transactivation, results from the time course analysis suggest that the post-translational modification of MEF2A, in response to GSK3β inactivation, has a stimulatory effect on its function.

The observation that GSK3β inhibition possibly leads to phosphorylation of MEF2A indicates that a GSK3β-responsive kinase targets MEF2A in the absence of GSK3β signaling. Data from our time course analysis show that the p38 MAPK is activated in response to GSK3β inhibition and that it targets downstream substrates such as ATF-2. Moreover, its activation coincides with the post-translational modification of MEF2A which leads us to postulate that it is the kinase that targets MEF2A. Indeed, p38 is known to phosphorylate MEF2 and to activate MEF2-dependent gene transcription (Ornatsky et al., 1999). Interestingly, p38 and JNK have been shown to be negatively regulated by GSK3β via MEKK4 in COS-7 cells (Abell et al., 2007). MEKK4 is a MAP3K that functions in neurulation and skeletal patterning during mouse embryogenesis (Abell et al., 2007). It phosphorylates and activates MKK3/MKK6 which is an upstream activator of p38. GSK3β binds to MEKK4 and blocks its dimerization, which is required for activation (Abell et al., 2007). This effectively inhibits MEKK4 stimulation of the p38 and JNK pathways. Inhibition of GSK3β activity results in enhanced MEKK4 kinase activity and increased p38 activation (Abell et al., 2007). Whether the MEKK4>MKK3/MKK6>p38 signaling axis exists in muscle cells, such as

C2C12s, remains to be determined. Nevertheless, this study raises the possibility that GSK3β inhibition stimulates p38 activation, through MEKK4 de-repression, which would result in MEF2A phosphorylation.

Based on our findings we propose a model wherein GSK3β inhibition prevents negative regulation of the C-terminus of MEF2A, which results in de-repression of MEF2A. Concurrent with GSK3β inhibition, activation of a GSK3β-responsive kinase, such as p38, leads to phosphorylation of MEF2A and augmentation of its transcriptional activity. It is conceivable that another GSK3β-responsive kinase besides p38 targets MEF2A, nevertheless p38 remains a leading candidate as it is known to phosphorylate MEF2 and to activate MEF2-dependent transcription (Ornatsky et al., 1999).

One limitation of the current study is that all data regarding inhibition of GSK3β were obtained by pharmacological inhibition using AR-A014418. Although AR is a potent and highly specific GSK3 inhibitor (Bain et al., 2007; Bhat et al., 2003) that was shown to significantly decrease GSK3 activity in our experiments, we cannot rule out nonspecific effects of AR on other kinases or on certain aspects of cell physiology. Furthermore, we do not know if AR can distinguish between the two major isoforms of GSK3, GSK3α and -β. The importance of isoform-specific effects in our data is thus unknown. Previous studies investigating the role of GSK3 in skeletal muscle all focused on the -β isoform, and certain key results (see p.22) suggest that it is the predominant isoform in that context. In a recent publication, Force et al. outlined some unique and overlapping functions of GSK3 isoforms in cell proliferation and differentiation as well

as in cardiovascular development (Force and Woodgett, 2009). Whether or not GSK3α has a role that overlaps with GSK3β in the regulation of muscle development is still unknown.

Several studies have identified GSK3β as a negative regulator of cardiac muscle hypertrophy. In neonatal rat cardiomyocytes, GSK3β is inactivated by hypertrophic stimuli via a PI3K-dependent protein kinase that phosphorylates GSK3β on ser 9 (Haq et al., 2000). However, adenovirus-mediated gene transfer of a constitutively active form of GSK3β (containing an S9A mutation which prevents inactivation by hypertrophic stimuli), markedly inhibits the hypertrophic response of cardiomyocytes (Haq et al., 2000). Consistent with these results, transgenic mice that express a constitutively active form of GSK3β, under control of a cardiac-specific promoter, are physiologically normal under non-stressed conditions, but their ability to mount a hypertrophic response to calcineurin activation (a potent inducer of myocardial hypertrophy) is severely impaired (Antos et al., 2002). Finally, cardiac-specific expression of activated GSK3β diminishes hypertrophy in response to chronic β-adrenergic stimulation and pressure overload (Antos et al., 2002). GSK3β is thought to regulate the hypertrophic response, at least in part, by modulating the nuclear/cytoplasmic partitioning of NFAT (Antos et al., 2002; Haq et al., 2000).

In contrast to GSK3β, MEF2 has been shown to mediate stress-dependent pathological cardiac hypertrophy, which is characterized by: hypertrophic growth of cardiomyocytes, assembly of additional sarcomeres to enhance contractility, and activation of a fetal cardiac gene program (Kim et al., 2008). Experiments using

transgenic mice harboring a MEF2-dependent reporter gene, have shown that hypertrophic stimuli augment MEF2 activity in the adult heart (Kim et al., 2008). Furthermore, stress-activated signaling pathways that culminate in the activation of CaMK and PKD, drive pathological cardiac remodeling and enhance MEF2 transcriptional activity by promoting the phosphorylation and nuclear export of class II HDACs (Vega et al., 2004).

Our study has implicated GSK3β in the negative regulation of MEF2 activity. Taking into account the findings just presented, our result may prove to have important ramifications in cardiac muscle. Since MEF2 mediates stress-dependent cardiac hypertrophy, according to our model, elevation of GSK3β levels in the heart could potentially repress MEF2 function and circumvent the chain of events that leads to pathological cardiac remodeling.

In summary, we have documented a novel convergence of GSK3β signaling on the MEF2A transcriptional regulator during skeletal myogenesis. GSK3β inhibition enhances MEF2A transcriptional activity, activates p38 MAPK and causes a post-translational modification of MEF2A (Fig. 8). Taken together, these results suggest that p38 targets MEF2A in the absence of GSK3β signaling. The role of GSK3β in relation to MEF2 may have important implications for skeletal muscle differentiation and also for cardiac hypertrophy.

Future work will be aimed at confirming our findings both in cell culture models and *in vivo* using a combination of genetic and pharmacological approaches. A crucial

step will be to ascertain whether MEF2 is in fact a physiological GSK3β substrate. In this regard, Cohen et al. have outlined a stringent set of criteria that help determine if a protein can truly be regarded as a substrate for GSK3β (Cohen and Frame, 2001). These criteria form the experimental framework for part of our future investigations.

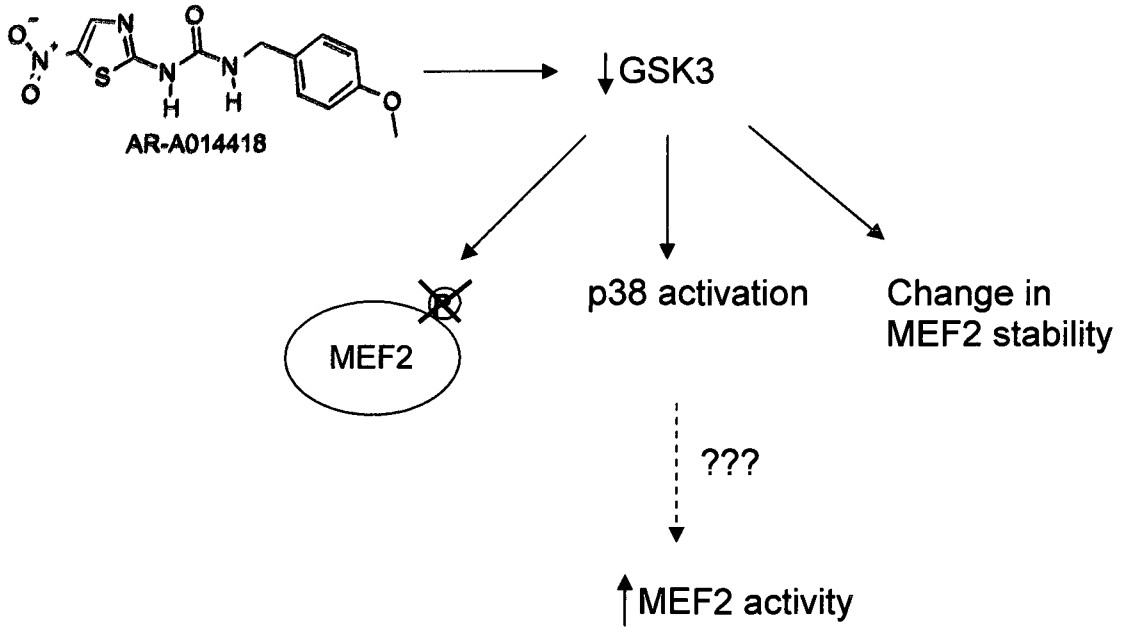

Figure 8: Summary. GSK3 inhibition by AR-A014418 results in increased MEF2 transcriptional activity through a mechanism that may involve the abrogation of MEF2 phosphorylation by GSK3, p38 activation or a change in MEF2 stability or a combination of the aforementioned.

Appendix: METHODS

Transformation of Bacterial Cells by Electroporation

NB: All Reagents should be kept on ice (except when growing bacteria). Reagents should be autoclaved. For higher efficiency transformation use freshly prepared competent cells.

Preparation of Competent Bacteria

1. Inoculate DH5α bacteria strain (single colony) into 2ml of LB media without antibiotics for 16h.
2. Add overnight culture to 100ml LB and incubate at 37°C with shaking for ~4h. (until OD600 = 0.4 - 0.6)
3. Transfer culture into two, sterile, 50ml falcon tubes.
4. Incubate on ice for 30min.
5. Pellet bacteria by centrifuging for 15min at 3600rpm and 4°C.
6. Discard supernatant, resuspend pellet first in 25ml ddH_2O and then add 25ml more ddH_20.
7. Repeat steps 5, 6 and 5 again.
8. Discard supernatant. Resuspend pellet in 25ml of 10% glycerol.
9. Repeat step 5.
10. Discard supernatant. Resuspend bacteria in 400-800µl of 10% glycerol.
11. Aliquot 50µl of competent cells into pre-chilled Eppendorf 1.5ml tubes.
12. Store bacteria at -80°C.

Clean centrifuge after all spins are done

Transformation by Electroporation

1. Pipette 1-3µl of DNA (10-100ng) into 50µl of ice-cold competent DH5α.
2. Apply correct settings to electroporator: Resistance = 200Ohms, Capacitance = 25µF, Voltage = 2.25kV.
3. Lay out materials in order to work efficiently. (Keep cuvette holder in freezer)

4. Pipette ~65µl into cuvette, wipe condensation, tap and put into holder.
5. Slide cuvette holder into place and hold red buttons until beeping sound. (Voltage between 4.85-5.00)
6. Add 1ml of LB to cuvette.
7. Remove LB with Pasteur pipette and place in culture tube.
8. Recover at 37°C with shaking for 1h. (warm plates for 1h)
9. Pellet cells by centrifugation, remove supernatant (leave 100µl) and, spread bacteria on warm LB-Agar plates.

Cell Culture

The C2C12 cell line was used in the aforementioned studies. What follows are general cell culture guidelines. Recommendations by the American Type Culture Collection (ATCC) were also adhered to.

Reagents:

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

NaCl 8g
KCl 0.2g
Na_2HPO_4 7H2O 1.44g
KH_2PO_4 0.24g
up to 800ml, pH to 7.4 with HCl, top to 1L

Versene

0.2g EDTA in 1L 1x PBS

0.125% Trypsin-EDTA (Gibco) diluted in Versene

DMEM supplemented with *Penicillin-Streptomycin* (Gibco) and *L-glutamine* (Gibco) added as required

Freezing medium: Growth medium in which the cells are normally cultured, supplemented with 10% DMSO; sterilize the freezing medium by passing through a 0.2μm filter.

FBS, heat inactivated at 56°C for 30min

HS, heat inactivated at 56°C for 30min

Methods:

Cell Passaging

1. Remove medium from dish.
2. Rinse the cell monolayer briefly with 5ml of Versene. (this step is necessary to remove any traces of serum, which could inactivate the Trypsin)
3. Add 0.5ml of 0.125% Trypsin-EDTA solution to 100mm dish, incubate at 37°C for 1-5min.
4. Inactivate the Trypsin by adding 4.5ml of DMEM.

5. Pipette the cells up and down several times to ensure complete removal of the cells from the dish and to dissociate clumps of cells; transfer cells to 15ml conical tube.

6. Pellet the cells by centrifugation for 5min at 1500rpm, aspirate the supernatant and vigorously flick tube to detach the cell pellet from the bottom of the tube.

7. Resuspend cells in 5-10ml of growth medium.

8. Count the cells in a haemocytometer (optional) and seed a dilution of cells that allows for future growth; incubate in new culture dishes.

Induction of Muscle Cell Differentiation

1. When cells have reached 60-80% confluence, deplete of growth factors by gently washing cells with PBS/DMEM and re-feeding with 5% HS in DMEM.

2. Incubate cells for desired time at 37°C and 5% CO_2.

Freezing Cells

1. Prepare a cell suspension and pellet the cells by centrifugation for 5min at 1500rpm.

2. Resuspend the cells in freezing medium at a concentration of $1x10^6$ - $8x10^6$ cells/ml.

3. Dispense 1ml of cell suspension into each freezing vial.

4. Place vials into a polystyrene box.

5. Place box in -80°C freezer overnight.

6. For long term storage place vials in liquid nitrogen.

Thawing Frozen Cells

1. Remove vial from the liquid nitrogen and thaw in 37°C water bath.

2. Dissociate clumps of cells using a Pasteur pipette.

3. Transfer to a 15ml conical tube containing 5ml of medium.

4. Centrifuge for 5min at 1500rpm, aspirate the supernatant and vigorously flick tube to detach the cell pellet from the bottom of the tube.

5. Resuspend cells in 5-10ml of growth medium.

6. Count cells in haemocytometer

7. Plate cells at 10^6 cells/100mm dish in 10ml of growth medium.

Transient Transfection of Mammalian Cells with DNA

Calcium/Phosphate-mediated transfections were performed in 35 or 100mm cell culture dishes. Below are guidelines for transfection in 100mm plates. Reagents were scaled in proportion to surface area for transfection in 35mm plates.

Reagents:

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

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

2.5M $CaCl_2$

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

Methods:

Transient transfection of adherent cells with Calcium

1. Plate cells 24h prior to transfection so that they are 30-50% confluent at time of transfection.

2. Re-feed cell cultures with growth medium 3h prior to addition of DNA.

3. Label sterile tubes and add 0.5ml of 2x HEBS to each tube.

4. Prepare DNA-$CaCl_2$ solution as follows: add 450µl ddH_20 to labelled Eppendorf tubes, add 25µg DNA, mix, add 50µl 2.5M $CaCl_2$, mix.

5. While vortexing HEBS at low speed, add DNA-$CaCl_2$ solution dropwise.

6. Add DNA mix dropwise to cell cultures.

7. 16h following addition of DNA, wash cells twice with 5ml PBS and re-feed with growth medium. Wait at least 4h before experimentally treating cells (e.g.: drug treatment).

Luciferase Assay

Luciferase assays were performed with commercially purchased substrate (Promega). All reporter assays were performed with cells cultured in 35mm dishes.

Reagents:

Lysis buffer

20 mM Tris, pH 7.4
0.1% Triton-X 100

Luciferase substrate (Promega)

Methods:

Harvesting

1. Remove medium from dish, wash adherent cells twice with 2ml ice-cold 1xPBS, aspirate remaining PBS.
2. Add 300µl of lysis buffer to each well.
3. Incubate dish on rocker at 4°C for 15min.
4. Gently scrape cells with rubber policeman and collect into labelled, pre-chilled Eppendorf tubes.
5. Incubate tubes on ice and vortex periodically over a period of 30min.
6. Spin tubes in microcentrifuge at 4°C for 10min at 13200rpm.
7. Transfer supernatant to new set of pre-chilled Eppendorf tubes.
8. Freeze cell lysates at -80°C until ready to be analyzed.
9. Thaw lysates on ice and transfer 100µl to luciferase assay tube.

β-Galactosidase Assay

Reagents:

ONPG (4 mg/ml in ddH_2O)

Z buffer

16.1 g Na2HPO4-7H2O (60mM)
5.5 g NaH2PO4-H2O (40mM
0.75 g KCl (10mM)
0.246 g MgSO4-7H2O (1mM)
Add 800 ml ddH_2O, pH to 7.0, bring volume up to 1L, 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 plus one blank.

1 M Na_2CO_3

Methods:

Aliquot 50-100µl of lysate into new set of Eppendorf tubes, add 600µl of β-Galactosidase reaction mix.

1. Incubate tubes at 37°C until a slight color change is apparent (yellow).
2. Add 300µl of 1M Na_2CO_3 to each tube to terminate reaction.
3. Transfer samples to spectroscopy cuvette and measure absorbance of samples at 420nm.

Harvesting Cells for Western Immunoblotting

Keep protein samples cold at all times (unless otherwise indicated). Whole cell extracts were prepared as follows:

Reagents:

1xPBS (keep cold)

Modified NP-40 Lysis Buffer (100ml)

5ml 1M Tris, pH 8.0
3ml 5M NaCl
0.4ml 0.5M EDTA
20ml 0.5M NaF
10ml 100mM Tetrasodium Pyrophosphate
0.5% NP-40
200µl 0.5M Na_3VO_4 (sodium orthovanadate)
200µl 0.5M PMSF (add fresh)
Protease inhibitor cocktail (add fresh, Sigma, P-8340)
Make up to 100ml with ddH_2O.

4xSDS sample buffer

Add β-mercaptoethanol right before use.

Methods:

1. Remove medium from dish, wash adherent cells twice with 5ml of wash solution (1xPBS containing 0.1M NaF and 0.5M NaV), aspirate remaining PBS.
2. Add 1ml wash solution to each dish.
3. Gently scrape cells with rubber policeman and collect in labelled, pre-chilled Eppendorf tubes.
4. Centrifuge tubes at 13200rpm for 10sec.
5. Aspirate supernatant, approximate volume of the cell pellet and dilute with five times the volume of lysis buffer.
6. Vortex tubes briefly every 5-10min for 30min.
7. Centrifuge tubes at 13200rpm for 10min at 4°C, transfer supernatant to new set or pre-chilled Eppendorf tubes.

8. Determine protein concentration by Bradford assay and dilute protein samples to equal concentration (0.5μg/μl – 2.0μg/μl) with equal amounts of 4x SDS sample buffer.

9. Boil samples for 5min (95-100°C), chill on ice for 1min, spin tubes for 10sec in table-top microcentrifuge.

10. If immediately loading gel, keep samples at RT. If samples are to be later analyzed, store at -80°C.

SDS-PAGE

Reagents:

PBS

10% Resolving gel (15ml)

ddH_20 5.9ml
1.5M Tris pH 8.8 3.8ml
30% acrylamide mix 5ml
10% SDS 0.15ml
10% APS 0.15ml
TEMED .006ml

Stacking gel (4ml)

ddH20 2.7ml
1.0 M Tris pH 6.8 0.5ml
30% acrylamide mix 0.67ml
10% SDS 0.04ml
10% APS 0.04ml
TEMED 0.004ml

10xLaemmli (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

Methods:

1. Prepare resolving gel and then top with stacking gel with appropriate comb inserted in Hoefer mini-gel apparatus.

2. Fill electrophoresis apparatus with 1xLaemmli buffer.

3. Load samples.

4. Run gel at 100V through stacking gel and 100-150V through running gel

Western Immunobloting

Reagents:

Transfer buffer (100ml)
Methanol 20ml
1X Laemmli 80ml

Prepare blocking buffer, washing solutions, ECL, and antibody diluent as per manufacturer's instruction.

Methods:

1. After SDS-PAGE, transfer protein to Immobilon-P (Millipore) membrane by wet transfer at 20V for 14-18h. Subsequently, increase voltage to 50V for 30min.
2. Block membrane with 5% (w/v) skim milk powder in 1xPBS/TBS for 1hr.
3. Incubate membrane O/N with primary antibody diluted 1:200-1:1000 in blocking buffer at 4°C on nutator.
4. Wash membrane with 1xPBS/TBST (3x 5min).
5. Incubate membrane with secondary antibody diluted 1:2000 in blocking buffer for 1h at RT.
6. Wash membrane with 1xPBS/TBST (3x 10min).
7. Develop blot with chemiluminescence reagent, expose blot to film, develop.

***In vitro* Kinase Assay**

Methods:

1. Mix 2.5μg of purified recombinant GST-MEF2A 1-507 with 0.5μg purified recombinant GST-GSK3β 1-433 (Cell Signaling) and with [γ-^{32}P]ATP or unlabeled ATP (for MS analysis) in 1.5ml Eppendorf tube.
2. Incubate for 30min at 37°C
3. Denature samples by heating for 5min at 95-100°C in SDS sample buffer
4. Separate protein samples by 10% SDS-PAGE and then either expose to X-ray film (Kodak X-Omat) for 1-74h to detect ^{32}P incorporation or stain with Coomassie blue to visualize proteins for MS analysis.

In-Gel Digestion of Proteins for Mass Spectrometry Analysis

Coomassie staining of polyacrylamide gels

1. Wash gel 3x 5min with ddH_2O.
2. Stain gel O/N in GelCode Blue (Pierce).
3. Destain gel 1-24h in ddH_2O.

Enzymatic Digestion

1. Use HEPA-filtered sterile hood.
2. Cut out desired band and dice into small pieces (approximately $1mm^3$)
3. Wash with 200µl of HPLC grade H_2O for a few minutes, remove water.
4. Add 200µl of 50% acetonitrile/25mM ammonium bicarbonate for 15min with shaking, remove solution.
5. Repeat two more times and remove solution.
6. Add 75µl of 50mM ammonium bicarbonate + 10mM DTT, incubate at 50°C for 30min.
7. Wash twice with acetonitrile, remove solution.
8. Add 75µl of 50mM ammonium bicarbonate + 55mM iodoacetamide (freshly made), incubate for 20min in the dark at RT.
9. Wash twice with acetonitrile until gel particles are white and stuck together.
10. Remove all solution and air dry in HEPA filtered hood (do not overdry).
    Make Trypsin solution:
    -12.5ng/µl trypsin (Promega sequencing grade) in 50mM ammonium bicarbonate
    -aliquot and freeze at –80°C
11. Add enough trypsin solution (~15µl) to rehydrate gel pieces. Rehydrate for 30-45min at 4°C.
12. Add enough buffer (25 mM ammonium bicarbonate) to keep gel pieces hydrated O/N.
13. Incubate at 37°C for 16h.

14. Wash outside of tube.

15. Transfer liquid to clean tube (keep pipette tip). Add 15µl of 3% formic acid to gel pieces and extract by shaking at 37°C for 15min (or heating at 75°C for 2min and shaking for 15min at RT).

16. Centrifuge at max speed for 2-5min.

17. Transfer extraction solution using same pipette for each sample and pool with liquid from step 17.

18. (OPTIONAL) Repeat 17-19.

19. Concentrate sample using a Zip tip and elute entire sample onto target.

Peptide Enrichment for Mass Spectrometry (ZIP TIP)

Reagents:

Activation Solution
60% ACN

Wash Solution
3 % Formic acid

Elution Solution
65 % ACN
10 mg/ml α-CN
3 µl/ml TFA

NB: Only pipette to first stop, do not introduce air bubbles into resin.

Methods:

1. Set up three microcentrifuge tubes for each sample.
2. Protein sample/tube 1, 100µl wash solution/tube 2, 10µl elution solution/tube 3.
3. Set pipette to 15µl and pipette Activation solution up and down 3x.
4. Pipette Wash solution up and down 3x.
5. Pipette protein sample up and down 10x.
6. Pipette Wash solution up and down 3x.
7. Pipette 1µl of elution solution, up and down on plate 3x (do not touch plate).

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