Glycogen Synthase Kinase 3β mediated regulation of skeletal myogenesis and alveolar rhabdomyosarcoma

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Abstract

Myogenic differentiation is a critical and highly regulated process that occurs during embryogenesis. Many studies have investigated the role of Muscle Regulatory Factor (MRF) and Myocyte Enhancer Factor 2 (MEF2) proteins in orchestrating myotube formation. More recently phosphorylation of these transcription factors has been shown to influence their ability to regulate muscle gene expression and hence control myogenic differentiation. One kinase that has been shown to prevent myogenic differentiation is GSK3β, although the precise mechanism by which this occurs is poorly understood. Alveolar rhabdomyosarcoma (ARMS) is a highly malignant mesenchymal tumor that originates from immature striated muscle tissue that does not properly differentiate despite the expression of MRF and MEF2 factors. The overall purpose of my research was to better understand how GSK3β regulates skeletal myogenesis and to explore the molecular basis for the lack of myogenic differentiation in ARMS. To achieve this objective, a variety of loss and gain of function studies were conducted in several tissue culture models, to assess the effect of GSK3β on MEF2 and MRF transcriptional activity as well as two MEF2 target genes: KLF6 and Myogenin, which are involved in myoblast proliferation and cell fusion (and hence differentiation) respectively.

Although an *in vitro* kinase assay revealed that MEF2A was not a GSK3β substrate, inhibition of GSK3 resulted in enhanced MEF2 activity and that regulation of MEF2 by GSK3β was indirect, through p38 MAPK: a potent activator of MEF2. This first study documented that cross-talk between p38 MAPK and GSK3β signaling converges on MEF2 activity in skeletal and cardiac muscle both *in vitro* and *in vivo*.

A paradoxical feature of ARMS is the expression of Myogenin, a MRF that regulates cell fusion and hence terminal differentiation. Upon confirming that GSK3β was predominantly un-

phosphorylated and hence de-repressed in PAX3-FOXO1 expressing ARMS cells, the second study revealed that Myogenin was also a GSK3β substrate *in vitro*. Mutation of the S160, S164 GSK3 phosphoacceptor sites resulted in (i) a decrease in a phosphorylated form of Myogenin by western blot analysis, (ii) de-repression of Myogenin transcriptional activity, and (iii) reduced the ability of ARMS-derived Rh30 cells to proliferate and form colonies in a colony formation assay.

The final study identified a novel, proliferative role for MEF2D target gene and GSK3β substrate, KLF6 in skeletal muscle. Further analysis revealed that TGFβ signaling and not MEF2 protein expression is required for KLF6 expression and induction of myoblast proliferation, in a Smad3-dependent manner. The data revealed that TGFβ signaling regulates myogenesis through two distinct pathways: (i) cell proliferation in a Smad3/KLF6 dependent manner and (ii) inhibition of myotube formation in an ERK1/2 MAPK dependent manner.

Dedication

To my parents, fiancé and sister: for your patience, love and support.

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1.1 Overview

Myogenesis is the process of skeletal muscle formation during embryonic development and is essential to all metazoan life. This complex multi-stage process is regulated by various transcription factors which produce developmental, stage-specific effector products in response to time dependent signalling cascades. This all begins with the Pax3/7 and Myf5 induced commitment of embryonic precursor cells to the myogenic lineage (Tajbakhsh et al., 1997; Daubas and Buckingham, 2013) followed by somitic migration and proliferation. The myoblast determination protein, MyoD, regulates the differentiation potential of the committed muscle cells by interacting with myocyte enhancer factor 2 (MEF2) proteins to up-regulate Myogenin and drive the formation of multinucleated myotubes (Figure 1.1). As the myotube matures and specializes for its particular function, the cytoplasm becomes predominantly occupied by contractile apparatus which can further grow and hypertrophy in response to intracellular stimuli and muscle-specific regulatory factor 4 (MRF4). The aforementioned myogenic transcription factors and hence the process of skeletal myogenesis is controlled by cell signalling cascades, a major component of which, are protein kinases: enzymes that catalyze the reversible process of phosphorylation. This review will survey the involvement of key protein kinases (particularly, p38 and GSK3β) that participate in myogenesis as well as the consequences of their dysfunction during the development of alveolar rhabdomyosarcoma (ARMS). ARMS is a highly malignant mesenchymal tumor that originates from immature, striated muscle tissue, resulting in dense aggregates of poorly differentiated cells that are separated by fibrous membranes. ARMS is the most common pediatric, soft tissue sarcoma accounting for 50% of all cases with extremely poor prognosis (Barr, 1997; Paulino and Okcu, 2008) and, is most commonly associated with the

chromosomal translocation t(2;13)(q35;q14) which results in the expression of a potent transcription factor, Pax3-foxo1a fusion protein (Biegel et al., 1995; Davis et al., 1995).

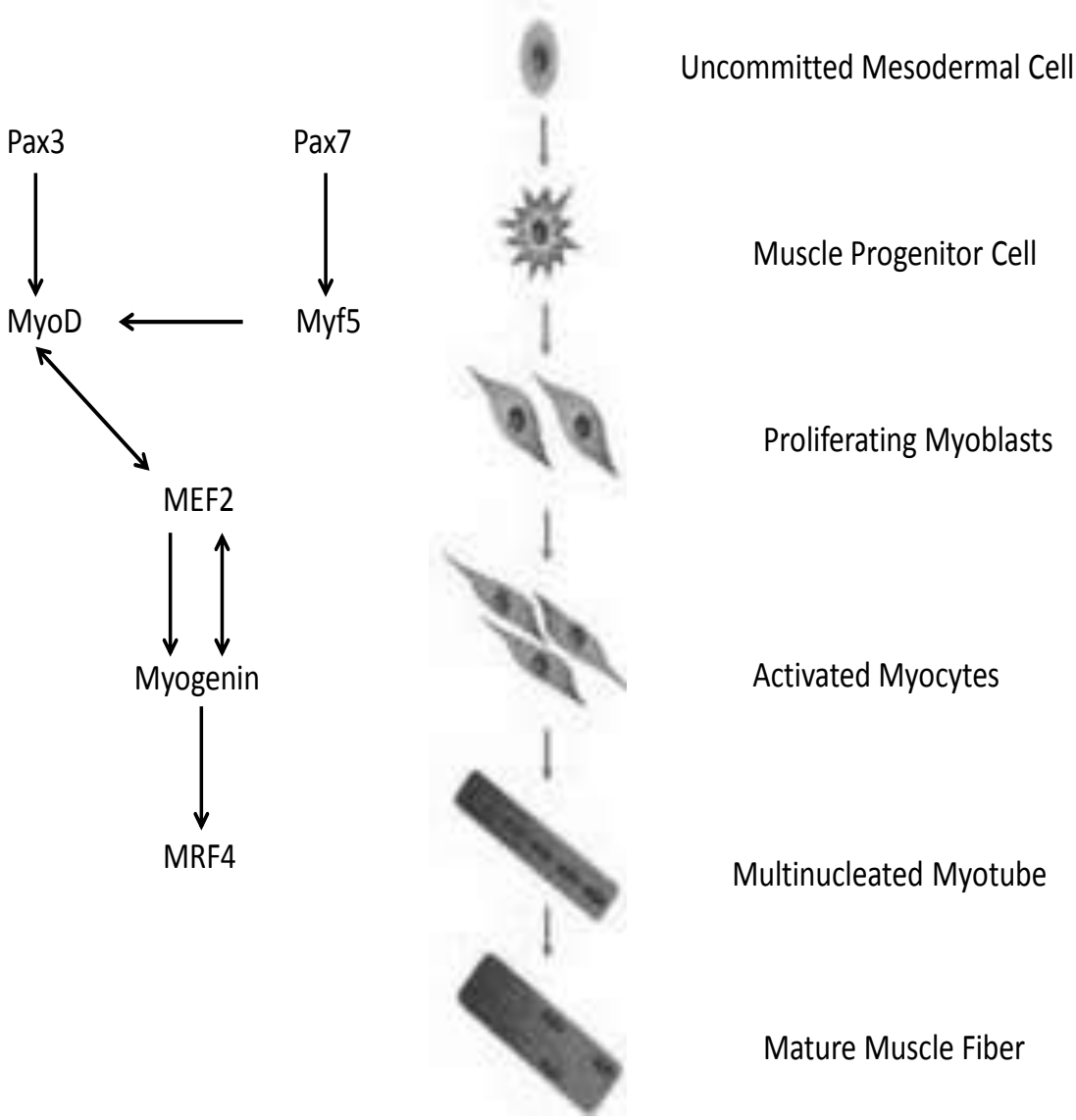


Figure 1.1: The role of key transcription factors at various stages of myogenic differentiation. The expression of Pax3 and Pax7 coincides with the commitment of precursor mesodermal cells to the myogenic lineage and are involved with the migration of these cells to the dermomyotome region of the somite where they proliferate in response to growth factors. Pax3 and Pax7 also potentiate the myoblast determination protein MyoD, either directly or indirectly through Myf5. Upon cell contact, signaling cascades promote the interaction of MyoD and MEF2 and subsequent transcription of downstream target genes such as Myogenin. Myogenin regulates cell fusion, structural proteins such as Myosin heavy chain, and hence myotube formation. MRF4 is involved with maintaining muscle structure integrity and muscle fiber function. This figure has been adapted from Zammit *et al*., 2006.

1.2 Skeletal Muscle Formation During Embryogenesis

Once the primitive streak appears at the posterior end of the embryo to mark the beginning of gastrulation (E6.5 in mice; week 3 in humans), it eventually expands in an anterior direction to form three primary germ layers which give rise to different organs (Tam and Behringer, 1997; Beddington and Robertson, 1999). One of these, the mesoderm compartmentalizes into the intermediate mesoderm, chordamesoderm, paraxial mesoderm and lateral plate mesoderm which eventually form the kidney, gonads, notochord, head, somite, circulatory system and body cavity (Tam and Behringer, 1997). The myotome or skeletal muscle is derived from the somite which comes from the paraxial mesoderm. The formation of the mesodermal layer between the endoderm and ectoderm occurs synchronously with the neural tube formation. As the primitive streak regresses, the neural folds gather at the middle of the embryo causing the paraxial mesoderm to separate into blocks of cells called somites (Tonegawa and Takahashi, 1998). Once they mature, somites develop three major compartments: the sclerotome, myotome and dermatome. This stage is referred to as the epithelial to mesenchymal transition (EMT) and is characterized by increased cellular mobility (Lim and Thierry, 2012). The cells in the two lateral portions of the epithelium constitute the primary myotome, which form a muscle-forming region by producing a second, lower layer of muscle precursor cells called myoblasts. Myoblasts which form adjacent to the neural tube form the intercostal musculature between the ribs and also the deep muscle of the back whereas myoblasts that form away from the neural tube develop into the limbs, body wall and tongue. A third population of myoblasts delaminate from the dermamyotome and join the primary myotome cells to make a secondary myotome (simply referred to as the myotome) and proliferate to make up the majority of myoblasts cells (Gros et al., 2005, Relaiz et al., 2005). Whilst the vast majority of these cells eventually differentiate,

some undifferentiated cells become satellite cells which surround the mature muscle fibres and become responsible for postnatal muscle growth and repair (Biressi et al., 2013; Sirabella et al., 2013). Signals and growth factors from the neural tube delay myoblast differentiation, hence allowing them to migrate away from the dorsal region to a more ventral position (Cossu et al., 1996, Dietrich et al., 1998). Once these are properly positioned in the somite, paracrine factors induce the Pax transcription factors in the dermamyotome region which in turn *trans*activates the *Myod* gene (Relaix et al., 2013; Hu et al., 2008). In the medial region which forms epaxial muscles, MyoD is induced by Myf5 (Tajbakhsh et al., 1997; Borycki et al., 1999) both of which belong to a basic helix-loop-helix (bHLH) family of transcription factors referred to as the myogenic regulatory factors (MRFs) which also include MRF4 and Myogenin. The MRFs all form transcriptionally active heterodimers with E-proteins and bind to the CANNTG consensus DNA binding element, the E-box which is found in the regulatory region of most muscle specific genes (Olson, 1990; Olson, 1992). MyoD establishes a temporal cascade of gene activation during the formation of skeletal muscles by direct activation of gene expression including its own gene (Zingg et al., 1994) and its cofactors such as MEF2 (Penn et al.,2004). This facilitates the binding to a new set of enhancer regions, activating a second set of muscle specific genes (Penn et al., 2004). Any cell that makes MyoD or Myf5 is committed to becoming a myoblast. As long as growth factors are present, myoblasts will remain proliferative. Once these are depleted, myoblasts stop dividing and adhere to each other through extracellular matrix (ECM) proteins (Menko and Boetiger, 1987; Boettiger et al., 1995). The next step is cell recognition and subsequent alignment of the myoblasts, which is mediated by cell glycoproteins including cadherins (Knudsen et al., 1990). Multinucleated myotubes that are characteristic of muscle tissue are a result of the aligned cell's cell membranes dissolving between them (Konigsberg 1963;

Mintz and Baker 1967). This cell fusion event is calcium ion dependent (Shainberg et al., 1969; David et al., 1981) and another bHLH protein, Myogenin, which is required to mediate differentiation by activating several muscle specific genes, becomes active (Bergstrom and Tapscott, 2001). Myogenin expression and activation is the first sign of myoblasts' ability to fuse and terminally differentiate with the exception of RMS. The role of Myogenin and other key transcription factors that are involved in skeletal myogenesis will be discussed in more detail below, and the regulation of Myogenin in alveolar rhabdomyosarcoma will be the main focus of Chapter 4.

1.3 Differential Gene Transcription

An intriguing feature in cellular biochemistry is the ability of different cell types to make different sets of proteins despite their genomes being identical, hence enabling for different tissue formation and function. There are multiple levels of regulation of gene expression such as selective nuclear RNA processing and translational control which are beyond the scope of this review, so the next few pages will focus on the role of transcription factors on differential gene regulation.

First it is important to understand that eukaryotic genes exist within a chromatin – DNA complex that is predominantly composed of histones. The basic unit of chromatin is the nucleosome which is made up of an octamer of histones that are wrapped with ~140bp DNA (Kornberg and Thomas, 1974). Because of this closed, compact conformation of chromatin, DNA is unexposed, and as a result tissue specific genes must become activated by disrupting this structure (Weintraub, 1985). Consequently, histone modification is a key component of regulation of gene expression. Histone acetyltransferases (HAT's) are enzymes that can loosen and thus expose genes, hence activating transcription by adding acetyl groups to histones; a process that

disrupts the compactness of their structure (Sterner and Berger, 2000). Conversely, histone deacetylases (HDAC's) stabilize nucleosomes by removing these acetyl groups, thus inhibiting transcription (Choudhary et al., 2009). Similarly, methyl transferases also enhance histone stability, further repressing transcription (Strahl and Allis, 2000; Cosgrove et al., 2004). These enzymes that affect the state of chromatin can be recruited by transcription factors to modify nucleosomes and affect gene expression (Zhu et al., 2011).

Adding to the complexity of eukaryotic gene expression, eukaryotic mRNA comes from noncontiguous regions on the chromosome, called exons which are separated by sequences called introns, which must be spliced out before translation and protein synthesis can occur. Before this can take place, RNA polymerases must bind to the promoter sequences on the DNA in order to initiate transcription of a particular gene, and synthesize mRNA. The promoter region is known as a TATA box which is typically flanked by GC rich regions, and is ~30bp upstream the transcription initiation site (Down and Hubbard, 2002). However, since RNA Polymerases cannot directly bind to DNA, this process is also regulated by a complex of transcription factors (Buratowski et al., 1989; Sopta et al., 1989). These include basal transcription factors which contain a TATA-binding protein (TBP), TBP-associated factors (TAF's) as well as a mediator complex which modulates RNA Polymerase II activity (Myers and Kornberg, 2000; Baek et al., 2002). Whilst most of these factors are ubiquitously expressed, cell specific transcription factors such as the Pax proteins activate genes by stabilizing this transcription initiation complex. In addition, enhancer regions on a gene regulate the rate and efficiency of transcription at a specific *cis*-linked promoter (Maniatis et al., 1987). Enhancer regions are necessary for temporal and tissue specific expression of genes and are bound by transcription factors (Maniatis et al., 1987). The ability of an enhancer to function at a distance from the promoter region ensures that multiple

signals determine whether a gene is transcribed or not. DNA – Protein interactions at these sites connect the enhancer and promoter regions resulting in direct regulation by either stabilizing the RNA Polymerase complex or through chromatin remodeling (Ogryzko et al., 1996). Conversely, transcription factors at silencer regions can inhibit transcription either by destabilizing the RNA Polymerase complex or by recruiting HDAC's.

Transcription factors are proteins that are characterized by three major domains: (i) a DNA-binding domain (DBD), which recognizes a particular DNA sequence (enhancer or promoter regions discussed above), (ii) a *trans*-activating domain which regulates the transcription of the gene it is bound to, and (iii) a protein – protein interaction domain, which modulates the transcription factor's ability to regulate the gene of interest. Protein – protein interactions allow for temporal and tissue specific effects of transcription factors. The same transcription factor might positively regulate a gene in one cell type but also inhibit this gene in another cell type according to its association with different co-factors in a context dependent manner.

With our current understanding of how transcription factors regulate promoter and enhancer regions, DNA sequences flanking a gene of interest can be cloned and fused to reporter genes whose products are easily identifiable and not usually made in the cells of interest. For instance, in the experiments described in the latter chapters of this thesis the *E.coli* gene for β-galactosidase or the bioluminescence, Luciferase gene were used as reporter genes that were fused to a generic promoter that can be activated in any cell type and an enhancer region that requires muscle-specific gene activation. Similarly, known enhancer regions were fused to reporter genes for either green or red fluorescent proteins (GFP and dsRed2 respectively) and used to test activity of myogenic transcription factors by transiently transfecting these transgenes

into immortalized, C2C12 myoblasts. In cells that have been transfected with a MEF2 enhancer region that are fused to a GFP reporter gene, GFP will only be expressed when MEF2 is transcriptionally active.

1.4 The Role of Key Transcription Factors in Skeletal Myogenesis

The myogenic differentiation program is controlled by muscle specific transcription factors which include members of the MRF and MEF2 protein families (Molkentin and Olson, 1996, Olson *et al*., 1995). Members of the MRF, bHLH superfamily of transcription factors include MyoD, Myf5, MRF4 and Myogenin which are tissue specific. Early MRFs, MyoD and Myf5 are regulated by the Pax3 transcription factor and then in turn these can up-regulate MRF4 and Myogenin. On the other hand the MEF2 transcription factors, MEF2A-D are also expressed in other cell types and are critical regulators of neurogenesis, cardiogenesis and vascular smooth muscle formation in addition to skeletal myogenesis. In myoblasts, MRF and MEF2 proteins synergize to regulate transcription of their downstream target genes (Molkentin and Olson, 1996) in a context-dependent, time-dependent manner by which they can recruit HATs and HDACs to turn transcription on or off respectively (Yuan *et al*., 1996, Lu *et al*., 2000, Mickensey *et al*., 2001).

The studies presented in this thesis focus on regulation of MRFs and MEF2 transcription factors and their downstream targets by protein kinases, with particular emphasis on GSK3β and p38 MAPK. These analyses compare these regulatory pathways during myoblast proliferation and differentiation as well as alveolar rhabdomyosarcoma, a disease that arises from impaired Pax3 transcriptional activity as a consequence of a chromosomal translocation that creates the Pax3-Foxo1a fusion protein.

Pax3 regulates muscle progenitor cell function

The Pax family of transcription factors are crucial regulators of tissue formation and organogenesis, characterized by a paired-domain (PD), that binds to a specifically to a GTTCC (Chalepakis *et al*., 1994) and GTTAT sequence (Phelan and Loeken, 1998). In addition, they may also have an octapeptide (OP) motif for protein interactions and "ATTA" sequence homeo-box DNA binding domain (HD) (Chi and Epstein, 2002). Nine *Pax* genes are identified based on these shared motifs and are grouped based on tissue specificity and spatiotemporal expression (Buckingham and Relaix, 2007). Pax3 and Pax7 are expressed in skeletal muscle progenitor cells and regulate myogenic cell fate; mutations of these are associated with alveolar rhabdomyosarcoma (Barr *et al*., 1999). Pax3 is already expressed in the presomitic paraxial mesoderm before segmentation and throughout the epithelial somite before being restricted dorsally to the dermomyotome (Buckingham, 2006). Functionally, Pax3 regulates the formation of hypaxial trunk muscle as well as delamination and migration of myogenic progenitor cells from the extremities of the dermomyotome to the underlying myotome and site of myogenesis (Tajbakhsh and Buckingham, 2000). Pax3 then directly binds to a regulatory sequence that promotes *Myf5* transcription in the trunk and limbs (Bajard *et al*., 2006), hence initiating the expression of muscle specific proteins. Myf5 is the first of four members of the bHLH superfamily of transcription factors that are activated to promote skeletal myogenesis. The second is MyoD which can be activated through Myf5 or Pax3 dependent mechanisms (Tajbakhash *et al*., 1997, Kassar-Duchossoy *et al*., 2004). Protein expression and activation of the MRFs in a timely manner is an absolute requirement for the differentiation of skeletal muscle.

In addition to their role in muscle development, a population of proliferating Pax3/7 positive cells was reported in developing skeletal muscle masses (Ben-Yair and Kalcheim 2005)

that were derived from the dermomyotome during somite maturation (Gros *et al*., 2005 Kassar-Duchossy *et al*., 2005) which can also give rise to myogenic cells thus providing a reserve of muscle progenitor cells during embryonic and fetal development. Loss of Pax function in these reserve cells prevents MyoD activation and hence entry of this population of cells into the myogenic program (Relaix et al., 2005). In the developing embryo Pax3/7 positive progenitor cells which arise beneath the basal lamina that forms around muscle fibers give rise to the satellite cells of the trunk (Gros *et al*., 2005) where as limb muscle satellite cells are derived from Pax3-expressing cells that migrate from the hypaxial dermomyotome (Schienda et al., 2006). Satellite cells are typically marked by the expression of Pax7 (Seale *et al*., 2000) although Pax3 can still be detected in these after birth. Satellite cells remain quiescent until the muscle gets injured and the lamina breaks down to activate their proliferation, and subsequent formation of muscle fibers (Montarras *et al*., 2005). Activation of satellite cells is accompanied by increased Myf5 and MyoD expression which results in downregulation of Pax7, subsequent Myogenin activation and hence new muscle fiber formation (Montarras *et al*., 2013). Asymmetric cell division allows for some cells to maintain high levels of Pax7 and lose MyoD, leading to self-renewal of a minority satellite cell population (Shinin *et al*., 2006).

Conflicting reports on the effects Pax3/7 overexpression on satellite cell differentiation have been reported and is probably dependent on protein levels (Buckingham and Relaix, 2007). On the other hand, loss of Pax3/7 function studies have resulted in repression of MyoD expression (Relaix *et al*., 2006, Zammit *et al*., 2006) but not prevention of myogenic differentiation. Instead, delayed Myogenin expression occurs in a Myf5 dependent manner since *Myf5* is already transcribed in most quiescent cells independently of Pax3/7 (Zammit *et al*., 2006). In satellite cells that do not express Myf5, loss of Pax3/7 prevents myogenesis in a MyoD

dependent manner (Relaix *et al*., 2006). In addition to their roles in regulating myogenic cell fate and muscle reserve cell populations, Pax3 and Pax7 are also involved in cell survival, proliferation and migration. In Pax3 mutant embryos, despite the expression of MyoD in the hypaxial somite, cell apoptosis is prevalent but not in the adjacent Pax7 expressing somite regions and dorsal neural tube where Pax3 would also be normally expressed (Borycki *et al*., 1999). These data suggest that Pax3 is a co-requisite for proper MyoD function and inhibition of apoptosis. In double Pax3/7 mutants, muscle specification is defective and also extensive cell death occurs (Relaix *et al*., 2005). During development, Pax3 and Pax7 are both expressed in proliferating muscle progenitor cells as well as in dividing cells that are present in the dermomyotome region of the somite. Pax3 expression coincides with regulation of fibroblast growth factors (Lagha *et al*., 2008) and may be important for the proliferative state of these cells (Conboy and Rando, 2002), although the precise mechanism by which this occurs is poorly understood (Diao *et al*., 2013).

Pax3 and Pax7 are crucial for enabling the formation of skeletal muscle most notably by directing progenitor cells into the myogenic program and assuring their survival. Mutational defects in these genes are associated with Waardenburg syndrome (Baldwin *et al*., 1995), melanoma (Vachtenheim and Novotna, 1999), neuroblastoma (Barr *et al*., 1999) and rhabdomyosarcoma (Barr *et al*., 1993, Shapiro *et al*., 1993).

The Muscle Regulatory Factors (MRFs)

The MRF superfamily of transcription factors share a highly conserved, ~70 amino acid basic helix loop helix (bHLH) region that is responsible for DNA binding and protein interactions. MRFs are key regulators of commitment to the myogenic lineage whose primary

role is to induce terminal cell cycle arrest during myogenic differentiation by increasing p21 expression and *trans*activating muscle specific genes (Halevy *et al*., 1995). MRF proteins dimerize with E-proteins, including E12, E47 and HEB, and these heterodimers preferentially bind to E-box DNA motifs (CANNTG consensus sequence), which are present in almost all muscle specific genes (Lassar *et al*., 1991). Interactions with other proteins such as MEF2 (Olson et al., 1995), AP-1 (Andreucci et al., 2002) and Smad (Liu et al., 2001) also influence MRF transcriptional activity either by binding to other regulatory motifs or by affecting MRF binding to E-box motifs. In addition to protein interactions, other post-translational modifications such as phosphorylation, acetylation and ubiquitination may regulate MRF transcriptional activity (Puri and Sartorelli, 2000). Due to their structural similarities the MRFs: MyoD, Myf5, MRF4 and Myogenin have similar target genes and hence certain functional redundancies. However, because of differences in their expression patterns there are also some unique characteristics of each MRF. The next section discusses the expression patterns, function and regulation of each MRF in more detail.

Expression Patterns of MRFs during mouse embryogenesis

As previously discussed, somites which give rise to myoblasts that become skeletal muscle of the head, trunk and limbs (Buckingham, 1992) begin to form at about day 7 p.c. in an anterior to posterior direction (Thelier *et al*., 1989). As the development program continues, somites undergo compartmentalization into dermomyotome and sclerotome regions (Langman and Nelson, 1968). The process of compartmentalization and subsequent determination of these cells into a myogenic lineage depends on MRF protein expression. *Myf5* and *MyoD* transcription occur in parallel to each other and are the first MRFs to be expressed in myogenic cells indicating

their role in initiating myogenesis (Cossu *et al*., 1996, Rudnicki *et al*., 1992). *Myf5* mRNA is first detected in somites at day 8 p.c. and is down-regulated at day 14 p.c. (Ott et al., 1991), whereas *Myogenin* transcripts aren't detected until about day 8.5 p.c and maintained throughout fetal development (Sassoon et al., 1989). *MyoD* mRNA is first detected in somites at around day 10.5 p.c and is expressed throughout development (Buckingham et al., 1992). Finally, *Mrf4* appears transiently between days 10-11 p.c. and re-appears at day 16 p.c. becoming most abundant after birth (Bober et al., 1991). The times at which these genes are expressed vary according to the region of the myotome which the myoblasts are derived from, however the sequence at which they are expressed is more or less the same, with *Myf5* and *MyoD* transcription preceding that of *Myogenin*, and with *MRF4* predominantly expressed after fusion. Based on the timing and complexity of MRF expression during embryonic development, there are likely overlapping phases of myogenic commitment and skeletal muscle differentiation that depend on differential expression of MRFs (Megeney and Rudnicki, 1995). In cultured myoblasts, Myf5 and MyoD are expressed before and after differentiation whereas Myogenin is only expressed upon myotube fusion and *MRF4* mRNA only shows up days after myotube formation (Rudnicki and Jaenisch, 1995). In addition to their expression patterns, phylogenic analysis of MRF amino acid sequences and their chromosomal locations suggest that MyoD and Myf5 are more similar to each other than either MRF4 or Myogenin and vice versa (Atchley et al., 1994). Collectively, these analyses suggest that the MRF family consists of two groups that reflect functional specialization. The first group is MyoD and Myf5 which are required for commitment of multipotent cells into the myogenic lineage whereas the second group consists of Myogenin and MRF4 which are involved in cell fusion, myotube formation and maintenance. The gene knockout studies described below

provide further insight to the individual function and relevance of the distinct MRF expression patterns.

MyoD gene ablation and functional analysis

MyoD (-/-) mice are viable and fertile however these mice are born 30% smaller than wild type mice (Rudnicki *et al*., 1992). Myf5 is up-regulated in these mice to compensate for the lack of MyoD expression, implicating an overlap in their functions in terms of cell determination to the myogenic lineage and myotube fusion (Rudnicki *et al*., 1992). Myf5 is able to potentiate MRF4 and Myogenin in the absence of MyoD and it was observed that MRF4 plays a more critical role in MyoD deficient mice suggesting that MRF4 may also be involved in cell commitment in addition to the maintenance of multinucleated myotubes (Rawls *et al*., 1995, Rudnicki *et al*., 1992). Reduced satellite cell proliferation and hence defects in muscle fiber regeneration have been observed in *MyoD* (-/-) mice (Rudnicki et al., 1992). However this may be more due to the compensatory up-regulation of Myf5 than lack of MyoD (Yoshida *et al*., 1998). *MyoD* and *Myf5* double knockout mice are lethal at birth and lack myoblasts and muscle fibers suggesting that expression of at least one of these two MRF proteins is absolutely essential for commitment to a myogenic lineage (Rawls *et al*., 1995, Rawls *et al*., 1998). Corroborating this idea, MyoD cDNA transfection alone is enough to convert fibroblasts to myoblasts in tissue culture (Davis et al., 1987).

Myf5 gene ablation and functional analysis

Similarly, *Myf5* (-/-) mice are also viable and fertile but with no significant loss of muscle mass compared to wild type mice despite delayed MyoD expression (Kaul *et al*., 2000). This

suggests that while Myf5 is clearly important in myoblast determination, its involvement in skeletal muscle development is probably more indirect by transcriptional activation of its downstream MRFs. In its absence, MyoD is up-regulated by Pax3 which compensates for the loss of Myf5 function in that regard (Buckingham, 2004, Tajbakhsh *et al*., 1997). Early suggestions that MRF4 was compensating for loss of Myf5 by up-regulating MyoD were not supported by subsequent studies which showed that Myf5/MRF4 double knockout mice had the exact same phenotype as Myf5 (-/-) mice (Rawls et al., 1995). Pax3/Myf5/MRF4 triple knockout mice on the other hand were unable to up-regulate MyoD expression (Kassar-Duchossoy *et al*., 2004, Tajbakhsh *et al*., 1997). Subsequent analysis revealed that Myf5 expression is downregulated in multinucleated myotube but maintained in a reserve cell population (Friday and Pavlath, 2001). Loss of Myf5 function studies revealed defects in satellite cell proliferation that were not compensated for by any of the other MRFs (Kaul *et al*., 2000). Triple MyoD/Myogenin/MRF4 knockout mice are the only viable knockout mice despite the absence of muscle fibers suggesting that physiological levels of Myf5 are insufficient to activate the differentiation program (Valdez *et al*., 2000) and may suggest that Myf5 is also involved in myoblast proliferation and cell survival.

Myogenin gene ablation and functional analysis

Of the four members of the MRF bHLH superfamily of transcription factors, Myogenin function is absolutely essential for muscle cell fusion and muscle fiber formation. Loss of Myogenin function cannot be compensated by any of the other MRFs (Hasty *et al*., 1993). *Myogenin* (-/-) mice are lethal at birth due to the absence of secondary muscle fiber formation despite an increase in the number of committed myoblasts (Hasty *et al*., 1993, Rawls *et al*., 1995,

Rawls *et al*., 1998). The elevated number of committed myoblasts indicated that Myogenin may also be involved in maintaining terminal cell cycle arrest by regulating $p21^{Kip2}$ expression (Andres and Welsh, 1996). Double knockout mice which include loss of Myogenin show no phenotypic difference to single *Myogenin* (-/-) mice corroborating the idea that Myogenin function might be irrelevant during myoblast determination but absolutely essential for myotube differentiation (Rawls *et al*., 1995, Rawls *et al*., 1998).

MRF4 gene ablation and functional analysis

MRF4 (-/-) mice showed no obvious differences to wild type mice: they are viable, fertile and produce committed myoblasts that can fuse to form functional secondary muscle fibers (Zhang *et al*., 1995). Although Myogenin protein expression levels are elevated in MRF4 knockout mice this study seems to suggest that MRF4 function is redundant during skeletal muscle formation (Zhang *et al*., 1995). However in the absence of MyoD, MRF4 is required for viability and muscle fiber formation suggesting that MRF4 function may overlap with MyoD function during skeletal muscle differentiation (Rawls *et al*., 1995). Also, early overexpression of MRF4 but not Myogenin rescues skeletal myogenesis in MyoD/Myf5 double mutant zebrafish embryos (Schnapp *et al*., 2009) suggesting that it may also be able to compensate for some of their cell determination properties whereas Myogenin cannot.

In summary, functional analysis of the MRFs reveals that they may be divided into two groups. The first group consists of MyoD and Myf5 which are primarily involved in commitment of pluripotent cells into the myogenic lineage whereas Myogenin functions as a differentiation factor which is required for terminal cell cycle arrest and myoblast fusion in order to get

functional muscle fibers. The role of MRF4 is less obvious and seems to be redundant as long as the other three MRFs are functioning properly. Under different conditions, it has been shown to function at both the commitment and differentiation stages. According to its late expression in the developing embryo and its maintenance during adulthood, it is highly likely that MRF4 is involved in maintaining muscle structure and that this feature would have been missed by the studies described above. Further analysis of MRF4 (-/-) mice did not reveal any muscle fiber defects, but instead multiple rib abnormalities were observed (Zhang et al., 1995). However, it is still unclear whether this means that loss of MRF4 function is directly involved in this birth defect.

Myocyte Enhancer Factor 2 (MEF2) proteins

In addition to MRFs, MEF2 transcription factors are critical regulators of myogenic differentiation (Black and Olson, 1998). There are four members of the MEF2 family, MEF2A-D in vertebrates with each gene located on different chromosomes (McDermott *et al*., 1993, Hobson *et al*., 1995). MEF2 protein belong to the MADS (MCM1, agamous, deficiens, serum response factor) superfamily of transcription factors which contain a highly conserved 57a.a MADS-box that is located directly N-terminal to a 29 a.a MEF2 domain (Molkentin *et al*., 1996). Together these two domains are involved in high affinity, DNA binding and protein interactions. MEF2 proteins also possess a divergent C-terminus which may allow for functional differences and unique interactions (Black and Olson, 1998). Despite their different C-terminal sequences, MEF2A/C/D possess a highly conserved C-terminal, nuclear localization sequence (NLS) (Yu et al., 1992). Since it was first identified on the muscle creatine kinase (MCK) enhancer region bound to a highly conserved A/T rich DNA sequence (Gossett et al., 1989), MEF2

(C/T)TA(A/T)$_4$TA(G/A) consensus DNA sequences have been found in the control regions of many striated and smooth muscle genes. Mutational analysis of these sites leads to reduced and even diminished loss of gene expression (Nurrish and Treisman, 1995). Interestingly, MEF2 must homo- or hetero- dimerize for DNA binding and these all have equivalent binding affinities with the exception of MEF2B (Pollock and Treisman, 1991, Yu et al., 1992, Molkentin et al., 1996).

MEF2 expression during mouse embryogenesis

Mef2 genes are expressed in distinct but overlapping temporal and spatial patterns during embryogenesis, particularly in the brain, heart and striated muscle. During mouse development *Mef2c* is first expressed at day 7.5 p.c in the developing myocardium, followed by *Mef2a* and *Mef2d* at day 8.5 p.c (Edmondson *et al*., 1994). In skeletal muscle, *Mef2c* expression precedes that of the rest at day 9 p.c. lagging *Myogenin* expression by a few hours (McDermott *et al*., 1993, Edmondson *et al*., 1994). Shortly after, *Mef2a* and *Mef2d* are expressed in the developing somite at 9.5. d.p.c and maintained throughout myogenic differentiation (Chambers *et al*., 1992). After day 12.5, MEF2 transcripts are detected at high levels in specific regions of the brain and in a wide range of tissue (Lyons *et al*., 1995, Black and Olson, 1998).

The generation of a mouse that contains multiple MEF2 binding sites that drive a *LacZ* indicator gene has been utilized experimentally, to show that MEF2 activity is induced selectively and enriched in the heart and somites despite MEF2 expression in numerous other tissues (Naya *et al*., 1999). This observation led to a hypothesis for the potential role of posttranslational regulation of MEF2 proteins, and these have been studied extensively since.

In immortalized C2C12 myoblasts, *Mef2* gene expression patterns differ to the *in vivo* gene expression patterns described above. Instead, MEF2A/D is expressed in proliferating

myoblasts prior to the onset of myogenic differentiation whereas MEF2C appears much later in the differentiation program, and only upon the formation of muscle fibers. Interestingly, despite the early expression of MEF2A and MEF2D in cultured myoblasts they are unable to initiate muscle specific gene expression in such a context further until p38 MAPK activation (Han *et al*., 1997, Rampalli *et al*., 2007) hence corroborating the importance of posttranslational regulation of these transcription factors.

MEF2 gene ablation and functional analysis

Since there is only one MEF2 gene (*D-mef2*) in *Drosophila*, analysis of this gene eliminates the issue of functional redundancy between the different vertebrate isoforms of MEF2. *D-mef2* homozygous null embryos are embryonic lethal displaying a loss of somatic, cardiac and visceral muscle differentiation despite the presence of cells that have already been committed to their respective myogenic lineages (Lilly *et al*., 1994, Lilly *et al*., 1995, Bour *et al*., 1995).

Analysis of MEF2 function through gene knockout studies in mice is obviously more complex due to overlapping expression and function of the four MEF2 isoforms. *Mef2c* (-/-) mouse embryos die at day 9.5 p.c as a result of defective cardiovasculature (Lin *et al*., 1998). Analysis of these embryos revealed diminished cardiac gene expression and phenotypically they lack right ventricle formation because the heart tube fails to undergo looping morphogenesis. *Mef2b* null mice are viable without any notable skeletal or cardiac muscle defects (Black and Olson, 1998) and to this date the role of MEF2B is still unclear. Similarly, *Mef2a* null mice are also viable but are highly susceptible to cardiac arrest within a week after birth due to a reduction in the size and number of cardiac mitochondria (Naya *et al*., 2002). Interestingly despite the importance of MEF2A regulation of skeletal myogenesis and muscle specific genes, *Mef2a* gene

ablation does not cause severe myogenic abnormalities (Naya *et al*., 2002). It is also the case that Mef2d (-/-) mice are viable without any apparent abnormalities and therefore it appears that the other isoforms can compensate for loss of a particular MEF2 isoform under normal conditions (Kim *et al*., 2008). Tissue specific gene deletions have provided more information on the individual functions of MEF2C/D (Pothoff *et al*., 2007, Kim *et al*., 2008). 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 muscle integrity (Pothoff *et al*., 2007). Loss of *Mef2d* function in the heart show an impaired response to stress signals that normally potentiate MEF2 *trans*activation of cardiac genes involved with hypertrophy and fibrosis (Kim *et al*., 2008). Thus although numerous lines of evidence point to a critical role for MEF2 during myogenic differentiation, redundancies in their functions and their roles in other developmental processes have made it difficult to pinpoint these functions *in vivo*. More recent tissue-specific gene ablation studies have proven useful, however these did not circumvent the compensatory effects of expressed MEF2 isoforms and therefore the generation of double and triple knockout mice in future studies may be more informative.

MEF2 and MRF transcription factors cooperate to regulate myogenic differentiation

Several studies have demonstrated that MEF2 factors and MRFs cooperate to activate skeletal muscle gene expression through direct protein-protein interactions between their respective DNA-binding domains (DBDs). Although the four bHLH MRFs can each activate the program for skeletal muscle differentiation in non-myogenic cell types (Olson 1990, Lassar and Munsterberg, 1994), MEF2 proteins cannot (Molkentin *et al*., 1995, Black and Olson, 1998). The bHLH domain of the MRFs mediates dimerization with HLH E-proteins, E12, E47 and HEB

resulting in a heterodimer complex that can bind to the E-box consensus DNA sequence (CANNTG) that is present in the regulatory region of most myogenic genes, as discussed above (Lassar et al., 1991). In non-myogenic cells, forced expression of MyoD or Myogenin induced MEF2 DNA-binding activity suggesting that it is an important downstream component of the myogenic program (Martin *et al*., 1993, Lassar *et al*., 1991). Corroborating this idea, co-transfection of a dominant negative version of MEF2A with MRFs in multipotent, non-myogenic cells prevents the initiation of the myogenic program (Ornatsky *et al*., 1997). Interestingly, MEF2 proteins have been shown to potentiate the *trans*activation of the E-box promoter despite the absence of a MEF2 binding site and alternatively MRFs can increase MEF2 transcriptional activity despite the absence of the E-box, suggesting that they additionally act as potent cofactors for each other's transcriptional activity (Molkentin *et al*., 1995). Even though MEF2 proteins cannot activate the expression of *Myod, MRF4* and *Myogenin* on their own despite the presence of MEF2 DNA-binding sites on their promoter regions, MEF2 is involved in the maintenance of their expression and is essential for *Myogenin* transcription in cultured myoblasts and mouse embryos, by interacting with these (Yee and Rigby, 1993). There are therefore four potential mechanisms by which MEF2 factors and MRFs cooperate to activate the skeletal muscle differentiation program: (1) MEF2 proteins are recruited to an E-box as part of an MRF/E-protein multimer or (2) MRF/E-protein heterodimers are recruited to a MEF2 box as part of a similar protein complex. (3) MEF2 and MRF transcription factors independently bind to DNA at adjacent consensus sites and then cooperate to activate transcription either by direct or indirect interactions. (4) MEF2 and MRF transcription factors independently bind to DNA at non-adjacent consensus sites and then bend the DNA to directly interact with each other (Molkentin *et al*.,

1995, Black and Olson, 1998). The mechanism by which MEF2 and MRF factors interact with each other varies according to the gene being activated and the context at which this occurs.

In addition to these modes of muscle gene activation other protein families including HDACs, HATs, AP-1 and Smads have also been shown to regulate myogenic differentiation either by directly binding to MEF2 and/or MRF proteins or by directly binding to muscle specific genes via their respective consensus DNA-binding sites. Adding to the complexity of an already complicated process, MRFs and particularly MEF2 proteins are controlled by multiple levels of regulation such as alternative splicing (Sebastian *et al*., 2013) and posttranslational modifications such as acetylation (Nebbioso *et al*., 2009), sumoylation (Gregoire *et al*., 2006) and phosphorylation (Han *et al*., 1997, Cox *et al*., 2003). While a lot of these are beyond the scope of this paper, the next section will focus on MRF and MEF2 phosphorylation and key signaling pathways that regulate MRF and MEF2 transcriptional activation with particular emphasis on two MEF2 target genes: Myogenin and Krüppel-like factor 6 (KLF6) which regulate myogenic differentiation and myoblast proliferation respectively.

Regulation of MRF and MEF2 factors by protein kinases

Overview

Skeletal muscle differentiation is regulated by many signaling pathways which affect the transcriptional activity of the MRF and MEF2 factors either positively or negatively. In both the developing somite and cultured myoblasts, two MRF proteins: Myf5 and MyoD as well as three MEF2 proteins, MEF2A/C/D are all expressed before the differentiation program in initiated (Rudnicki *et al*., 1993, Tajbakhsh and Cossu, 1997). Therefore the influence of external signals is critical for the spatial and temporal activation of MRF and MEF2 factors and their ability to

regulate processes such as irreversible cell cycle arrest and terminal differentiation through muscle specific target genes (Nadal-Ginard, 1978, Lassar *et al*., 1994). In particular, signaling pathways propagated by growth factors such as fibroblast growth factors (FGFs), insulin-like growth factor 1 (IGF1), transforming growth factor beta (TGFβ) and platelet-derived growth factor (PDGF) have all been shown to influence the skeletal myogenic program (Florini *et al*., 1991, Husmann *et al*., 1996). Despite the knowledge that extracellular signals were regulating myogenic differentiation in somites, the intracellular mechanisms by which these signals were being transmitted to the transcriptional machinery in order to modulate gene expression and thus differentiation, were less clear. Because of their integral roles in myogenic differentiation, posttranslational regulation of MRF and MEF2 proteins in response to extracellular signals that emanate from adjacent tissues and cell-cell contact have been the focus of research over the last 15 years. The next section profiles some of the key intracellular effectors of these signals and their role in regulating skeletal myogenesis through MRF and MEF2 proteins.

Fibroblast growth factor receptor-1 (FGFR1) signaling and regulation of MRF and MEF2 proteins

Fibroblasts growth factors are key regulators of muscle progenitor cell migration and proliferation that are transcribed in response to Pax3 (Webb *et al*., 1997, Lagha *et al*., 2008). Despite this, it is not until bFGF are down regulated that terminal differentiation can occur even though MRF and MEF2 proteins are expressed (Hannon *et al*., 1996, Itoh *et al*., 1994). One kinase that has been shown to be activated by bFGF is protein kinase C (PKC) which can phosphorylate a conserved threonine in the basic region of MRF proteins (Li *et al*., 1992b). MyoD has been shown to be phosphorylated at this site in proliferating myoblasts and

rhabdomyosarcoma (Liu *et al*., 1998) resulting in reduced DNA binding and hence transcriptional activity. Despite the conservation of this PKC phosphoacceptor site amongst the MRF family of transcription factors, PKC has been shown to inhibit MRF4 transcriptional activity without phosphorylating it (Hardy *et al*., 1993) therefore the precise mechanism by which bFGF/PKC signaling inhibits MRF activity is still unclear. Adding to the complexity, nine PKC isoforms have been identified and classified into three groups: conventional, novel and atypical (Mellor and Parker, 1998) of which novel PKCs have been shown to phosphorylate the MEF2A *trans*activation domain (TAD), enhancing its transcriptional activity (Ornatsky *et al*., 1999). More recently a bFGF has been shown to elevate intracellular cyclic AMP (cAMP) and protein kinase A (PKA) activity in both muscle and non-muscle cells (Motamed *et al*., 2003, Barraud *et al*., 2010). PKA has also been shown to phosphorylate the basic region of MRFs at a site different to the PKC sites previously described, however these were not essential for PKA-mediated inhibition of MRF transcriptional activity (Li *et al*., 1992a). Instead, PKA inhibition of skeletal myogenesis was found to be via a bipartite mode of MEF2D regulation, the first is by direct phosphorylation at S121 and S190 and the second was by enhancing nuclear localization of HDAC4 and subsequent MEF2D binding (Du *et al*., 2008, Salma and McDermott, 2012). Given the importance of MRF and MEF2 factor interactions in enhancing their target gene expression; this would also explain PKA mediated repression of MRF activity (Figure 1.2).

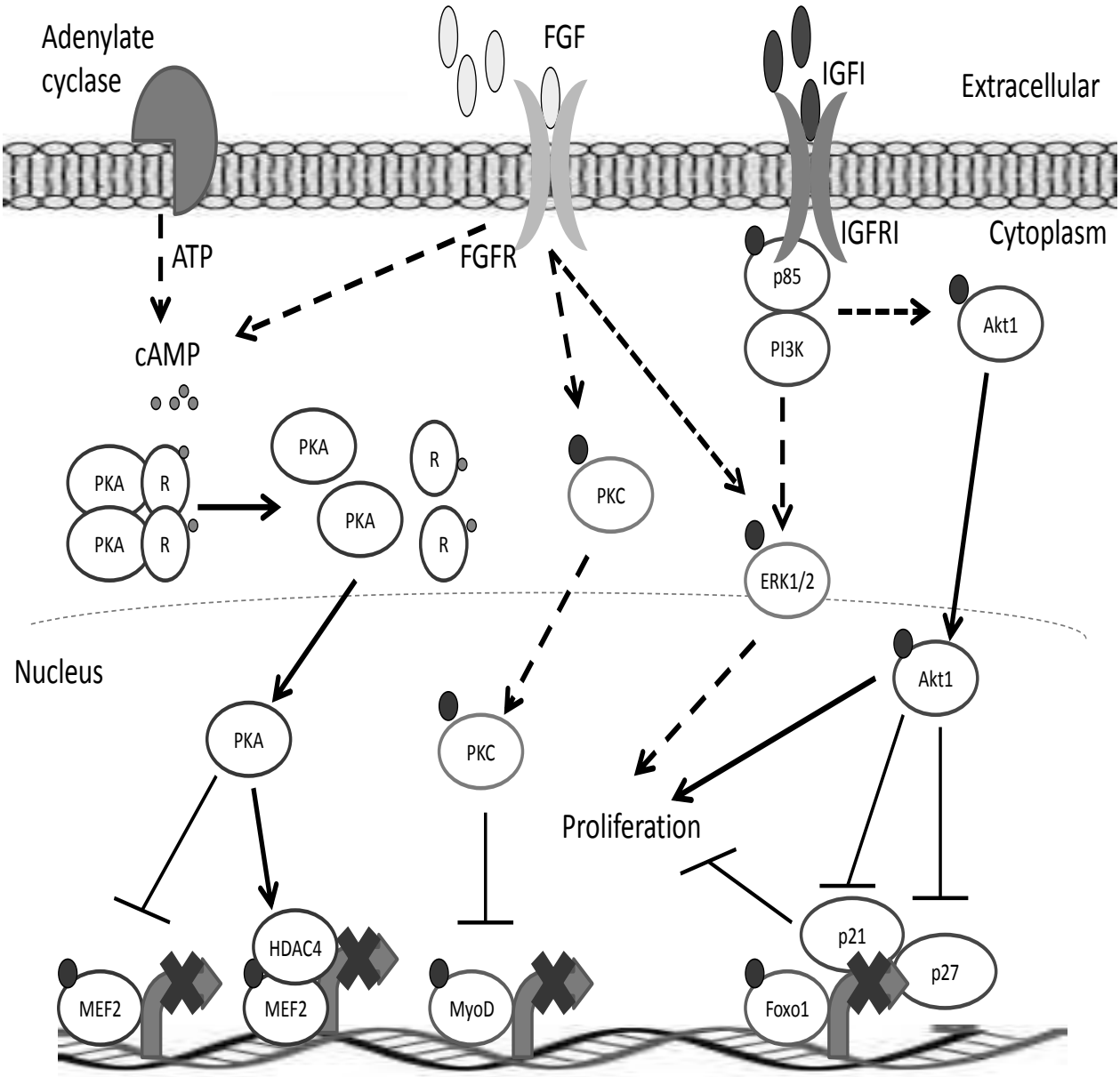


Figure 1.2: Early stage regulation of myogenesis by extracellular signaling cascades. The figure illustrates how the growth factors discussed in the text regulate myoblast proliferation and prevent muscle differentiation. Upon receptor-mediated activation of cyclic AMP as indicated, cAMP binds to the regulatory subunits (R) of PKA releasing the catalytic subunits which can then translocate into the nucleus. Nuclear PKA inhibits MEF2 transcriptional activity directly through phosphorylation, and also indirectly through HDAC4 as depicted. Even though PKA potentiates MyoD expression, MyoD remains inactive in the presence of growth factors such as fibroblast growth factor (FGF) which can indirectly repress its DNA-binding properties through PKC. The extracellular regulated kinase (ERK1/2) is also activated by growth factors such as FGF and insulin-like growth factor (IGF) and represses myogenic differentiation as discussed later in the next section of this chapter. IGFI also activates Akt1 and stimulates cell proliferation

when myoblasts are sub-confluent by directly phosphorylating Foxo1 causing it to exit the nucleus thus blocking the expression of genes such as p21 and p27 which regulate cell cycle exit. This figure has been adapted from Knight and Kothary, 2011.

Transforming growth factor β (TGFβ) regulation of MRF and MEF2 proteins

The TGFβ superfamily of cytokines mediate several cellular and physiological processes including epithelial-to-mesenchymal transitions (EMT), cell proliferation, differentiation and apoptosis (Duband *et al*., 1995, Pampusch *et al*., 1990, Massague and Xi, 2012, Rotello *et al*., 1991). TGFβ ligand binding to either type I or type II receptors facilitate the formation of a heteromeric TGFβ ligand-receptor complex that allows a constitutively active type II receptor kinase to phosphorylate and activate the type I receptor (Feng and Derynck, 2005, Shi and Massague, 2003). In a myogenic context the active type I receptor can phosphorylate and activate receptor mediated Smad2/3 factors which translocate into the nucleus upon dimerizing with Smad4, where it regulates muscle gene expression (Figure 1.3). More commonly, Smad proteins affect transcription of TGFβ target genes by acting as cofactors rather than by directly binding to the gene itself (Derynck *et al*., 1998). Subsequently, Smad3 has been shown to inhibit MyoD transcriptional activity by binding to the bHLH region which is required for its dimerization with E-proteins and hence DNA (Liu *et al*., 2001). Furthermore Smad2 and Smad3 have both been shown to interact with the MEF2 domain to promote or inhibit MEF2 transcriptional activity in a context dependent manner (Quinn *et al*., 2001, Liu *et al*., 2004). A second pathway that is directly activated by TGFβ ligands is the Extracellular-signal regulated kinase (1/2) MAPK through its upstream MAPKK, MEK1/2 (Yue and Mulder, 2000). Phosphorylation of MEK1/2 results in inhibition of skeletal myogenesis through two known mechanisms (Figure 1.3). In the first, phosphorylated MEK1/2 translocates into the nucleus

where it can bind to and destabilize MyoD – DNA interaction (Miyake *et al*., 2009). The second mechanism involves MEK 1/2 activation of ERK 1/2 which can activate AP-1 family members such as Fra1/2, c-Jun and c-Fos (Gruda et al., 1994, Alli *et al*., 2013). AP-1 proto-oncogenes promote cell proliferation and in a myogenic context repress MyoD transcriptional activity by direct interaction (Bengal et al., 1992). More recently, pharmacological inhibition of ERK has been shown to rescue Myogenin transcriptional activity and the authors speculate that ERK-mediated inhibition of Myogenin might be by direct phosphorylation (Penna *et al*., 2010), although they do not provide any direct evidence of this. Regardless, there is strong evidence supporting the repressive effect of TGFβ signaling on skeletal muscle differentiation through two molecular pathways: Smad mediated activation of cell proliferation and ERK1/2-dependent repression of myogenic genes.

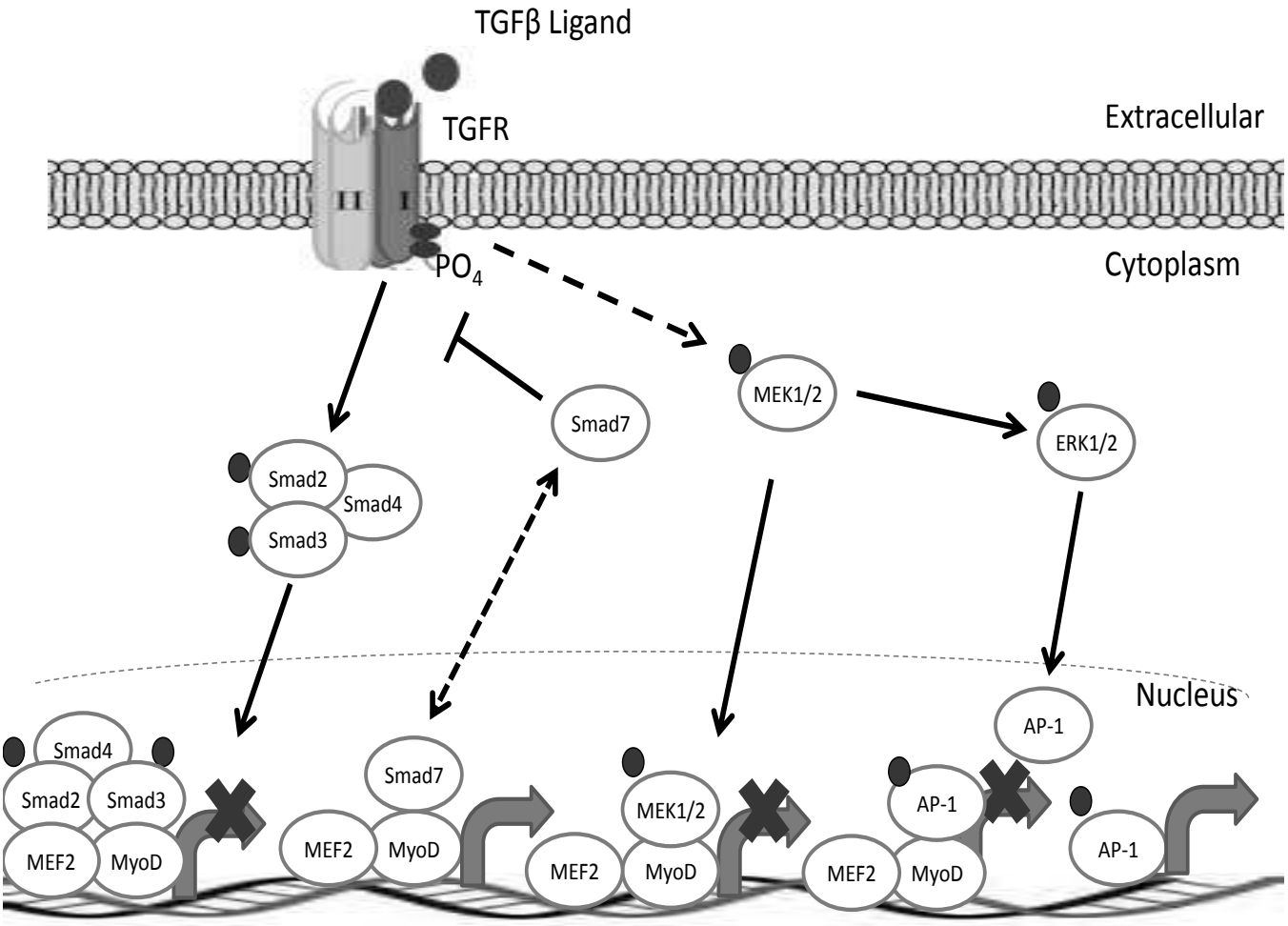


Figure 1.3: The TGFβ signaling pathway and repression of muscle-specific gene expression. TGFβ ligand association with the appropriate Type I / Type II receptor complex results in auto-phosphorylation, and subsequent phosphorylation of receptor-mediated Smads 2/3. These can then interact with Smad4 and translocate into the nucleus to activate target gene expression either directly or indirectly by interacting with MEF2 and MyoD. In addition, TGFβ signaling represses muscle gene expression by activating MEK(1/2) signaling. Phosphorylated MEK1/2 can translocate into the nucleus where it interacts with and destabilizes MyoD – DNA binding. Both of these mechanisms are antagonized by Smad7 which competes with the receptor-mediated activation of Smad2/3 and stabilizes MyoD / DNA-binding in the nucleus, as depicted. Subsequent activation of the downstream MEK1/2 target, ERK1/2 results in AP-1 activation which in turn represses differentiation by abrogating MyoD and can also promote cell proliferation through AP-1 target gene expression.

Insulin-like growth factors (IGFs)

The IGF family of growth factors can be subdivided into two subgroups, IGF-I and IGF-II (Husmann *et al*., 1996). Unlike other growth factors IGFs are involved in both cell proliferation and differentiation (Ewton and Florini, 1980, Ewton and Florini, 1981). During the onset of myogenic differentiation, IGF-I levels are reduced and this is coupled with a 15-fold increase of IGF-II expression suggesting that IGF-I might initially delay the onset of myogenesis before it can act as a stimulator (Rosenthal *et al*., 1991, Ewton *et al*., 1994). In proliferating myoblasts there is evidence that other mitogens such as FGF might be up-regulating IGF-I receptors by down-regulating IGF-II protein levels supporting the idea that a subtle balance between FGF and IGF-II levels might be important for initiating differentiation (Rosenthal *et al*., 1991). Furthermore, IGFs are secreted by satellite cells in regenerating muscle, inducing differentiation of myoblasts by increasing IGF-II levels implying that IGF-II may also act as a trophic factor during muscle regeneration (Jennische *et al*., 1987, Florini *et al*., 1991). From these studies it is clear that IGFs play an important role in the onset of skeletal muscle differentiation depending on their interactions with other factors, whereas all other growth factors inhibit muscle differentiation. IGF-I receptor null mice had under-developed muscle and there was also a significant decrease in skeletal muscle in zebrafish that had IGF-IR knocked down (Liu *et al*., 1993, Schlueter *et al*., 2006). Similarly, loss of IGF-I or IGF-II function in myoblasts resulted in defective muscle regeneration following injury and inhibition of myogenic differentiation, respectively (Lefaucheur and Sebille, 1995, Carter *et al*., 2009).

Upon binding to the type I IGF receptor, IGFs activate multiple intracellular signaling pathways including the MEK/ERK-1/2 (Figure 1.2 and 1.3) and phosphatidylinositol 3-kinase (PI3-K) / Akt (PKB) cascades (Figure 1.4). The latter being required for up-regulation of IGF-II

upon cell contact, by creating a positive feedback loop that regulates activation of both PI3-K and IGF-II as well as inducing p38 MAPK (Lovett *et al*., 2010, Lovett *et al*., 2006, Wilson *et al*., 2004). Since p38 MAPK and PI3-K/Akt signaling are all positive regulators of myogenesis the next sections will discuss their roles in promoting differentiation converging at MRF and MEF2 transcriptional activity.

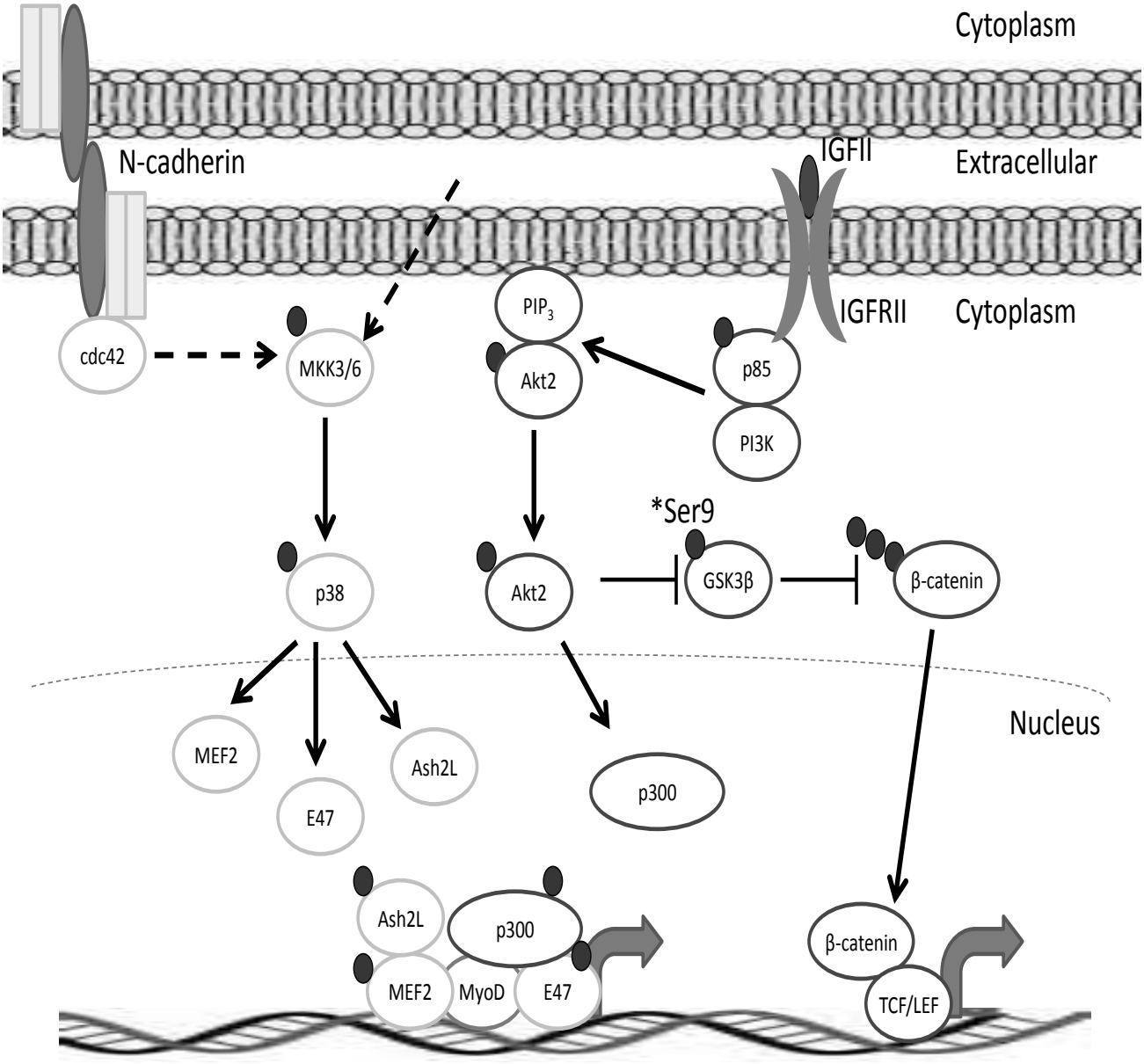


Figure 1.4: Kinase-mediated control of key transcription factors during myogenic differentiation. Differential gene expression and subsequent muscle differentiation begins with cell – cell contact and N-cadherin ligation which is thought to activate MKK3/6 (MAP2K) in conjunction with extracellular mitogens although the mechanism is poorly understood. P38 induces cell cycle exit and promotes differentiation by phosphorylating MEF2, E47 and Ash2L that form part of a myogenic transcriptional complex together with MyoD. This complex does not become active until Akt2 phosphorylates the transcriptional co-activator and histone acetyltransferase, p300. Akt2 activation in response to insulin-like growth factor II is therefore essential in initiating differentiation, and can also promote muscle hypertrophy through several other mechanisms, one of which is inhibition of GSK3β at Ser 9 as illustrated above. Un-

phoshorylated and hence active GSK3β represses its substrates such as β-catenin and NFAT3, by sequestering them in the cytoplasm and preventing their nuclear accumulation and activation of key transcription factors such as TCF/LEF. This figure has been adapted from Knight and Kothary, 2011.

P38 Mitogen Activated Protein Kinase (MAPK)

P38 MAPK is perhaps the single most crucial kinase activator of skeletal myogenesis. Defect in this pathway blocks *in vivo* embryonic MEF2 activation as well as in somites, concomitantly inhibiting myogenic differentiation (de Angelis *et al.,* 2005). P38 was originally found to interact with and phosphorylate MEF2C at three residues, T293, T300 and S387 with mutation of these sites to alanines, reducing MEF2C transcriptional activity (Han *et al*., 1997). Subsequent analysis revealed that p38 could also phosphorylate and potentiate MEF2A and MEF2D activity in both myoblasts and fibroblasts (Zhao *et al*., 1999, Penn *et al*., 2004, Rampalli *et al*., 2007). Deletion of a highly conserved p38 docking site located at the MEF2 TAD abrogates MEF2 responsiveness to p38 (Yang *et al*., 1999). In addition to its role in phosphorylating MEF2, p38 also phosphorylates E47 promoting the formation of MyoD/E-protein complexes that are essential for muscle specific gene expression (Simone *et al*., 2004, Lluis *et al*., 2005). This would explain the effect of p38 activation on MyoD transcriptional activity without ruling out the possibility of a direct effect despite the lack of strong supporting evidence (Puri *et al*., 2000). Furthermore p38 facilitates myogenic signaling by phosphorylating a SWI/SNF subunit initiating chromatin remodeling by targeting the complex to myogenic loci (Simone *et al*., 2004, Rampalli *et al*., 2007). This process also involves MEF2D phosphorylation

by p38 and functional interdependence with the PI3-K/Akt pathway (Serra *et al*., 2007, Keren *et al*., 2006). Finally p38 activity can also regulate Myogenin transcriptional activity in differentiating myocytes either indirectly through MEF2 / E-proteins or by direct phosphorylation. Defect in this pathway is strongly associated with rhabdomyosarcoma (Puri *et al*., 2000). Despite the multiple mechanisms by which p38 directly regulates myogenic genes and hence differentiation, the primary stimuli responsible for activating p38 MAPK have not been identified, although it is believed to be through a cell contact dependent mechanism (Lovett *et al*., 2006) as illustrated in figure 1.4.

Glycogen synthase kinase 3β (GSK3β) and myogenic differentiation

Although it was originally identified for its role in glycogen synthesis, as its name implies, this fails to adequately represent the many functions of GSK3 which include cell fate specification during embryonic development, cell proliferation, apoptosis and microtubule function (Cohen and Frame, 2001). The GSK3 family of serine/threonine kinases consists of two closely related isoforms, α and β, which are 98% identical within their kinase domains but differ in their N- and C-termini (Force and Woodgett, 2009). GSK3 kinases are expressed in all tissue types and are particularly critical in the brain, heart, liver, pancreas and skeletal muscle. Unlike most protein kinases, GSK3 is constitutively active in unstimulated cells and is inhibited in response to cell signaling (Force and Woodgett, 2009). Furthermore, GSK3 preferentially targets substrates that are phosphorylated by another kinase at a serine or threonine that is located four amino acids C-terminal to the GSK3 phosphorylation site, referred to as a "priming phosphate" (Doble and Woodgett, 2003). In contrast to most kinases, subsequent phosphorylation by GSK3 usually results in their inactivation and in many cases proteasomal degradation.

Recent GSK3α (-/-), GSK3β (-/-) and double knockout studies revealed a functional redundancy between the two isoforms, particularly as downstream regulators of Wnt/β-catenin signaling (Doble *et al*., 2007). Therefore, while most attention has focused on GSK3β due to the Zeste-white 3 (GSK3) mutation in Drosophila (Ruel *et al*., 1993, Siegfried *et al*., 1992) the effect of GSK3α on some of these studies cannot be ruled out (Force and Woodgett, 2009, Doble *et al*., 2007). GSK3α and –β can both form multimeric protein complexs with axin, APC protein and β-catenin resulting in β-catenin phosphorylation by GSK3 and its subsequent proteasome-mediated degradation (Cohen and Frame, 2001). Wnt signaling disrupts this complex formation resulting in stabilization and de-phosphorylation of β-catenin. Another mode of GSK3 regulation is by IGF binding to their receptors which leads to activation of PI3-K/Akt signaling (Figure 1.4). Akt (PKB) phosphorylates the N-terminal S9 and S21 residues of GSK3 α and β respectively resulting in their deactivation (Cohen and Frame, 2001).

Despite their similarities in function and regulation there is also evidence of tissue specificity between the two isoforms. GSK3β(-/-) mice are embryonic lethal with liver and heart defects in particular (Hoeflich *et al*., 2000, Kerkela *et al*., 2008). On the other hand, GSK3α (-/-) mice are viable with no notable heart defects and with increased glucose and insulin sensitivity accompanied by reduced fat mass (Patel *et al*., 2011, MacAulay *et al*., 2007). Interestingly these studies revealed no difference in glycogen synthesis in the liver or skeletal muscle and collectively the data suggest that GSK3β and not GSK3α might be important in skeletal and cardiac muscle (Patel *et al*., 2011). Corroborating this idea, loss of GSK3β function studies in cultured C2C12 myoblasts using RNAi stimulates myogenic differentiation with no evidence of any compensation by GSK3α (van der Velden et al., 2008). Similarly, IGF1 mediated induction

of muscle hypertrophy required GSK3β inactivation at S9, and pharmacological inhibition of GSK3 resulted in an increase in muscle specific gene expression (van der Velden et al., 2006).

To date there are no known GSK3β substrates that are absolutely essential for myogenic differentiation to occur therefore it begs the question whether GSK3β can indeed directly target and inactivate MEF2 or MRF factors. Interestingly, *in silico* analysis reveals several highly conserved GSK3 phosphoacceptor sites amongst MEF2 proteins, and since MEF2 is critically involved in various aspects of skeletal muscle development it represents a possible target for GSK3β mediated repression of this process particularly in ARMS where GSK3 activity is abnormally high (Zeng *et al*., 2010, Annavarapu *et al*., 2013).

Phosphatidylinositol 3-kinase (PI3-K) / Akt (Protein kinase B (PKB)) and muscle regulation

The effects of PI3-K/Akt activation on myoblast proliferation and differentiation as a result of IGF signaling are well characterized in the literature (Figure 1.2 and 1.4). Initially, activation of this pathway enhances cell cycle progression by down-regulating $p27^{Kip1}$ (Chakravarthy *et al*., 2000, Machida *et al*., 2003) however prolonged PI3-K/Akt signaling resulted in myotube differentiation and induced hypertrophy (Glass, 2010.) possibly through GSK3β inactivation (van der Velden *et al*., 2007). There are multiple mechanisms by which PI3-K/Akt signaling can regulate myogenesis, both negatively and positively in time and context dependent manners. More recently Akt has been shown to directly regulate MEF2 and MRF transcriptional activity. Whilst the effect of p38 MAPK phosphorylation on MEF2 transcriptional activity is well characterized, there is strong evidence that PI3-K is an important co-requisite for this crucial step in skeletal muscle differentiation (Rampalli *et al*., 2007). There is no evidence in the literature supporting the idea that Akt can directly phosphorylate MEF2 however there is

strong evidence that PI3-K/Akt signaling is required for p38 MAPK to directly phosphorylate MEF2 (Keren *et al*., 2006). Alternatively, this effect could be through inhibition of GSK3β and subsequent de-repression of the p38/MEF2 signaling pathway (Dionyssiou and Nowacki *et al*., 2013). In addition, PI3-K/Akt activity is required for chromatin remodeling and MEF2/MRF binding as well as acetylation of muscle specific genes (Rampalli *et al*., 2007), which also offers an explanation for the observation that Akt can potentiate MyoD and Myogenin transcriptional activity (Kaneko *et al*., 2002, Sumitani *et al*., 2002). Although Akt may not directly influence MEF2 or MRF proteins there is evidence that a downstream target, GSK3β might be able to do so. Akt is able to directly phosphorylate S9 and inhibit GSK3β activity (Rommel *et al*., 2001) and GSK3β inhibition alone is able to promote myotube formation (van der Velden *et al*., 2006). Despite these reports, the mechanism by which GSK3β can inhibit skeletal myogenesis is poorly understood. Since GSK3β is constitutively active in proliferating myoblasts and defect in this pathway has been implicated in many muscle-related diseases, including alveolar rhabdomyosarcoma, the rest of this thesis will focus on the role of GSK3β in both skeletal muscle and ARMS.

Rhabdomyosarcoma: Pathology and Regulation

Overview

Rhabdomyosarcoma (RMS) is a highly malignant mesenchymal tumor that originates from immature striated muscle tissue, resulting in dense aggregates of poorly differentiated cells that are separated by fibrous membranes. It accounts for nearly half of all pediatric soft tissue sarcomas and about 5% of all pediatric cancers (Paulino and Okcu, 2008). There are two main subtypes: embryonal (ERMS) which accounts for roughly two thirds of all RMS cases and

alveolar (ARMS) which accounts for less (~30%) but has a poorer prognosis due to an extremely high rate of metastasis. Like neuroblastoma, Ewing's sarcoma and lymphoma, immunhistochemical staining of RMS using muscle-specific markers such as myosin, desmin or MyoD reveal Actin/Myosin bundles by electron microscopy (Tonin *et al*., 1991). More recently, Myogenin has been implicated as a more specific RT-PCR marker for RMS because it is predominantly expressed in RMS but not neuroblastomas or Ewing's sarcomas (Michelagnoli *et al*., 2003). While these protein markers link RMS to a myogenic lineage it is still possible for them to sometimes originate from a different lineage (Hatley *et al*., 2012). Corroborating this idea is the development of RMS tumors at non-myogenic sites such as the genitourinary tract (Grimsby and Ritchey, 2012) and parameninges (Wijnaendts *et al*., 1994).

While the cytogenetic characterization of ERMS is currently unclear, tumors of this subtype comprise a mixture of dense spindle areas and loose myxoid foci, and have also been associated with a loss in heterozygocity at the 11p15 chromosomal locus (Scrabble *et al*., 1987). Tumors of this histology are typically found in the head, neck and genitourinary regions. In addition, botryoid variants of ERMS tumors resemble clusters of grapes and form in hollow organs such as the vagina, nasopharynx and urinary bladder (Paulino and Okcu, 2008), and typically have better prognosis (Al-Daraji *et al*., 2009). The inability of cells of the ERMS subtype to undergo myogenic differentiation despite the expression of the myogenic bHLH superfamily of transcription factors, the MRFs and MEF2 proteins has been widely attributed to poor MyoD/E-protein complex stability (MacQuarrie et al., 2013, Yang *et al*., 2009) and lack of p38 MAPK activation (Puri *et al*, 2000). Activation of p38 MAPK by manipulation of various signaling pathways has been shown to rescue the myogenic program *in vitro* ERMS systems by inducing MyoD transcriptional activity (Puri *et al*., 2000, Rossi *et al*., 2011).

In contrast, ARMS is typically associated with two chromosomal translocations t(2;13)(q35;q14) and t(1;13)(q36;q14) which fuse the *Pax3* and *Pax7* genes to *Foxo1a* resulting in potent transcription factors: Pax3-Foxo1a and Pax7-Foxo1a, 55% and 22% of the times respectively (Galili *et al*., 1993, Davis *et al*., 1994, Barr, 1997). ARMS subtypes without these fusion proteins do exist albeit less frequently and they exhibit characteristics that are more common to ERMS subtypes (Williamson *et al*., 2010). The rest of this chapter will focus on the pathology and biomolecular mechanisms associated with Pax3-Foxo1a expressing ARMS which have the lowest survival rate at 8% (Sorensen *et al.*, 2002).

The break points that occur during the fusion at intron7 of Pax3 and intron1 of Foxo1a result in a chimeric protein that contains the Pax3, DNA-binding, paired-box and homeodomains as well as the forkhead (FKHR) transactivation domain from Foxo1a. In addition, this fusion results in the loss of a C-terminal, Pax3 mono-ubiquitination site that is involved in its degradation (Boutet *et al*., 2007), as well as the disruption of a Foxo1a, PKB/Akt dependent phosphoacceptor site which is involved in its nuclear export (Schwab *et al*., 2005, Brunet *et al*., 1999, del Peso et al., 1999). These combined characteristics make Pax3-Foxo1a a much more potent transcription factor than either Pax3 or Foxo1a alone and can regulate the expression of all of the Pax3 target genes and many more (Zhang *et al*., 2009).

Gene Regulation and Mechanisms that Prevent RMS from Achieving Terminal Differentiation

It is well documented that ectopic expression of Pax3-Foxo1a is repressive to myogenic differentiation in various cellular systems as well in Pax3-Foxo1a expressing transgenic mice (Calhabeu *et al*., 2013, Roeb *et al*., 2007). Pax3-Foxo1a is 100 fold more transcriptionally active than wild type Pax3 (Fredericks *et al*., 1995) and several studies support the transforming

properties of Pax3-Foxo1a including increased proliferation rates and accelerated tumor formation both *in vitro* and *in vivo*. Contributing to its potency, Pax3-Foxo1a is expressed in high quantities as it is able to regulate its own transcription via a positive feedback loop (Zeng et al., 2010, Davis and Barr, 1997). In addition to enhancing cell proliferation, Pax3-Foxo1a regulates anti-apoptotic properties genes and suppressing terminal differentiation despite the expression of MRFs and MEF2 proteins (Bernasconi *et al*., 1996, Khan *et al*., 1999). A central common theme that underlies the inability of Pax3-Foxo1a expressing and non-expressing subtypes to undergo terminal differentiation is the deregulation of MyoD activity despite its expression. A second commonality that is predominantly associated with ERMS but not ARMS, is the lack of p38 MAPK induction, a potent activator of MEF2 and key requisite for chromatin remodeling and transcriptional control of muscle specific genes (Cox *et al*., 2003, Ramapalli *et al*., 2007). The intent of this section is to review the various mechanisms by which Pax3-Foxo1a de-regulates myogenic differentiation and how it cross-talks with various signaling pathways to achieve this.

The mechanisms by which the bHLH MRFs regulate skeletal myogenesis have been discussed in the previous section. With the basis that MyoD is a master switch, skeletal muscle specific transcription factor that controls myogenic differentiation, most studies regarding impaired differentiation in RMS have focused on MyoD. These studies revealed that even though MyoD can bind to its consensus DNA binding site, it exhibits poor *trans*activation in RMS (Tapscott *et al*., 1993). One mechanism by which this occurs is through inhibition of EGR1 dependent transcription of $p57^{Kip2}$ (Roeb *et al*., 2007). $P57^{Kip2}$ plays a dual role in skeletal myogenesis: 1. It is a cell cycle inhibitor, 2. It stabilizes the MyoD / E-protein heterodimer interaction with DNA. Pax3-Foxo1a inhibits EGR1 by promoting its proteasomal degradation (Roeb *et al*., 2007). Another, Pax3-Foxo1a dependent mechanism that interferes with MyoD

transcriptional activity is through the up-regulation of histone methyltransferases such as KMT1A (Lee *et al*., 2011). MyoD is known to interact with histone acetyltransferases (HATs) and histone deacetylases (HDACs) to regulate the temporal expression of muscle specific genes (Sartorelli and Puri, 2001). Pax3-Foxo1a promotes the expression of Jarid2 and KMT1A in ARMS cells (Walters *et al*., 2013, Mungamuri *et al*., 2012) and these target genes promote trimethylation of lysine 9 of histone 3 (H3K9me3) which suppresses the transcription of several MyoD target genes that promote skeletal myogenesis such as Myogenin and Myosin heavy chain. The effect of KMT1A can be reversed by p53 (Mungamuri *et al*., 2012) but since p53 is also defective in most RMS cases, most MyoD target gene promoter regions are predominantly trimethylated and hence repressed in Pax3-Foxo1a expressing ARMS. Despite trimethylation of the Myogenin promoter region, Myogenin is still highly expressed in RMS implicating a non-canonical MyoD independent mechanism for its transcriptional control.

Cross-talk between Pax3-Foxo1a and Cell Signaling Pathways

In addition to its ability to deregulate MyoD transcriptional activity, genome wide studies in a variety of cell types have revealed both direct targets of Pax3-Foxo1a as well as a plethora of genes that are regulated as a consequence of the ARMS causing fusion protein. These are summarized and discussed in a well written review by Amy D. Marshall and Gerard C. Grosveld (Marshall and Grosveld, 2012). Interestingly it appears that ~95% of these genes are up-regulated by Pax3-Foxo1a and the few that are down-regulated are involved with cell cycle arrest or myotube formation. Since key transcription factors that are required for myogenic differentiation to occur are all expressed in RMS, it is likely that key post-translational modification of these are

defective. The next part of this section will discuss cross-talk between Pax3-Foxo1a expression and various critical signaling pathways involved in myoblast proliferation and differentiation.

Pax3-Foxo1a and Tyrosine Receptor Mediated Growth Factors

Even though Pax3-Foxo1a down regulates Pax3 expression, it still up-regulates many Pax3 target genes and with greater potency (Keller *et al*., 2004, Kikuchi *et al*., 2008). One such target gene is fibroblast growth factor 4 (FGF4) which promotes cell proliferation (Figure 1.2), and tumorigenesis in both ERMS and ARMS (Cao *et al*., 2010). Although FGF4 is a requisite regulator of myoblast migration and proliferation through its tyrosine receptor kinase, during embryonic development (Webb *et al*., 1997), loss of FGF4 function is necessary for terminal differentiation to occur (Itoh *et al*., 1996). In ARMS, loss of FGF4 function reduces proliferation and tumorigenicity despite the expression of Pax3-Foxo1a (Cao *et al*., 2010). A second key tyrosine kinase receptor mediated growth factor that influences Pax3-Foxo1a induced tumorigenicity is Insulin-like growth factors 1/2 (IGF1/2), the receptors of which are direct Pax3-Foxo1a targets (Makawita et al., 2009, Engert *et al*.,1996). Initially, IGF regulates myoblast proliferation (Figure 1.2) but subsequently promotes the expression of muscle specific genes as illustrated in figure 1.4 (Engert *et al*., 1996). Maintained IGF receptor signaling results in myotube hypertrophy in normal myogenic cells (Barton *et al*., 2010) however, in RMS the expression of key muscle genes that promote differentiation are not regulated through the same pathway despite IGF receptor activity (Xu and Wu *et al.*, 2000), resulting in enhanced cell proliferation.

Pax3-Foxo1a and TGFβ signaling

TGFβ signaling inhibits skeletal myogenesis through two distinct mechanisms that promote cell proliferation and inhibit gene expression of key muscle regulatory factors such as Myogenin (Figure 1.3). It is therefore not surprising that the key effectors of TGFβ signaling Smad3/4 and ERK1/2 have been implicated and targeted in RMS (Wang *et al*., 2010, Guo *et al*., 2007). Loss of Smad and ERK function studies have been shown to reduce tumorigenicity in non-Pax3-Foxo1a ERMS cell types and promote apoptosis (Wang *et al*., 2010, Ye *et al*., 2006). Myostatin, a member of the TGFβ superfamily that is heavily linked with muscle related diseases inhibits myogenic differentiation through a Smad-dependent pathway (Kollias *et al*., 2006) is also highly active in RMS (Ricaud *et al*., 2003, Langley *et al*., 2004). Prevention of Myostatin secretion or binding to its activin-like receptor promotes differentiation of both Pax3-Foxo1a expressing and non-expressing RMS subtypes, by reducing translocation of Smad proteins into the nucleus and by activating p38 MAPK (Ricaud *et al*., 2003, Rossi *et al*., 2011). Repression of myogenic differentiation in ERMS is attributed to their inability to activate P38 MAPK (Puri *et al*., 2000). In addition to being a key activator of MEF2 factors, P38 MAPK also plays a key role in regulating cell cycle arrest (Perdiguero et al., 2007). Subsequent studies have shown that indirect stimulation of p38 MAPK through competitive inhibition of Myostatin, or more recently through the Notch signaling pathway, promotes cell cycle exit and reduces the tumorigenicity of RMS derived cells (Raimondi *et al*., 2013).

Pax3-Foxo1a, Akt and GSK3β

Akt is an intriguing regulator of RMS because in functional myoblasts, Akt is absolutely required for activation of muscle specific genes and prolonged induction of Akt results in myotube hypertrophy (Barton *et al*., 2010). Paradoxically, Akt has been shown to promote tumorigenicity in RMS despite its maintained activity by enhancing cell proliferation and more recently by increasing apoptosis resistance in hypoxic conditions (Wan and Helman, 2003, Kilic-Eren *et al*., 2013). Cross-talk between Akt and a wide range of signaling pathways seems to affect its role in a myogenic context. For instance, TGFβ induces cell proliferation through PI3-K/Akt dependent phosphorylation and hence inhibition of cell cycle regulators, p21 and p27 (Fang *et al*., 2012, Suwanabol *et al*., 2012). Once TGFβ signaling and possibly that of other growth factors ceases in myoblasts, p21/p27 activate cell cycle arrest despite the maintained activity of PI3-K/Akt implicating that the effects of Akt are context dependent and that molecular pathways involving kinases and their substrates are complex and interlinked. ARMS is a great model to demonstrate this co-dependency. In ARMS, Akt phosphorylates and activates NFκB to promote cell proliferation (Sizemore *et al*., 1999, Wang *et al*., 2008) whereas another substrate GSK3β, which is anti-myogenic remains predominantly un-phosphorylated at S9 (Zeng *et al*., 2010), and hence constitutively active. These examples indicate how Akt preferentially activates proteins that enhance cell proliferation and survival in ARMS while ignoring its well documented pro-myogenic targets.

GSK3β has been extensively discussed in a previous section and its activity is specific to Pax3-Foxo1a expressing ARMS subtypes (Zeng *et al*., 2010). GSK3β has already been shown to phosphorylate the chimeric Pax3-Foxo1a oncogenic protein (Zeng et al., 2010), however it is unclear if this phosphorylation increases its transcriptional activity, potency or even creates a positive feedback loop that maintains GSK3β activity. Since pharmacological inhibition of

GSK3β reduced cell proliferation and tumorigenicity of RH30 cells whilst promoting the myogenic program, we decided to further investigate its role in both the contexts of myogenic differentiation and ARMS. Both studies are documented and discussed in the next two chapters.

Pax3-Foxo1a loss of function

In ARMS, the chimeric Pax3-Foxo1a has been shown to affect the process of skeletal muscle differentiation by disrupting a huge variety of signaling pathways and their downstream effectors, promoting malignant phenotypes such as cell proliferation, motility, and to suppress differentiation. Loss of Pax3-Foxo1a function studies using RNAi reduced the number of proliferating cells by causing them to accumulate in the G1 phase of the cell cycle, as well as decreased motility and hence mesenchymal-to-epithelial transition (Kikuchi et al., 2008). In addition, these studies also revealed increased levels of myogenic proteins involved in muscle differentiation and morphology, including Myogenin (Kikuchi et al., 2008).

Statement of Purpose

Skeletal myogenesis is a critical and highly regulated process during embryonic development, and is primarily orchestrated by MRF and MEF2 transcription factors. The overall purpose of my research has been to better understand the molecular basis for alveolar rhabdomyosarcoma, a highly malignant mesenchymal tumor that originates from poorly differentiated, immature, striated muscle tissue and occurs despite the expression of MRF and MEF2 proteins. Recently, cell signaling by extracellular stimuli such as growth factors have been shown to regulate MRF and MEF2 transcriptional activity by causing posttranslational modifications. Protein kinases are key effectors of these signaling cascades, and phosphorylation of their substrates affects their ability to bind DNA or protein cofactors, hence influencing their transcriptional activity.

Previous studies have shown that enhanced GSK3β activity inhibits myogenic differentiation and additionally, GSK3β activity has been strongly associated with alveolar rhabdomyosarcoma. Since GSK3β phosphorylation of its substrates is typically inhibitory, usually leading to their proteasomal degradation, I wanted to investigate whether GSK3β could directly phosphorylate MEF2 and/or MRF transcription factors.

Research in our lab has systematically focused on documenting MEF2 phosphorylation patterns in mammalian systems. These efforts have resulted in the discovery of a crucial phosphoacceptor site, S255 that regulates MEF2A stability and function (Cox *et al*., 2003). The ubiquitous phosphorylation of S255 in several cell types, coupled with its role in MEF2A stability, points to a role for GSK3β. To address this hypothesis, a series of experiments were conducted to determine whether or not GSK3β could indeed regulate MEF2A function and

expression in skeletal muscle, either directly by phosphorylation or indirectly. The findings and implications of this study are discussed in Chapter 2.

A paradoxical feature of alveolar rhabdomyosarcoma is the expression of Myogenin. *Myogenin* is a downstream MEF2 and MyoD target gene, and its expression functions to promote cell fusion by activating structural muscle genes and hence promoting myogenic differentiation. Despite Myogenin expression, ARMS cell types do not undergo terminal differentiation, therefore we hypothesized that something was inhibiting Myogenin transcriptional activity. Since GSK3β has already been implicated in alveolar rhabdomyosarcoma, coupled with the presence of several GSK3 phosphoacceptor sites on Myogenin, we wanted to examine whether or not Myogenin was indeed a GSK3β substrate and if this abrogated Myogenin function. A series of experiments which included gain or loss of function assays as well as mutational analysis of these proteins were conducted to address this hypothesis. The findings and implications of this study are discussed in Chapter 3.

Finally, our lab has identified a novel MEF2 target gene, the tumor suppressor protein, KLF6 (Salma and McDermott, 2012) that is also a GSK3β substrate in hepatocellular carcinoma (Okcu et al., 2013), and that has also recently been identified in the skeletal muscle transcriptome (Blais *et al*., 2005). Since GSK3β and MEF2 have opposite effects on skeletal myogenesis and since the role of KLF6 in a myogenic context was unknown, we wanted to examine KLF6 regulation and function in myoblasts using loss of function assays. The findings and implications of this study are discussed in Chapter 4.

The following chapter has been published as a research article in the Journal of Molecular and Cellular Cardiology in a slightly modified format (Jan 2013). Contributing authors: MG Dionyssiou, NB Nowacki, S Hashemi, J Zhao, A Kerr, RG Tsushima, JC McDermott.

The experimental design, figures and data presented in this chapter are mostly of my own efforts with the following exceptions. NB Nowacki who is an equal co-author made the initial observation that MEF2A was regulated by pharmacological inhibition of GSK3 and produced the data in figures 2.1B, 2.2A-C. Animal husbandry was performed by S Hashemi who also provided the data in figures 2.4A-C, as well as J Zhao who provided the data in figure 2.5. Heart samples from a cardiac-specific Cre-Lox excision of exon 2 of the GSK3β gene were provided by A Kerr and RG Tsushima and used in figures 2.4A and 2.4C. JC McDermott assisted with experimental design and with editing the manuscript. Catherine Chan provided technical support throughout the study.

Cross-talk between glycogen synthase kinase 3β (GSK3β) and p38MAPK regulates myocyte enhancer factor 2 (MEF2) activity in skeletal and cardiac muscle

2.1 Abstract

Glycogen synthase kinase 3β (GSK3β) is a known regulator of striated muscle gene expression suppressing both myogenesis and cardiomyocyte hypertrophy. Since myocyte enhancer factor 2 (MEF2) proteins are key transcriptional regulators in both systems, we assessed whether MEF2 is a target for GSK3β. Pharmacological inhibition of GSK3β resulted in enhanced MEF2A/D expression and transcriptional activity in skeletal myoblasts and cardiac myocytes. Even though *in silico* analysis revealed GSK3β consensus (S/T)XXX(S/T) sites on MEF2A, a subsequent *in vitro* kinase assay revealed that MEF2A is only a weak substrate. However, we did observe a posttranslational modification in MEF2A in skeletal myoblasts treated with a GSK3β inhibitor which coincided with increased p38MAPK phosphorylation, a potent MEF2A activator, indicating that GSK3β inhibition may de-repress p38MAPK. Heart specific excision of GSK3β in mice also resulted in up-regulation of p38MAPK activity. Interestingly, upon pharmacological p38MAPK inhibition (SB203580), GSK3β inhibition loses its effect on MEF2 transcriptional activity suggesting potent cross-talk between the two pathways. Thus we have documented that cross-talk between p38MAPK and GSK3β signaling converges on MEF2 activity having potential consequences for therapeutic modulation of cardiac and skeletal muscle gene expression.

2.2 Introduction

Mitogen activated protein kinase (MAPK) signaling pathways are prominently involved in many cellular processes including cell proliferation and growth [1], development, migration [2] and differentiation [3,4]. Deregulation of MAPK signaling almost invariably leads to developmental defects and diseases including cardiac hypertrophy, muscular atrophy and cancer [5]. Proper regulation of this pathway in the context of elaborate and highly complex signaling networks within the cell is strongly dependent on communication with other signaling molecules, resulting in either synergistic or antagonistic relationships that produce a spectrum of biological outcomes. Understanding the nature of cross-talk between signaling pathways is indeed a major hurdle to understanding the molecular basis of all cellular processes. In the studies described here, we take advantage of the convergence of several signaling pathways on the MEF2 family of transcriptional regulators in order to gain insight into how cross-talk between GSK3β and p38MAPK signaling influence a single effector molecule which functions as a signaling conduit for the control of cardiac and skeletal muscle gene expression.

MEF2 proteins belong to the MADS (MCM1, agamous, deficiens serum responsive factor) superfamily of transcription factors. There are four isoforms of MEF2 in vertebrates, MEF2A-D that contain a highly conserved 57aa MADS-box domain at their amino-termini immediately adjacent to their 29aa MEF2 domain. Collectively these two domains are involved with DNA-binding, dimerization and interaction with co-factors. MEF2 factors regulate transcription as homo- or heterodimers by binding to the consensus DNA sequence (C/T)TA(A/T)$_4$TA(G/A) found in the regulatory regions of most cardiac and muscle specific genes [9,10]. Less conserved amongst the MEF2 isoforms are the C-termini which are subject to alternative splicing [11,12]

and a variety of posttranslational modifications such as acetylation [13-15], sumoylation [16,17] and phosphorylation, many of which have proved important in regulating MEF2.

Previous studies have identified several kinases that regulate MEF2 *trans*activation properties. Casein kinase II (CK2) phosphorylates MEF2C at serine 59 enhancing its DNA binding capacity and hence transcriptional activity [18]. ERK5 interacts with the N-termini of MEF2A/C/D [19] and can phosphorylate serine 387 in the transactivation domain [20]. Of the kinases that target MEF2, p38MAPK has been most extensively studied and deemed to be a key requisite for skeletal and cardiac muscle differentiation. Defects in this pathway have also been associated with muscle related diseases, such as embryonal rhabdomyosarcoma (ERMS) [21]. MEF2A has multiple p38MAPK phosphoacceptor sites as indicated by mass spectrometric analysis [8] and all four isoforms have been repeatedly demonstrated to be activated by this kinase. During embryogenesis, p38MAPK activation of MEF2 is necessary for proper heart development [23] and is also involved in cardiac hypertrophy in adult heart tissue, both *in vivo* and *in vitro* [22,23]. Similarly, as well as being a key regulator of skeletal myogenesis *in vitro,* p38MAPK critically interacts with and activates MEF2 in the somite myotome during development [24]. Thus MEF2 is a key convergence point for several cellular signaling pathways in the control of striated muscle gene expression.

Two kinases that actively repress skeletal and cardiac muscle differentiation are PKA and GSK3β. Whilst the effect of PKA has been shown to be mediated through repression of MEF2 transactivation properties [25], the effect of GSK3β on this process is less clear. GSK3β is involved in multiple cellular processes including glycogen metabolism, embryonic development,

cell proliferation and apoptosis [26,27]. Several unique features distinguish GSK3 from other protein kinases; it is constitutively active in unstimulated cells and paradoxically, it is inhibited in response to cellular signals such as growth factors [28]. More importantly phosphorylation of its substrates often leads to their subsequent ubiquitylation followed by proteasomal degradation [26]. GSK3β usually targets proteins that have already been phosphorylated by another kinase at a serine or threonine residue located four amino acids C-terminal to a consensus (S/T)XXX(S/T)-PO_4 motif [26,27]. In addition to this canonical consensus recognition sequence, GSK3β has been shown to phosphorylate KSP motifs in neurofilament proteins [29] and microtubule associated proteins [30] leading to their inactivation.

GSK3β has been studied extensively in insulin and Wnt signaling. Upon insulin binding to its receptor, activation of the phosphtidylinositol-3-kinase (PI3K) pathway occurs, leading to phosphorylation and hence inactivation of serine 9 on GSK3β via protein kinase B (PKB) [31] as well as C-terminal phosphorylation on serine 389 by p38MAPK [38]. In a myogenic context, PI3K activation has been shown to lead to cardiac and skeletal muscle hypertrophy [6,7,26,28,32,33] as well as being an activator of p38MAPK [34] and a co-requisite for p38MAPK induced chromatin remodeling [35,36]. Defects in the PI3K pathway lead to activation of GSK3β and consequently repression of myogenic differentiation [35]. In kidney cells induction of GSK3β results in the repression of JNK and p38MAPK through the inhibition of their upstream mitogen activated protein kinase kinase kinase (MAPKKK), MEKK4 [37]. Thus there is considerable circumstantial evidence suggesting an intersection between GSK3β and p38MAPK signaling pathways.

In this report, based initially on informatics analysis, we hypothesized that GSK3β is involved in the regulation of cardiac and skeletal muscle gene expression either by directly phosphorylating and hence destabilizing MEF2, or indirectly abrogating MEF2 activity through inhibition of p38MAPK. Whilst we report that MEF2A is a weak substrate of GSK3β *in vitro* we document that GSK3β activity represses MEF2 transactivation properties in both skeletal and cardiac myocytes both *in vitro* and *in vivo*. Pharmacological inhibition of GSK3β resulted in (i) increased MEF2 activity and (ii) de-repression of p38MAPK. Heart specific excision of GSK3β also resulted in up-regulation of p38MAPK activity. Gain of function assays using constitutively active GSK3β (S9A) repressed MEF2 activity which can be counteracted by exogenous activation of p38MAPK. Based on these data we propose integration of GSK3β and p38MAPK into the signaling network converging on the MEF2 transcription factors regulating both skeletal and cardiac gene expression.

2.3 Materials and Methods

Plasmids

MEF2 and MCK reporter constructs (pMEF2, pMCK, pMCKΔMEF2) in pGL3 and expression vectors for MEF2A in pMT2 were used in reporter gene assays. The Gal4-MEF2A fusions have been described previously [43]. HA tagged Pax3-fkhr was cloned into pcDNA3.1 and kindly donated by Dr. Malkin and Adam Durbin at MaRS, Toronto. HA tagged GSK3β(S9A) was cloned in pcDNA3 ORF 995-2305. p38 and MKK6(EE) expression vectors were previously described [8]

Antibodies

Anti-MEF2A rabbit polyclonal antibody was produced with the assistance of the York University Animal Care Facility; anti-MEF2D (1:1000; BD Biosciences); β-catenin, phospho-β-catenin, p38,

phospho-p38, ATF2, phospho-ATF2 and GSK3β (1:1000; Cell Signaling); actin, α/β-tubulin (1:2000; SantaCruz) were used for immunoblotting experiments.

Cell culture and transfection

C2C12 and RH30 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^{o}C incubator with a 5% CO_2 atmosphere. For transfections, cells were seeded 1 day prior to transfection and transfected according to the standard calcium phosphate method previously described by Perry *et al*. 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. (2001) [54]. Neonatal Cardiomyocytes were isolated from 2- to 5-day old rats. Whole hearts were separated and minced in a buffer solution (calcium and Bicarbonate Free Hanks with Hepes) and then dissociated into single cells by trypsin enzyme (Gibco) during repeated digestion with slow stirring. 10% FBS (Sigma) DMEM F12 (w/1% Penicillin/Streptotocin, 50 mg/L gentamycin sulfate) (Invitrogen) was added to the suspended cells and centrifuge for 10 mins in 1200 rpm. The pellet was resuspended in medium. The isolated cells were plated for 30 to 60 minutess at 37^{o}C, allowing differential attachment of non-myocardial cells. The cardiomyocyte cells were counted and transferred to pre-gelatin coated plates.

The HL1 cardiac cell line was cultured in Claycomb Medium (Sigma Aldrich) supplemented with 100µM norepinephrine (Sigma Aldrich), 10% FBS and 4mM L-glutamine (invitrogen). Cells were maintained in a humidified 37°C incubator with 5% CO_2. The HL-1 cell line was originally

established from an AT-1 subcutaneous tumor excised from an adult female Jackson Laboratory inbred C57BLy6J mouse.

Transient transfections in neonatal cardiomyocytes and HL-1 cells were performed using lipofectamine 2000. A 1:2.5 mixture ratio of DNA to lipofectamine in 250 µl Opti-Medium (Gibco) was prepared for a 4h incubation.

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).

Independent two sample t-tests of all quantitative data were conducted using R software. P-values are indicated with respect to controls where appropriate.

In vitro kinase assay

2.5μg of purified recombinant GST-MEF2A (1-507) was mixed with 0.5μg purified recombinant GST-GSK3β (1-433; Cell Signaling) and with [γ-^{32}P] ATP and incubated for 30min at 37^{o}C. Samples were denatured for 5min at 95^{o}C in SDS sample buffer. Protein samples were then separated by 10% SDS-PAGE and exposed on X-ray film (Kodak X-Omat) for 21hrs to detect ^{32}P incorporation.

Animal treatment with GSK3 inhibitor in vivo

LacZ-MEF2 Transgenic mice were used in this study. Two groups of male mice (n=4/each group) at 3 months old were used. The mice received i.p. injections of 0.6 M LiCl or 10mM sterile PBS daily for 30 days. Mice were sacrificed by cervical dislocation. The apexes of heart and 10μm transverse sections of the skeletal muscles were fixed with 2% paraformaldehyde in PBS for 30 min. After being washed three times with PBS, the samples were incubated with X-Gal solution (5mM ferrocyanide, 5mM ferricyanide, 2mM $MgCl_2$, and 1 mg/ml X-Gal) at 37°C.

Embryo treatment with GSK3 inhibitor in vitro

The MEF2-LacZ transgenic mouse embryos were dissected at 9.5 dpc from timed pregnant mice and cultured in a 24-well plate with 1 ml DMEM/10% FBS containing 50μM GSK3β inhibitor TD-8 or solvent only (served as the control) at 37°C for 24h. After incubation, the embryos were fixed with 2% paraformaldehyde for 30 min. The embryos were rinsed twice with PBS, and then immersed in X gal staining solution (5mM ferrocyanide; 5mM ferricyanide; 2mM $MgCl_2$; 1mg/ml X gal) at 37°C.

2.4 Results

Pharmacological inhibition of GSK3β enhances MEF2 transcriptional activity in skeletal myoblasts

Given that GSK3β suppresses myogenic differentiation [39,40] and that phosphorylation by GSK3β is generally inhibitory to protein function, it was hypothesized that the pharmacological inhibition of GSK3β might regulate MEF2A transcriptional activity. To test this hypothesis, C2C12 myoblasts were transfected with MEF2A and were treated with increasing concentrations (1-50μM) of AR-A014418, a GSK3 inhibitor [41]. MEF2 activity was assessed using a 3x MEF2-Luciferase construct, a reporter gene containing three copies of the MEF2 *cis* element. These data illustrate enhanced transcriptional activity of both endogenous ($p<0.001$) and ectopically expressed MEF2 (supplementary data) in a dose dependent manner upon GSK3β inhibition. The optimum concentration of AR-A014418 treatment was determined to be 10μM ($p<0.001$) and, the effect of this treatment on MEF2A transcriptional activity is depicted in figure 2.1a. To further corroborate this, the effect of GSK3β on the muscle creatine kinase (MCK) enhancer, a physiological MEF2 target, was also analyzed (Fig. 2.1b). The MCK enhancer is useful in studying muscle specific gene expression as it is highly dependent on MEF2, CARG-box and E-box *cis* elements during myogenesis. Therefore, myoblasts were co-transfected with MEF2A and either the wild type MCK-Luciferase construct or an alternate version containing mutated MEF2 binding sites (MCK-Luc ΔMEF) and treated with 10μM AR for 19h. Data revealed that GSK3β inhibition enhanced MCK-Luc activity ($p<0.001$) and that this effect was abrogated when the MEF2 sites are mutated, hence demonstrating that the effect of GSK3β is primarily through the MEF2 cis element (Fig. 2.1b). As a consequence it was hypothesized that the effects of GSK3β inhibition were mediated by modulation of MEF2A *trans*activation

properties. GSK3β is predicted to phosphorylate the first serine or threonine in the consensus sequence (S/T)XXX(S/T) of its substrates preferably with the +4 serine or threonine already primed by phosphorylation by a different kinase [26,27], although this is not absolutely required. In human MEF2A, *in silico* analysis revealed several potential GSK3β phosphoacceptor sites. To begin to determine whether MEF2A is indeed a substrate for GSK3β, an *in vitro* kinase assay was performed using GST-MEF2A 1-507, purified GST-GSK3β and γ-^{32}P ATP. Bands were resolved using SDS-PAGE and revealed radio-labeled bands for autophosphorylated GSK3β and MyBP (a positive control). A very weak radio-labeled band for MEF2A was detected compared to the positive control (MyBP) and we therefore concluded that MEF2A was at best a weak GSK3β substrate *in vitro*. We subsequently hypothesized that any effects caused by manipulation of GSK3β on MEF2A activity were indirect.

Figure 1.

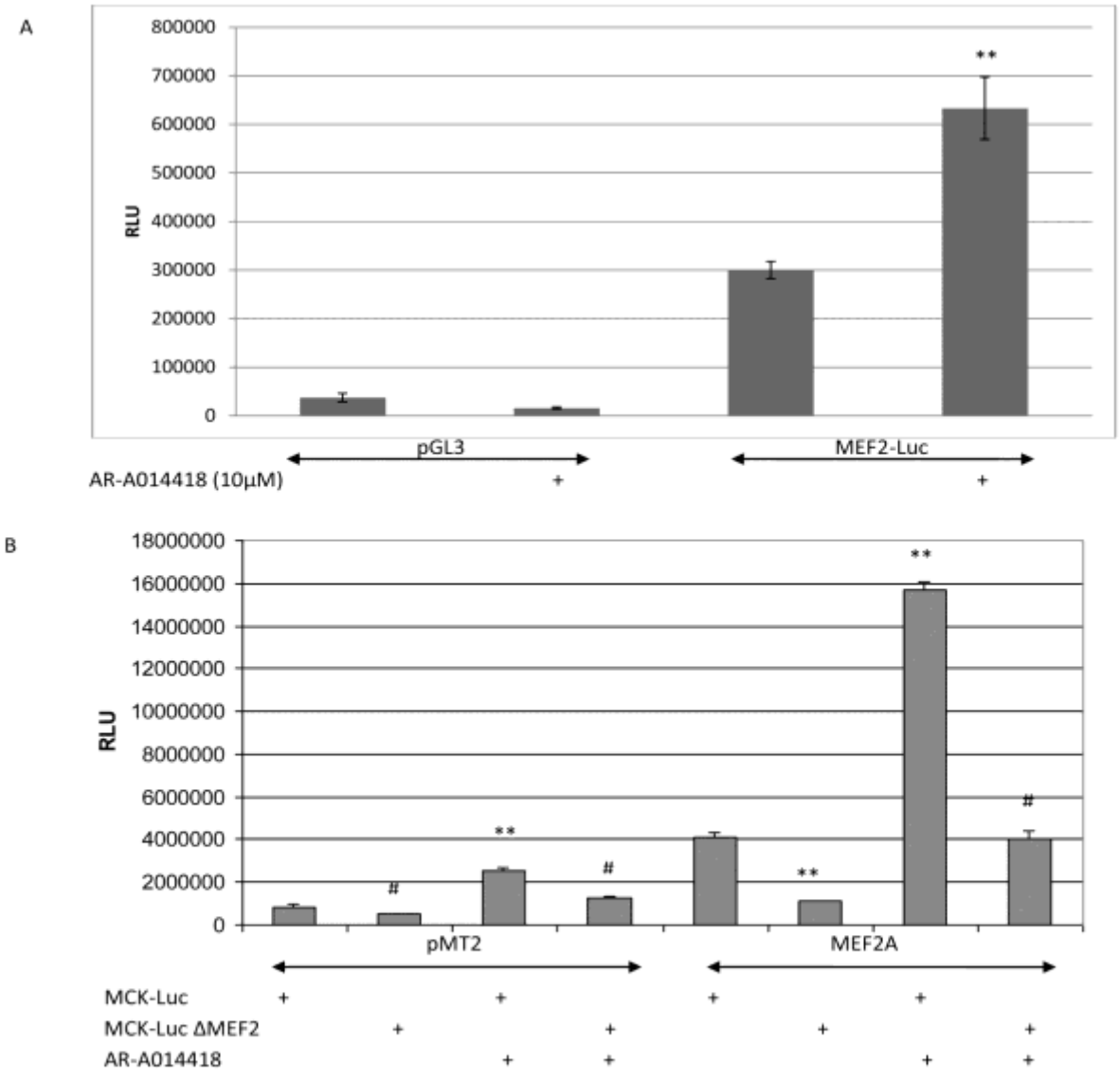


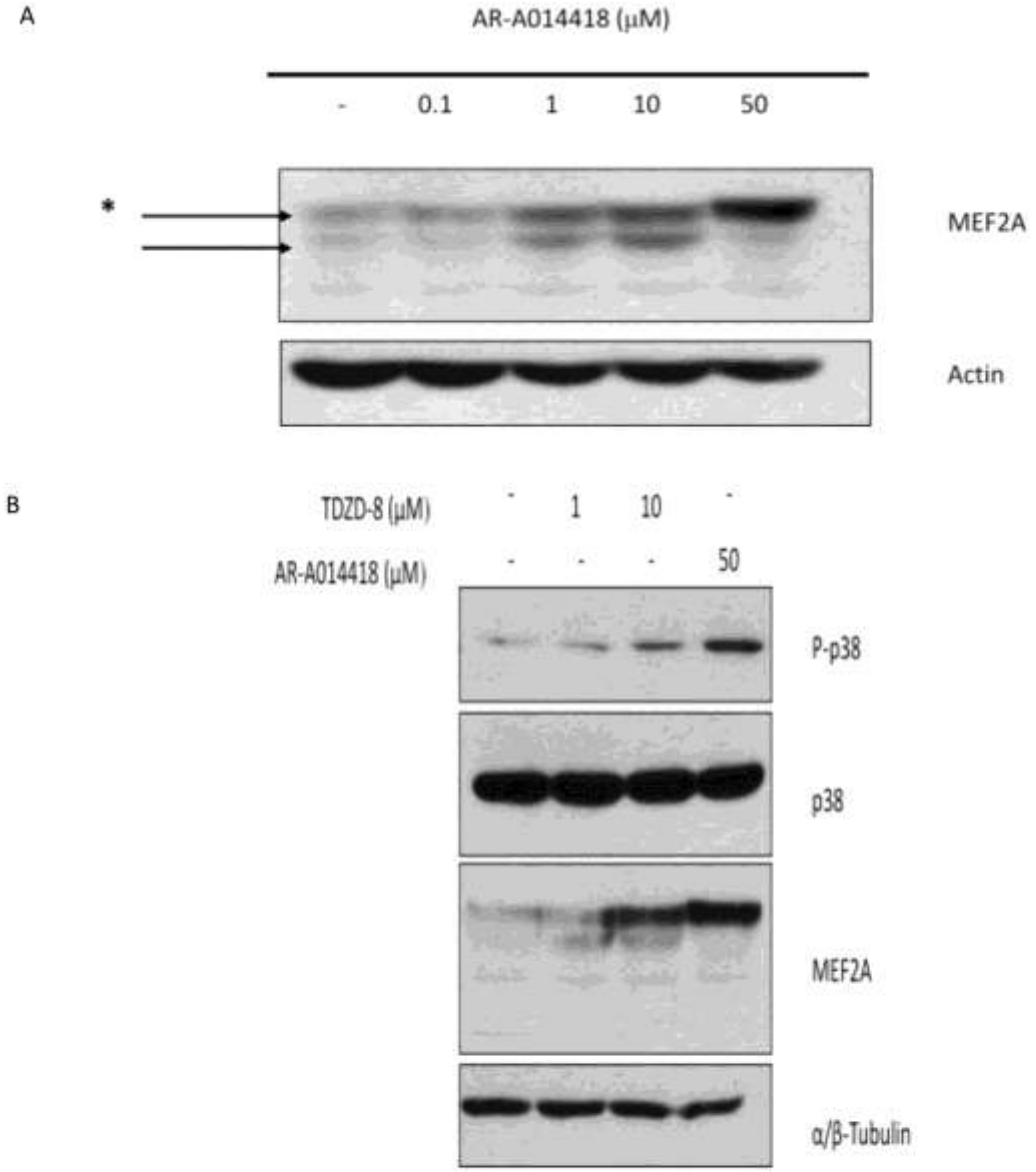
Figure 2.1: **Pharmacological GSK3β inhibition enhances (a) MEF2-Luc and (b) MEF2A transactivation of the MCK enhancer**. C2C12 myoblasts were maintained in GM and transiently transfected with either pMT2 or pMT2-MEF2A and MCK-Luc with or without the MEF2 binding sites mutated. Luciferase activity was assessed using the respective reporter genes mentioned above and normalized to β-galactosidase activity. Cells were either treated with 10μM AR-A014418 (unless otherwise depicted) or solvent (DMSO) for 19h prior to harvesting. Data are the mean + S.E. (n=3). ** indicates a significant difference with respect to the control ($p<0.001$) and # indicates no significant change.

GSK3β inhibition causes a post translational modification in MEF2A and this correlates with increased p38MAPK *phosphorylation in skeletal myoblasts.*

Since a common mode of GSK3β regulatory activity is through modification of protein stability (e.g. β-catenin) [42], we also tested the effect of GSK3β on MEF2A protein expression levels. If GSK3β can indeed directly phosphorylate MEF2A, then GSK3β inhibition should either result in an increase in MEF2A expression or enhanced stability. To test this hypothesis, skeletal myoblasts were cultured and treated with increasing concentrations (0.1-50mM) AR-A014418 for 4h. Western immunoblotting revealed increasing MEF2A expression levels with increasing concentration of GSK3β inhibition, 0-50μM AR-A014418 and that also corresponded with an obvious change in MEF2A gel migration (Fig. 2.2a). A lower mobility, high molecular weight form of MEF2A that is post-translationally modified has already been identified [43] and at 50μM AR-A014418, the faster migrating band shifted and merged with this slower migrating band forming a single, high molecular weight MEF2A band. This strongly suggests that MEF2A underwent a post-translational modification [8,43]. Modification of MEF2A was obviously not due to GSK3β phosphorylation (since it was inhibited) and was reminiscent of effects that we have observed and documented before with p38MAPK [8]. Therefore we went on to test the possibility that GSK3β inhibition might cause p38MAPK phosphorylation using 50μM AR-A014418 as well as a second GSK3β inhibitor, TDZD-8 [44]. The results are depicted in figure 2.2(b) and illustrate that the MEF2A band shift observed at 50μM AR-A014418 corresponds with increased levels of phospho-p38MAPK. Similarly increasing concentrations (1-10μM) of TDZD-8 resulted in increased phospho-p38MAPK protein levels as well a MEF2A band shift (Fig. 2.2b). Finally, we conducted a time course experiment with 50μM AR-A014418 treatment for 0-24h and looked at the effect of GSK3β inhibition on (i) p38MAPK activity (ii) ATF (a known

p38MAPK substrate) and, (iii) β-catenin (a known GSK3β substrate, Fig. 2.2c). The results show activation of p38MAPK within 30min of GSK3β inhibition and subsequent phosphorylation of its substrate, ATF which also coincided with the observed MEF2A band shift. These effects become increasingly prominent with time. De-phosphorylation of the GSK3β target, β-catenin was also observed at 2-24h indicating that the drug treatment worked.

Figure 2.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AR-A014418 (μM)
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MEF2A
Actin
B
TDZD-8 (μM)
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AR-A014418 (μM)
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P-p38
p38
MEF2A
α/β-Tubulin

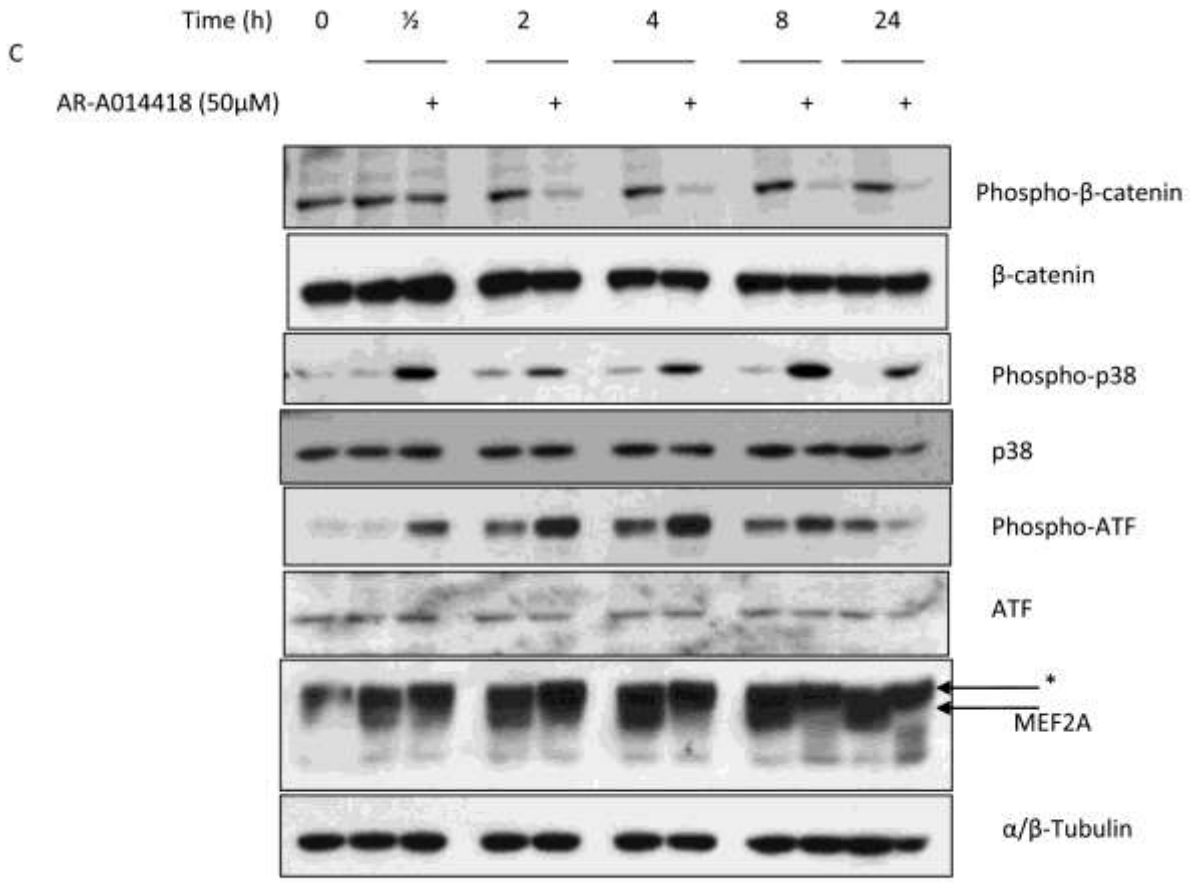


Figure 2.2: **GSK3β inhibition results in a (a) posttranslational modification of MEF2A in skeletal myoblasts which coincides with (b) the activation of p38MAPK and (c) activation of p38MAPK substrates.** C2C12 myoblasts were maintained in growth conditions for 48h and subsequently treated with GSK3β inhibitors, AR-A014418 (0.1-50μM), TDZD-8 (1-10μM) or solvent (DMSO) as indicated above. Following drug treatment, cells were 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. α-Actin and α/β-Tubulin were used as loading controls for figures 2.2(a) and 2.2(b) respectively. The arrows in figure 2.2(a) indicate MEF2A; the arrow with an asterisk points to a low mobility, high molecular weight form of MEF2A that has undergone a posttranslational modification. Figure 2.2(b) shows that this posttranslational modification occurs upon treatment with either AR-A014418 or TDZD-8 and that this coincides with enhanced *p38* phosphorylation. (c) Total and phosphorylated protein levels of GSK3β and p38MAPK substrates were compared 0-24h upon 50μM treatment of AR-A014418. Activation of p38MAPK coincides with subsequent activation of its substrate, ATF as well as the observed MEF2A band shift. Similarly, GSK3β repression causes de-phosphorylation of its substrate, β-catenin.

Pharmacological inhibition of p38MAPK antagonizes the enhancement of MEF2 transcriptional activity caused by GSK3β inhibition in myoblasts.

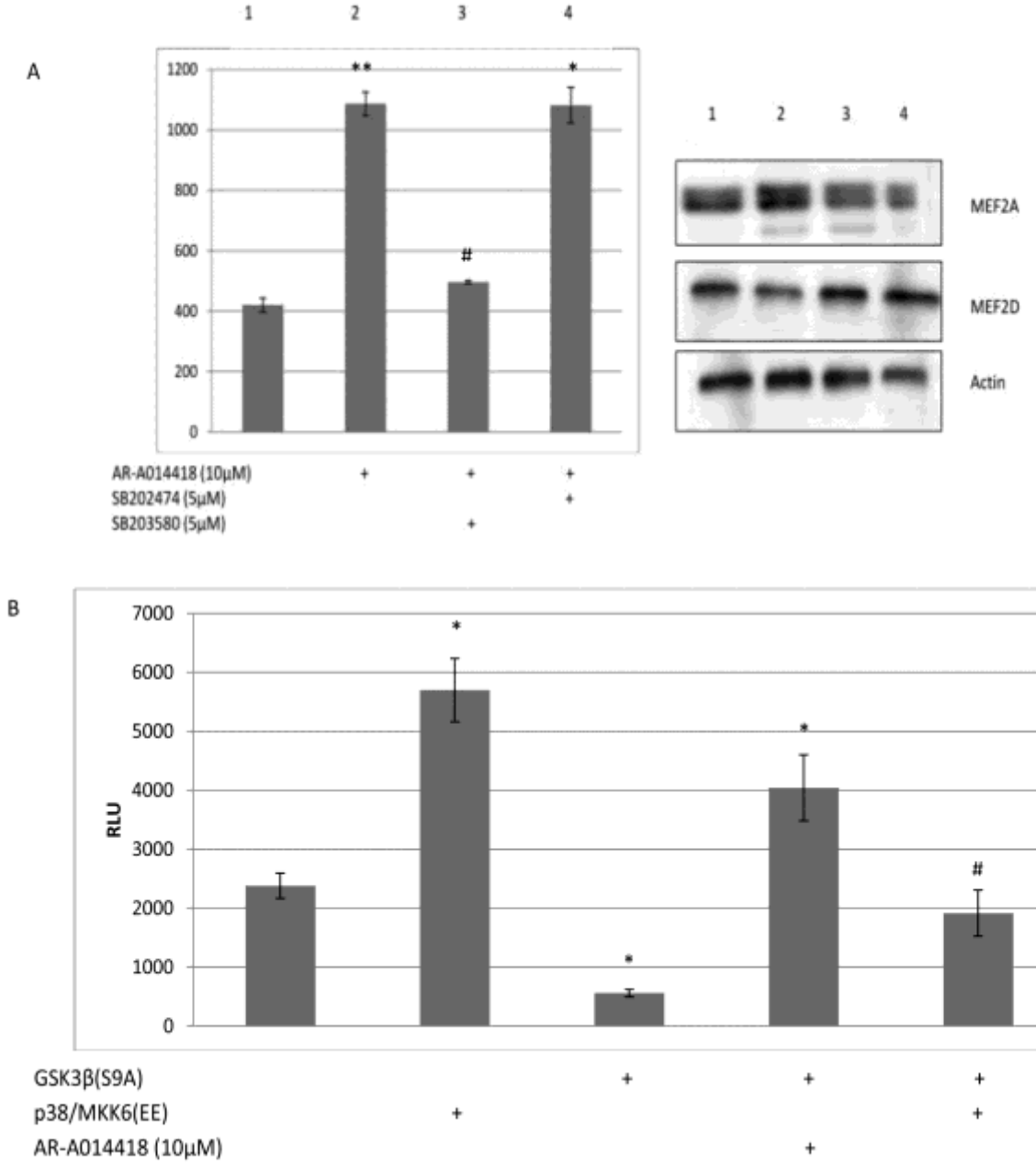
Based on our observations we next tested the possibility that GSK3β inhibition indirectly activates MEF2 through a corresponding de-repression of p38MAPK. To test this idea, we transfected C2C12 myoblasts with MEF2A, using 3xMEF2-Luc to assess MEF2 transcriptional activity and β-galactosidase activity as a control for transfection efficiency. We then treated the transfected cells with or without 10μM AR-A014418 with either 5μM p38MAPK specific inhibitor, (SB203580) or its negative control, (SB202474) for 6h prior to harvesting. We hypothesized that if GSK3β indeed enhances MEF2 transcriptional activity through p38MAPK, then AR-A014418 would lose its effect when p38MAPK is inhibited. The results show that a 6h treatment with 10μM AR-A014418 enhances exogenous MEF2A transcriptional activity ($p<0.001$) and that effect is reduced in the presence of 5μM SB203580 (Fig. 2.3a). This effect is clear considering that in the presence of 5μM SB202474 (which was used as a negative control) 10μM AR-A014418 still enhanced MEF2 transcriptional activity ($p>0.01$). To confirm that indeed this effect was caused by MEF2 transactivation and not an increase in protein levels, western blot analysis was included and revealed no enhanced MEF2A/D protein expression levels.

Constitutively active GSK3β(S9A) mutation reduces MEF2 transcriptional activity and this effect is rescued by p38MAPK

Since MEF2 activation by GSK3β inhibition was determined to be dependent on p38MAPK activity, we wanted to determine whether p38MAPK could rescue MEF2 inhibition by GSK3β. Myoblasts were therefore co-transfected with either pMT3 or p38/MKK6(EE) and pcDNA3.1 or GSK3β(S9A). Endogenous MEF2 activity was assessed using MEF2-Luc as

described above. The data revealed that MEF2 transcriptional activity was potentiated by p38/MKK6(EE) ($p<0.01$) and repressed by GSK3β(S9A) ($p<0.01$, fig. 2.3b). Interestingly, the combination of the two activated kinases resulted in neither activation nor repression of MEF2 compared to the control. This could mean one of two things: either activation of p38MAPK partially rescues MEF2 repression by GSK3β or that GSK3β represses p38MAPK induced activation of MEF2. Regardless, cross-talk between these two signaling pathways converges at MEF2 activity. However to address this question, p38MAPK protein expression and phosphorylation levels were analyzed under these conditions (Fig. 2.3c). MKK6(EE) increased phosphorylation of p38 as expected (lane 2), however in the presence of active GSK3β(S9A), MKK6(EE) lost its ability to phosphorylate p38 (lane 3). This effect was only rescued upon 4h treatment with 50μM AR-A014418 (lane 4). GFP was used as a control for transfection efficiency and actin was used as a loading control. If GSK3β inhibited the p38MAPK pathway upstream of MAP2K as suggested in the literature [37] then ectopically expressed activated MKK6(EE) should have rescued p38 phosphorylation. However based on these data, this was not the case and therefore we conclude that regulation of p38 phosphorylation by GSK3β is at the MAPK level.

Figure 3.


A
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800
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#
AR-A014418 (10μM)
SB202474 (5μM)
SB203580 (5μM)
MEF2A
MEF2D
Actin
B
RLU
7000
6000
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4000
3000
2000
1000
0
GSK3β(S9A)
p38/MKK6(EE)
AR-A014418 (10μM)

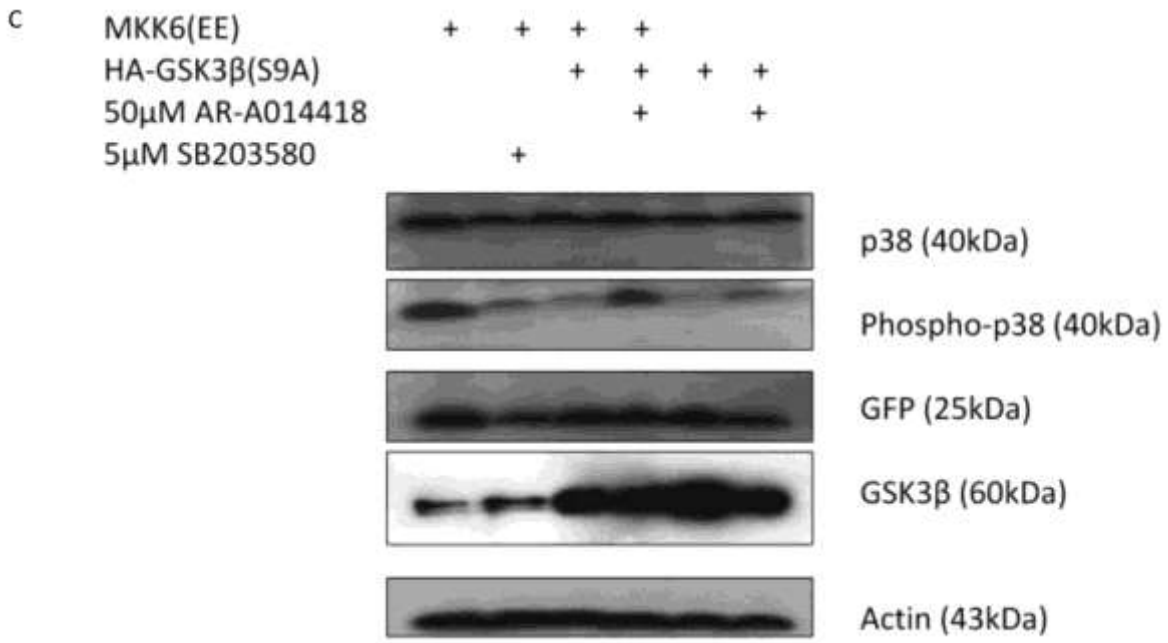


Figure 2.3: MEF2-Luc assays: **(a) Inhibition of p38MAPK reduces MEF2A transcriptional activity mediated by GSK3β inhibitor**. C2C12 myoblasts were treated with different combinations of 10μM AR-A014418 or solvent DMSO with or without 5μM of p38 inhibitor, SB203580 or its negative control, SB202474 for 6h prior to harvesting. The data is coupled with western blot analysis of MEF2A and D protein expression levels under the same conditions with actin used as a loading control. **(b) MEF2 activity is repressed by constitutively active GSK3β(S9A) and enhanced by activated p38MAPK.** Myoblasts were co-transfected with either pMT3 or p38/MKK6(EE) and pcDNA3.1 or GSK3β(S9A) and subsequently treated with either 10μM AR-A014418 or DMSO as indicated, 19h prior to harvesting. Data are the mean + S.E. (n=3). ** indicates a significant difference with respect to the control ($p<0.001$), *indicates a significant difference with respect to the control ($p<0.05$) and, # indicates no significant change. **(c) Activated GSK3β(S9A) de-phosphorylates MKK6(EE) activated p38.** Myoblasts were transfected with different combinations of active MKK6(EE), GSK3β(S9A) or both and treated with or without 50μM AR-A014418 and 5μM SB203580 as indicated.

GSK3β inhibition enhances endogenous MEF2 activity in primary neonatal cardiomyocytes and HL-1 cells.

Since MEF2 proteins are crucial regulators of cardiac morphogenesis [45], vascular development [46], cardiac remodeling [47,48] and cardiac hypertrophy [49] we hypothesized that GSK3β might regulate the effect of MEF2 in the heart as well. To test this idea we used two *in vitro* models. In the first, neonatal cardiomyocytes were extracted, isolated and cultured before being transfected with pGL4-3xMEF2 Luciferase to assess MEF2 transcriptional activity upon 19h treatment with increasing concentrations (0-10μM) of AR-A014418 (data not shown). In the second model, endogenous MEF2 activity was measured in HL-1 immortalized cardiac cells treated with either solvent (DMSO) or 10μM AR-A014418 (data not shown). In both cell culture models, MEF2 transcriptional activity is enhanced by GSK3β inhibition in a dose dependent manner similar to the data observed in our skeletal myoblast model.

Pharmacological GSK3β inhibition or CreLox mediated GSK3β excision in the heart causes increased p38MAPK *activity in cardiac myocytes both in vitro and in vivo.*

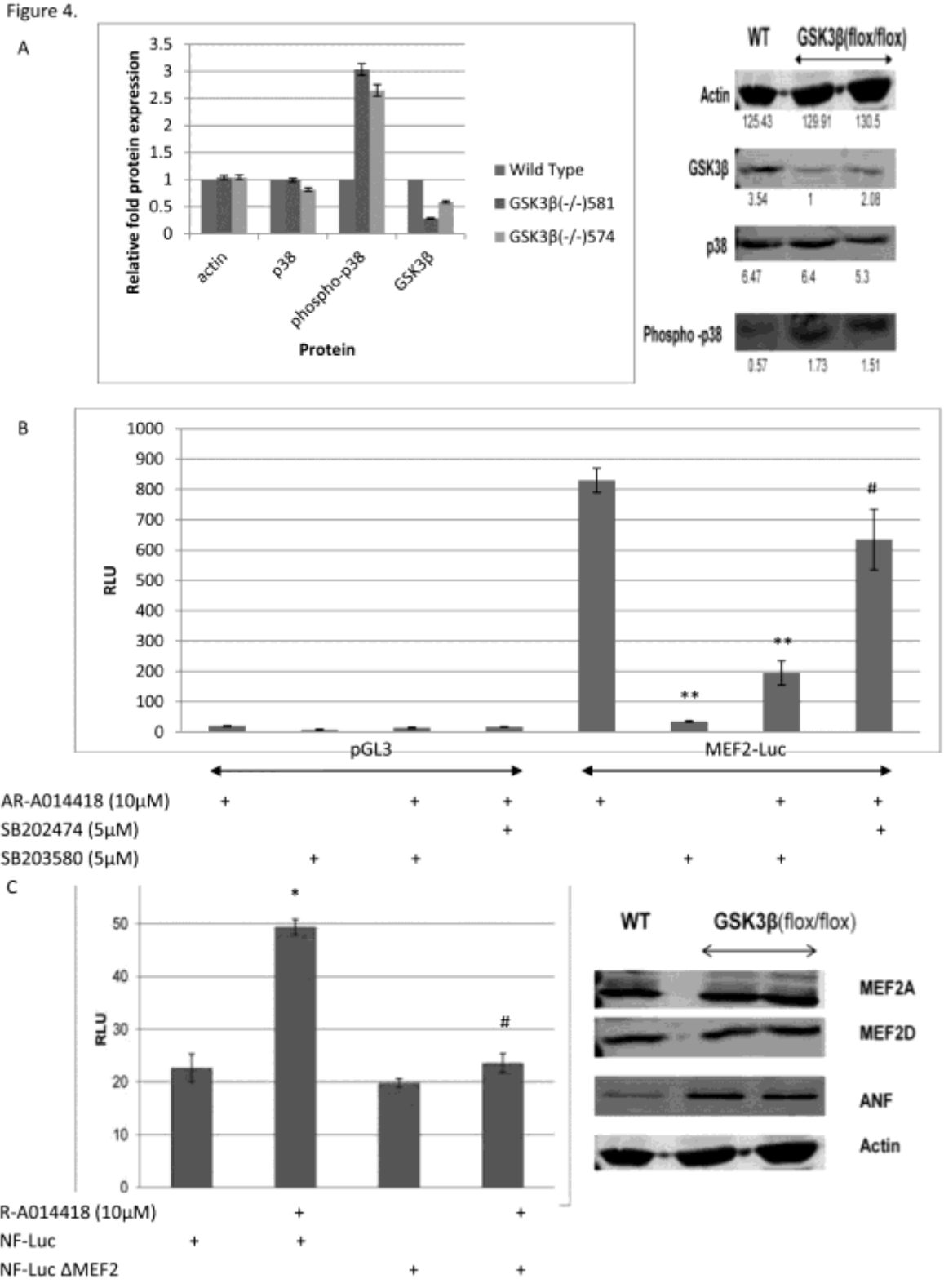
To assess whether GSK3β suppression of MEF2 in cardiomyogenesis is through the regulation of MEF2A/D protein expression or indirectly through p38MAPK, we analyzed endogenous protein levels by a standard western immunoblotting technique upon 4h treatment with increasing concentrations (0-20μM) of AR-A014418 in primary neonatal cardiomyocytes. The data clearly depicts enhanced phosphorylation of p38MAPK in a dose dependent manner up to the 10μM drug treatment but no further elevation at 20μM (data not shown). *In vivo* analysis of GSK3β effects on p38MAPK activity in cardiac tissue was achieved by analyzing cardiac specific Cre-Lox excision of exon 2 of the GSK3β gene. The Cre recombinase was flanked by a tamoxifen-inducible mutated estrogen receptor under the control of an α-MyHC promoter to

render it cardiac specific and Cre expression was activated at 10-12 weeks by treating the mice with 20mg/kg tamoxifen citrate for four consecutive days. Heart tissue from these mice was then isolated and lysed for protein analysis by western immunoblotting and protein levels were quantified using quantitative western blotting (LiCor odyssey system). Data analysis revealed a substantial decrease in GSK3β in three independent floxed tamoxifen treated mice; residual GSK3β likely emanates from non-cardiac cells such as fibroblasts (in which Cre would not be activated). This cardiomyocyte excision of GSK3β resulted in a 2.5-3 fold increase in phosphorylated p38 (Fig. 2.4a).

GSK3β inhibition enhances MEF2 transcriptional activity through p38MAPK *in cardiomyocytes as well as downstream target, ANF promoter activity and expression.*

Primary neonatal cardiomyocytes and HL-1 cells were treated with 10μM AR-A014418 and either 5μM p38MAPK specific inhibitor, SB203580 or SB202474 (an inactive analog) as described above, for 6h before measuring MEF2 transcriptional activity. P38MAPK inhibition reduces the enhancement of MEF2 activity by 7-fold ($p<0.001$) in primary cardiomyocytes (fig. 2.4b) resulting from GSK3β inhibition. Furthermore, we assessed that GSK3β inhibition enhanced ANF-Luc promoter activity ($p<0.01$) but not an alternate version containing mutated MEF2 sites (ANF-Luc ΔMEF2). Protein samples from GSK3β floxed mice revealed enhanced ANF protein levels but no change in MEF2A/D protein expression levels, supporting the idea that GSK3β regulates MEF2 transactivation properties and not protein expression levels (Fig. 2.4c).

Figure 4.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Relative fold protein expression
3.5
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actin
p38
phospho-p38
GSK3β
Protein
Wild Type
GSK3β(-/-)581
GSK3β(-/-)574
WT
GSK3β(flox/flox)
Actin
125.43
129.91
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GSK3β
3.54
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Phospho -p38
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ANF-Luc
ANF-Luc ΔMEF2
WT
GSK3β(flox/flox)
MEF2A
MEF2D
ANF
Actin

Figure 2.4: **(a) *In vivo*, phospho-p38 protein levels are elevated 3-fold in GSK3β knockout mice.** Cardiomyocytes from GSK3β (-/-) mice were 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 as indicated and quantified using the Odyssey system (n=3). **(b) Inhibition of p38MAPK reduces MEF2A transcriptional activity mediated by GSK3β inhibitor**. Primary neonatal cardiomyocytes were treated with different combinations of 10μM AR-A014418 or solvent DMSO with or without 5μM of p38 inhibitor, SB203580 or its negative control, SB202474 for 6h prior to harvesting. Data are the mean + S.E. (n=3). ** indicates a significant difference with respect to the control ($p<0.001$), *indicates a significant difference with respect to the control ($p<0.05$) and, # indicates no significant change. **(c) GSK3β inhibition enhances (i) transactivation of ANF promoter through MEF2 and (ii) ANF protein expression levels.** The effect of 10μM AR-A014418 or solvent DMSO was assessed on ANF-Luc and an analog with the MEF2 site mutated (ANF-Luc ΔMEF2) together with ANF and MEF2A/D expression levels in cardiomyocytes from GSK3β (-/-) mice.

In vivo inhibition of GSK3β enhances MEF2 activity in the hearts of MEF2-LacZ transgenic mice.

To further investigate this possible cross-talk between GSK3β and p38MAPK we used a third known GSK3β inhibitor, lithium chloride (LiCl) and administered either 0.6M LiCl or solvent (PBS) intraperitoneal daily for 30 days to MEF2 LacZ sensor mice [53, 24]. Mice were sacrificed 1h after the last treatment before (i) the apexes of heart (Fig. 2.5a) and, (ii) 10μm transverse sections of the skeletal muscles (Fig. 2.5b) were fixed with 2% paraformaldehyde for 30 minutes. The tissue samples were then stained with X-Gal overnight and visualized for MEF2 activity. The data depicts a small qualitative enhancement of MEF2 activity in the hearts and skeletal muscles, illustrated by the dark blue stains in both sets of tissue samples. Lithium is already used to treat psychiatric disorders such as depression [54] and Alzheimer's [55] but our data implies that lithium treatment may also influence gene expression in cardiac and skeletal muscle. Since MEF2 is also a key player in neuronal function, lithium treatment could also influence these disorders through MEF2 activity.

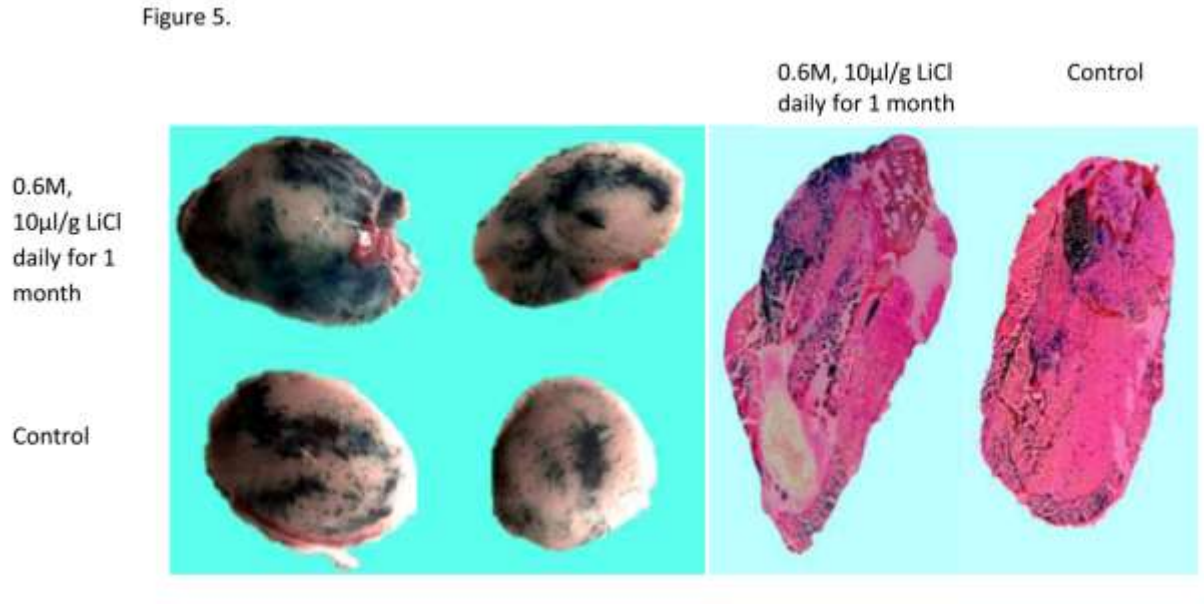

Figure 2.5**: Animal treatment with GSK3β inhibitor *in vivo***. Two groups (n=4/group) of male LacZ-MEF2 transgenic mice were used in this study. The mice received i.p. injections of either 0.6 M LiCl (10µl/g) or sterile 10mM PBS daily for 30 days. Mice were sacrificed 1h after treatment and (a) the apexes of heart and (b) 10µm transverse sections of the skeletal muscles were fixed with 2% paraformaldehyde in PBS for 30 minutes. The samples were then incubated with X-Gal solution overnight and visualized for MEF2 activity. The dark blue stain indicates MEF2 activity which is enhanced with GSK3β inhibition in both the heart and skeletal muscle tissue samples.

2.5 Discussion

In this report, we document GSK3β as a negative regulator of MEF2 transcriptional activity in skeletal and cardiac muscle. This effect is mediated indirectly through repression of the p38MAPK pathway, a known positive regulator of MEF2 activity. A variety of loss of function approaches have revealed that abrogation of GSK3β signaling leads to enhanced MEF2 transcriptional activity, both *in vitro* and *in vivo* in skeletal myoblasts and cardiac myocytes. Furthermore, inhibition of GSK3β enhances p38MAPK phosphorylation *in vitro* in skeletal myoblasts, HL-1 and neonatal primary cardiomyocytes as well as *in vivo*, in heart restricted GSK3β excised mice. Thus, several lines of evidence reveal cross-talk between GSK3β and

p38MAPK in the control of MEF2 activity in cardiac and skeletal muscle. Our conclusions are summarized in figure 2.6, adding a further level of complexity regarding the modulation of p38MAPK activation for the control of striated muscle gene expression through the MEF2 *cis* element. Numerous previous studies have documented a profound role of pharmacological inhibition of p38MAPK for gene expression in these cell types and it is unequivocal that a major target for p38MAPK is MEF2 [8,22-24,35,36]. Interestingly GSK3β has long been known to be an important regulator of cardiac [45-46] and skeletal muscle gene expression although its targets are much less clear. Studies reported here indicate a reciprocal relationship between GSK3β and p38MAPK at the MEF2 *cis* element that in many cases explains the phenotypic impact of modulation of GSK3β signaling in these tissues. In skeletal myogenesis p38MAPK activation is required for differentiation and inhibition abrogates myogenesis. Consistent with this, constitutive GSK3β activation inhibits myogenesis and pharmacological inhibition potentiates myogenesis. Likewise, effects of GSK3β in the heart are consistent with the modulation of p38MAPK signaling to MEF2. Constitutive activation of GSK3β in the heart protects against cardiac hypertrophy [50-51] and p38MAPK regulation of MEF2 has been strongly implicated in the hypertrophic program [22]. Thus, the idea of reciprocal antagonism between GSK3β and p38MAPK at the MEF2 cis element is supported by a variety of experimental strategies in both cardiac and skeletal muscle. Cardiac infarction caused by myocardial ischemia and reduced oxygen supply has been shown to be reduced in dnGSK3β hearts and that preconditioning loses its protective effect in when GSK3β is constitutively active [52]. It is highly likely based on our data that during oxygen deprivation GSK3β is antagonizing p38MAPK signaling in response and that upon inhibition of GSK3β, p38MAPK can carry out its stress response.

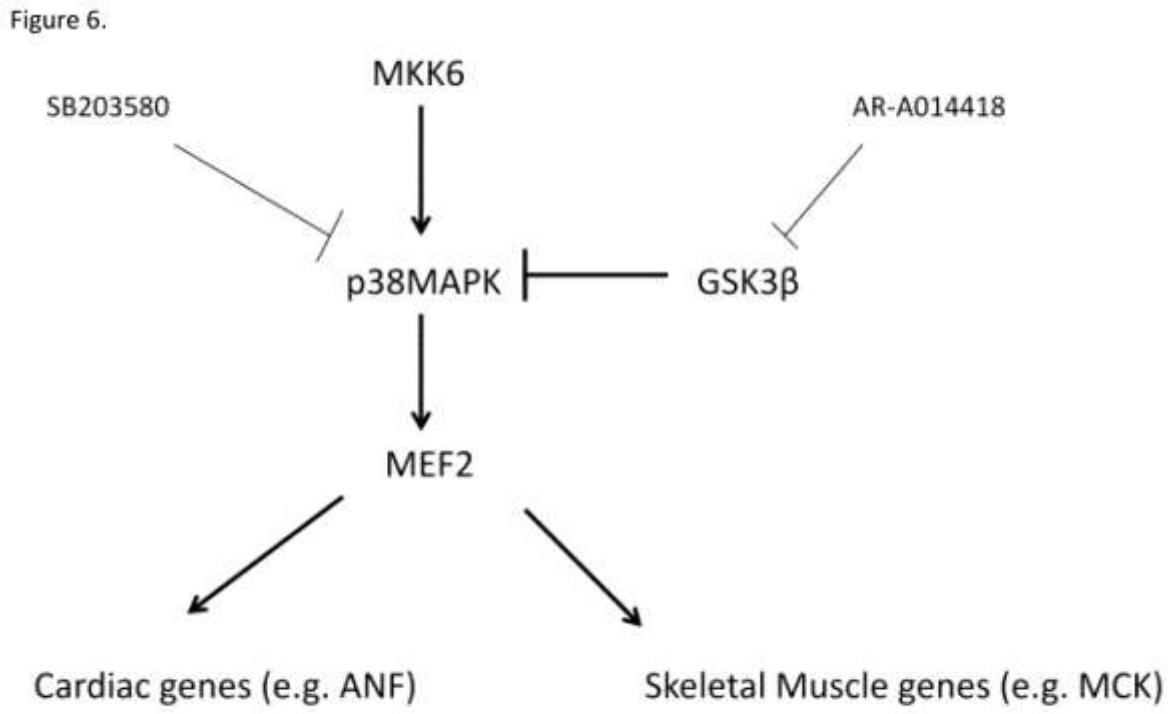

Figure 2.6: **Summary.** Our data demonstrates that MKK6 phosphorylates p38MAPK which subsequently activates MEF2 and hence downstream cardiac genes such as ANF in cardiomyocytes and, MCK in skeletal myoblasts, hence promoting differentiation. This pathway is antagonized by GSK3β which suppresses p38MAPK activity. Pharmacological treatment with (a) SB203580 and (b) AR-A014418 results in inhibition of p38MAPK and GSK3β respectively. The latter, resulted in increased p38 phosphorylation and enhanced MEF2 activity.

Collectively our findings display an important level of reciprocal antagonism between GSK3β and p38MAPK signaling in cardiac and skeletal muscle. In view of the central role of both signaling pathways in a variety of developmental, physiological and pathological processes in both tissue types, these findings will have important therapeutic implication for the treatment of striated muscle pathology.

2.6 References

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The experimental design, figures and data presented in this chapter are mostly of my own efforts with the following exceptions. S Ehyai assisted with the cloning and purification of Myogenin and mutated versions of Myogenin which were used throughout the chapter. E Avrutin and MK Connor made the initial observation that electrical stimulation of C2C12 myoblasts and alveolar RMS derived RH30 cells altered Akt activity and performed the experiments presented in figures 3.7A-E. JC McDermott assisted with experimental design and with editing the manuscript. Catherine Chan provided technical support throughout the study.

Glycogen synthase kinase 3β represses Myogenin function in Alveolar Rhabdomyosarcoma

3.1 Abstract

Myogenin is a member of the muscle regulatory factor family that orchestrates an obligatory step in myogenesis, the terminal differentiation of skeletal muscle cells. A paradoxical feature of alveolar rhabdomyosarcoma (ARMS), a prevalent soft tissue sarcoma in children arising from cells with a myogenic phenotype, is the inability of these cells to undergo terminal differentiation despite the expression of Myogenin. The chimeric Pax3-foxo1 fusion protein which results from a chromosomal translocation in ARMS has been implicated in blocking cell cycle arrest, preventing myogenesis from occurring. We report here that Pax3-foxo1 enhances glycogen synthase kinase 3β (GSK3β) activity which in turn represses Myogenin activity. Myogenin is a GSK3β substrate *in vitro* based on *in vitro kinase* assays and Myogenin is phosphorylated in ARMS-derived RH30 cells. Constitutively active GSK3β(S9A) increased the level of a phosphorylated form of Myogenin based on western blot analysis and this effect was reversed by neutralization of the single consensus GSK3β phosphoacceptor site by mutation (S160/164A). Congruently, GSK3β inhibited the *trans*-activation of an E-box reporter gene by wild type Myogenin, but not Myogenin with the S160/164A mutations. Functionally, GSK3β repressed muscle creatine kinase (MCK) promoter activity, an effect which was reversed by the S160/164A mutated Myogenin. Importantly, GSK3β inhibition or exogenous expression of the S160/164A mutated Myogenin in ARMS reduced the tumorigenic potential of RH30 cells in colony formation assays. Thus, sustained GSK3β activity represses a critical regulatory step in the myogenic cascade, contributing to the undifferentiated, proliferative phenotype in alveolar rhabdomyosarcoma (ARMS).

3.2 Introduction

Rhabdomyosarcoma (RMS) is the most common pediatric solid tumor accounting for 5% of all pediatric cancers and approximately 50% of soft tissue sarcomas [1-3]. There are two main subtypes: embryonal and alveolar RMS and while embryonal RMS is more common, alveolar RMS is considered to carry a worse prognosis. A somatic mutation resulting in the t(2;13)(q35;q14) chromosomal translocation fuses PAX3 and FOXO1 to create a potent transcription factor (Pax3-foxo1) which is a predominant causative genetic lesion for the development of alveolar rhabdomyosarcoma (ARMS) [1]. ARMS is a highly malignant mesenchymal tumor that has properties of immature striated muscle tissue resulting in dense aggregates of poorly differentiated cells that are separated by fibrous membranes resulting in a loss in cellular cohesion [2,3]. Pax3 is a key determinant of somatic myogenesis and, is involved in the migration of progenitor cells to the dermomyotome region of the somite where they grow and divide in the presence of growth factors [4]. Pax3 is also required to activate the myogenic determination gene, *MyoD* [5]. MyoD is one of four myogenic regulatory factors (MRFs, which include Myf5, MRF4 and Myogenin) from the basic helix-loop-helix superfamily of transcription factors which interact with myocyte enhancer factor 2 (MEF2) proteins in the hierarchical control of muscle specific gene expression [6]. Two kinases that potently exert effects on this myogenic regulatory cascade are p38 mitogen activated protein kinase (MAPK) and glycogen synthase kinase 3β (GSK3β). p38 MAPK is a key regulator of skeletal myogenesis that critically interacts with and activates MEF2 in the somite myotome during development [21-23]. Conversly, GSK3β activation leads to a repression in skeletal and cardiac muscle differentiation, in part by antagonizing p38 MAPK mediated activation of MEF2 [25,39]. GSK3β usually targets proteins that have already been phosphorylated by another kinase at a “priming” serine or threonine

residue located four amino acids C-terminal to a consensus (S/T)XXX(S/T)-PO_4 motif [26,27]. Regulation of MEF2 and the MRFs leads to morphological changes including epithelial to mesenchymal transition, cell alignment and fusion to form multinucleated myotubes that eventually develop into functional, contractile muscle fibers. In particular, cells that express MyoD and Myogenin are typically fusion competent [7,8] with the exception of ARMS cell types. To date, lack of myogenic differentiation of Pax3-foxo1 expressing ARMS cells has been attributed to their inability to up-regulate $p57^{Kip2}$ activity, hence destabilizing the DNA binding affinity of MyoD transcription complexes [9]. Dysfunctional MyoD/E-protein complex association and transcriptional control is a common feature between ARMS and the non Pax3-foxo1 expressing embryonal rhabdomyosarcoma (ERMS). Subsequent restoration of the MyoD/E12 complex has been shown to switch ERMS cells from an arrested myofibroblast phase to a more differentiated state [10]. Similarly p38 MAPK activity can potentiate myogenic differentiation in ERMS cells by enhancing MyoD *trans*activation properties [11]. Therefore, it is fairly clear that in both rhabdomyosarcoma subtypes the ability of MyoD to potentiate transcription is compromised. However, the role of Myogenin in RMS is more equivocal. For normal myogenesis to occur, *both in vitro and in vivo,* an absolute requirement for Myogenin is evident, Thus, Myogenin activity constitutes a pivot point for irreversible commitment to terminal differentiation [18,19]. The combination of data from gene targeting studies of the MRFs [12,13] supports the prevailing consensus that while the other three MRFs can compensate each other's functional roles [14-17], Myogenin is absolutely essential for skeletal muscle fiber formation [19]. Despite its expression in RMS, the paradox as to why Myogenin cannot mediate competence for differentiation is unknown.

Here, we examined the post-translational regulation of Myogenin in ARMS. Based on the *in silico* prediction of a single consensus phosphorylation site for GSK3β on the Myogenin protein and also high levels of GSK3β activity in these cells, we determined that Myogenin function is potently repressed by GSK3β activity in ARMS. Moreover, pharmacological inhibition of GSK3β results in a profound decrease in size and, to a certain extent, number of RMS colonies in a colony formation assay. This effect is mimicked by introduction of Myogenin bearing neutralizing mutations in the GSK3β consensus site. In combination, these data reveal Myogenin as a key target of GSK3β activity in ARMS, indicating that pharmacologic manipulation of this signaling axis may provide an opportunity for therapeutic intervention.

3.3 Materials and Methods

Plasmids

E-box, Myogenin and MCK reporter constructs in pGL3 and expression vectors for Myogenin in EMSV were used in reporter gene assays. HA tagged Pax3-foxo1 was cloned into pcDNA3.1 and kindly donated by Dr. Malkin at MaRS, Toronto. HA tagged GSK3β(S9A) was cloned in pcDNA3 ORF 995-2305.

Antibodies

Anti-Myogenin and anti-HA mouse monoclonal antibodies as well as anti-MEF2A rabbit polyclonal antibody were produced with the assistance of the York University Animal Care Facility; anti-Pax3 (1:250; Cell Signaling) GSK3β, phospho-GSK3β (1:1000; Cell Signaling); actin, MyoD, Myf-5, GFP, dsRed2 (1:2000; SantaCruz) were used for immunoblotting experiments.

Cell culture and transfection

C2C12, Cos7 and RH30 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^{o}C incubator with a 5% CO_2 atmosphere. For transfections, cells were seeded 1 day prior to transfection and transfected according to the standard calcium phosphate method previously described by Perry *et al*. 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. The cells were counted and transferred to pre-gelatin coated plates.

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). Independent two sample t-tests of all quantitative data were conducted using R software. P-values are indicated with respect to controls where appropriate.

In vitro kinase assay

3μg of purified recombinant GST-Myogenin was mixed with either 0.5μg purified recombinant GST-GSK3β (1-433; Cell Signaling) and with [γ-^{32}P] ATP and incubated for 30min at 30^{o}C. Samples were denatured for 5min at 95^{o}C in SDS sample buffer. Protein samples were then separated by 10% SDS-PAGE and exposed on X-ray film (Kodak X-Omat) for 21hrs to detect ^{32}P incorporation. The lanes containing GST-Myogenin are elongated because 2 lanes were pooled to fit a higher total reaction volume, in order to accommodate for the low concentration of purified GST-Myogenin (0.06μg/μl). All lanes contain equal total amounts of proteins (3μg)

Electrical Stimulation

Cells were plated onto 0.1% gelatin coated 6 well plates. The lids of the plates were fitted with two parallel platinum wire electrodes, placed at the opposite ends of each well and extending into the media. The wires from all wells were arranged in parallel and connected to an electrical stimulator (Harvard Apparatus Canada, Saint-Laurent, Quebec, Canada). Cells were stimulated at 5V and a frequency of 5Hz for 4 hours/day and allowed a subsequent 20 hour recovery period. Cells were harvested following the recovery period throughout the 4 days of the protocol.

Soft Agarose Colony Formation Assay

Materials: 0.7% (w/v) DNA grade Agarose, 1% (w/v) DNA grade Agar, 0.005% Crystal Violet (Sigma-Aldrich), 2X Media + 20% (v/v) FBS. After 48h of transfection with Myogenin containing the S160/164A mutations or empty vector, RH30 cells were assayed for their capacity

to form colonies as previouslt described [41]. A total of 1 x 10^4 cells were suspended on a layer of 0.35% Agarose in DMEM (10% FBS) with or without 10μM AR-A014418, in 6-well plates. Medium was refreshed every 3-5 days as needed and on the 22^{nd} day the amount of colonies were counted using a contrast phase microscope. The relative colony sizes were calculated using ImageJ software. Four independent experiments were carried out in triplicate.

3.4 Results

Myogenin is expressed in Pax3-foxo1 expressing RH30 cells.

Serum (10% FBS) contains growth factors that repress the transcriptional activity of MRFs and also stimulate cell cycle progression hence rendering C2C12 myoblasts proliferative. In tissue culture, serum withdrawal (2% HS) results in activation of MEF2 and MRFs causing cell alignment and fusion to form multinucleated myotubes. Initially, in order to investigate the effect of Pax3-foxo1 on this differentiation program, proliferating C2C12 myoblasts were transiently transfected with CMV-dsRed2, MCK-eGFP, and either HA-Pax3-foxo1 or pcDNA3.1 control vector. Growth media (GM) was replaced with differentiation media (DM) 19h after transfection and cells were allowed to differentiate for 96h. SDS PAGE samples were prepared from populations of myoblasts that either expressed or did not express Pax3-foxo1, (a) before serum withdrawal (time = 0; GM = 10% FBS) and (b) at 24 hour increments upon serum withdrawal (days 1-4; DM = 2% HS). Protein expression levels of these samples were then compared with protein samples from Pax3-foxo1 expressing RH30 cells in GM and DM, by western blotting. These data indicate that despite the expression of Pax3-foxo1, Myogenin protein expression is maintained in human ARMS derived, RH30 cells (Figure 3.1a). In addition, Pax3-foxo1 repressed myotube formation in C2C12 myoblasts (Figure 3.1a, 3.1b). Detection of myogenic

differentiation using an MCK promoter driving GFP expression [20] revealed GFP expressing, multinucleated, myotubes in the controls but not in cells expressing Pax3-foxo1 (Fig. 3.1b).

It is well documented that MRFs and MEF2 proteins are highly sensitive to pro-myogenic kinases such as p38 MAPK [23,31-33] and also kinases such as GSK3β which are repressive to myogenesis [25,34]. Therefore we tested for GSK3β activity under conditions when myogenesis is suppressed. Since GSK3β is constitutively active until it is repressed by phosphorylation at serine 9 (by PKB), we assessed both total GSK3β protein expression levels and S9 phosphorylation levels using appropriate antibodies as indicated. We document that GSK3β is expressed in proliferative C2C12 myoblasts, Pax3-foxo1 expressing ARMS cells (RH30) and, non-Pax3-foxo1 ERMS cells (RD). However only in Pax3-foxo1 expressing RH30 cells, is GSK3β predominantly in its un-phosphorylated form (at serine 9) and, hence fully active state (Figure 3.1c). In addition, ectopic expression of Pax3-foxo1 increased GSK3β activity in C2C12 myoblasts by reducing GSK3β phosphorylation at serine 9 (Figure 3.1d).

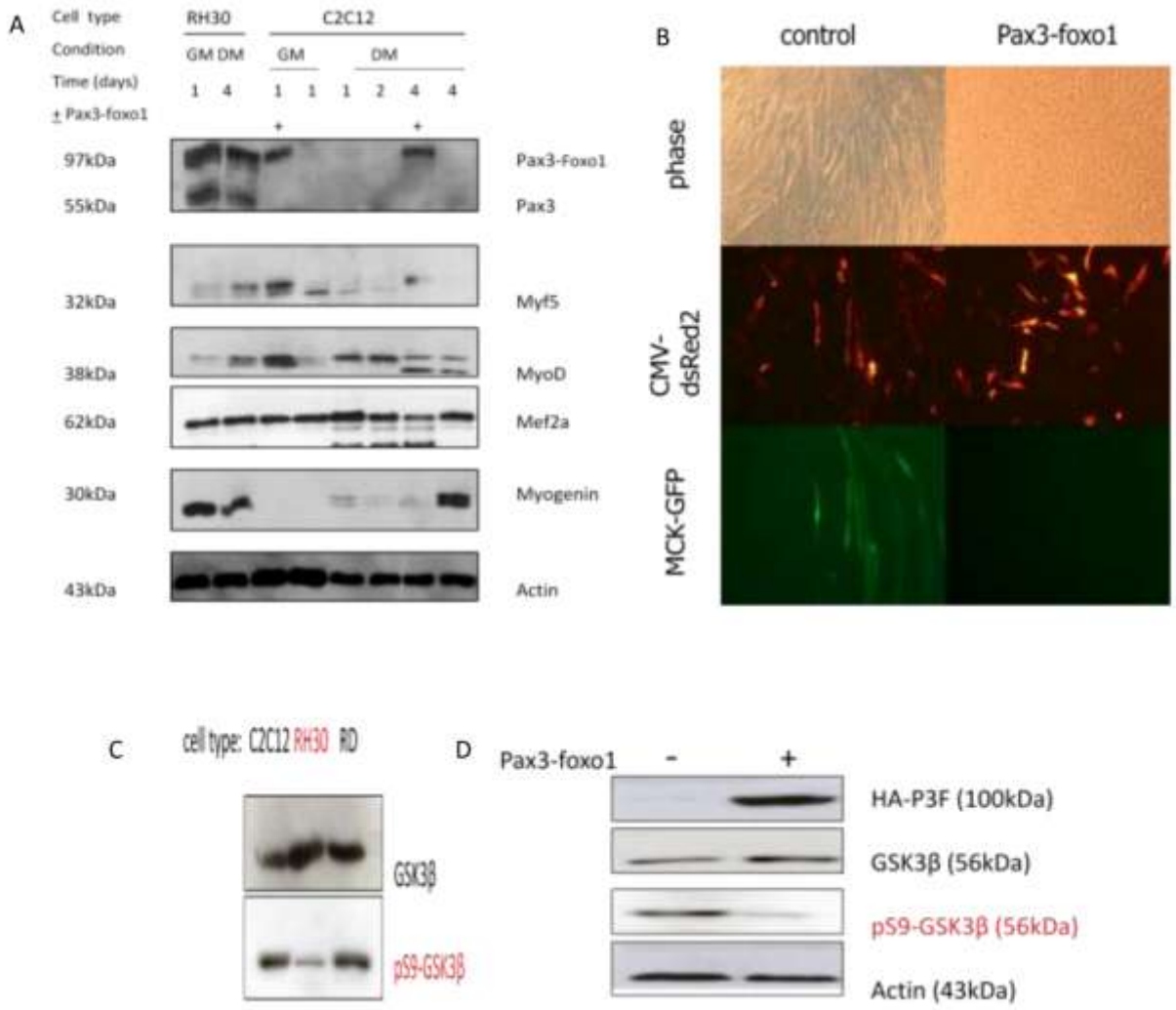


Figure 3.1. Myogenin protein expression and GSK3β activity are both maintained in ARMS: (a) C2C12 myoblasts were transfected with HA-Pax3-foxo1 or pcDNA3.1 control plasmid for 1 day before extraction or serum withdrawal and then extraction at 1 day increments for up to 4 days as indicated. Protein levels were compared to protein extracts from Pax3-foxo1 expressing Rh30 cells 1 day in growth media (GM) and 4 days in differentiation media (DM). The results show that despite the expression of Pax3-foxo1, Rh30 cells also express myogenin. On the other hand, HA-Pax3-foxo1 overexpression in C2C12 inhibits myogenin expression and subsequent myogenic differentiation. (b) C2C12 myoblasts were transfected with CMV-dsRed2, MCK-eGFP and, either HA-Pax3-foxo1 or pcDNA3.1 control plasmid. HA-Pax3-foxo1 overexpression repressed the formation of multi-nucleated myotubes. (c) Endogenous GSK3β protein levels and phosphorylation at serine 9 were compared in C2C12 myoblasts, Rh30 and, ERMS RD cells. Whilst GSK3β is expressed in all three cell types, it is predominantly phosphorylated and hence inactive in C2C12 myoblasts and RD cells but not Pax3-foxo1 expressing Rh30 cells. (d) C2C12 myoblasts were transfected with HA-Pax3-foxo1 or pcDNA3.1 control plasmid for 1 day before

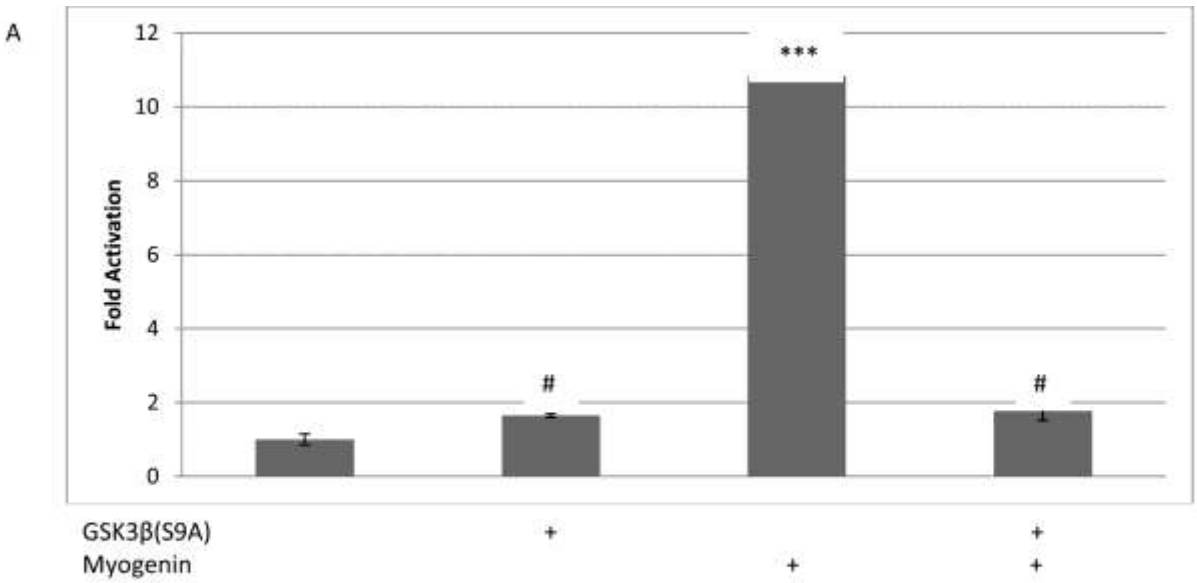
extraction. Overexpression of HA-Pax3-foxo1 resulted in decreased phosphorylation of GSK3β at serine 9 indicating its activation.

Myogenin transactivation function is repressed by GSK3β

To assess the effect of GSK3β activity on Myogenin function, *trans*-activation of a 4x E-box Luciferase construct was measured in proliferating C2C12 myoblasts that were transfected with different combinations of constitutively active GSK3β(S9A) and Myogenin as indicated in figure 3.2a. The data indicate that Myogenin potentiates the 4x E-box Luc reporter gene and that GSK3β(S9A) abrogates this effect ($p<0.001$) indicating repression of Myogenin by active GSK3β.

GSK3β directly phosphorylates Myogenin in vitro

In order to determine whether Myogenin is a substrate for GSK3β, an *in vitro* kinase assay was performed using GST-Myogenin (1-225), purified GST-GSK3β and γ-^{32}P ATP. Bands were resolved using SDS-PAGE and subsequent autoradiography showed ^{32}P labelled bands for Myogenin, autophosphorylated GSK3β and MyBP (positive control, Figure 3.2b). In addition, Coomassie Blue staining revealed a lower mobility band indicative of phosphorylation (Figure 3.2b). To further test the idea that the lower mobility band is hyperphosphorylated we used calf intestinal phosphatases on RH30 cell lysates and found that the low mobility band was eradicated (Figure 3.2c). Collectively these data suggest that Myogenin is a GSK3β substrate *in vitro*.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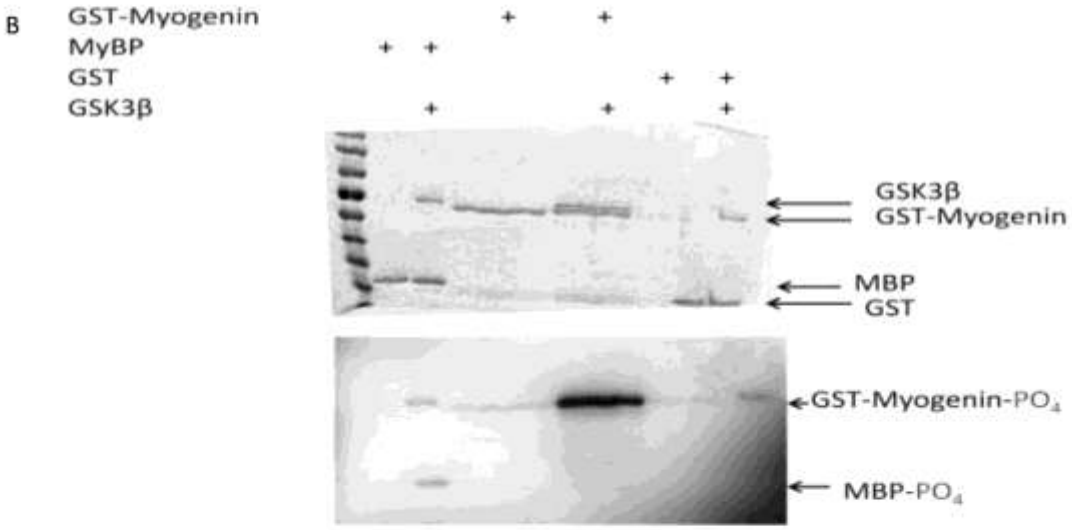
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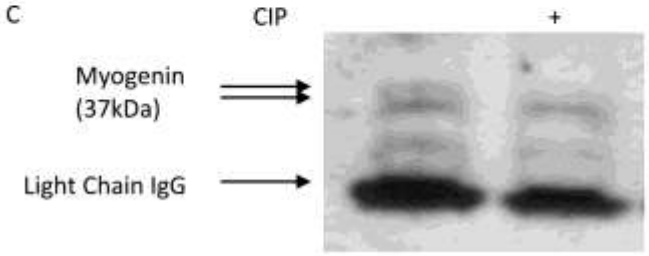
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Figure 3.2. Overexpressed, constitutively active GSK3β (S9A) represses myogenin *trans*activation of E-box. (a) C2C12 myoblasts were transfected with 4x E-box-Luc reporter and different combinations of HA-GSK3β(S9A) and myogenin or pcDNA3.1 control plasmid as indicated. Overexpressed HA-GSK3β(S9A) repressed myogenin transcriptional activity ($p<0.001$). (b) GSK3β directly phosphorylates myogenin *in vitro*: Purified GST-Myogenin was incubated *in vitro* with GST-GSK3β and [γ-^{32}P]ATP. GST and MBP proteins were used as negative and positive control respectively as indicated. Bands were resolved using SDS-PAGE and visualized by Coomassie Blue staining (top panel). Gels were dried and exposed to X-ray film for 21h after the assay (bottom panel). (c) Calf-intestinal phosphatase (CIP) treatment of immunoprecipitated Myogenin that was obtained from 1000μg of Rh30 protein extract. The data shows that CIP treatment causes a loss of a high-molecular weight, phosphorylated form of Myogenin.

Pharmacologic manipulation of GSK3β activity alters Myogenin properties

To further investigate the effect of GSK3β on Myogenin, Cos7 cells were co-transfected with Myogenin and GSK3β(S9A) and, then treated with or without 10μM GSK3β inhibitor, AR-A014418, as indicated in figures 3.3a and 3.3b. Western blot analysis revealed two predominant forms of Myogenin, a low mobility hyper-phosphorylated isoform and a high mobility, hypo-phosphorylated isoform (Figure 3.3a, lane 2). The lower mobility, hyper-phosphorylated band is reduced upon pharmacological treatment with AR-A014418 as indicated (Figure 3.3a, lane 3). This corresponded with a significant increase in *trans*-activation of an E-box *cis* element driven reporter gene ($p<0.001$, Figure 3.3b). In contrast, constitutively active GSK3β(S9A) without pharmacological inhibition resulted in an increase in the low mobility, hyper-phosphorylated band (Figure 3.3a, lane 4) which corresponded to a decrease in E-box luciferase activity in reporter gene assays ($p<0.05$, Figure 3.3b).

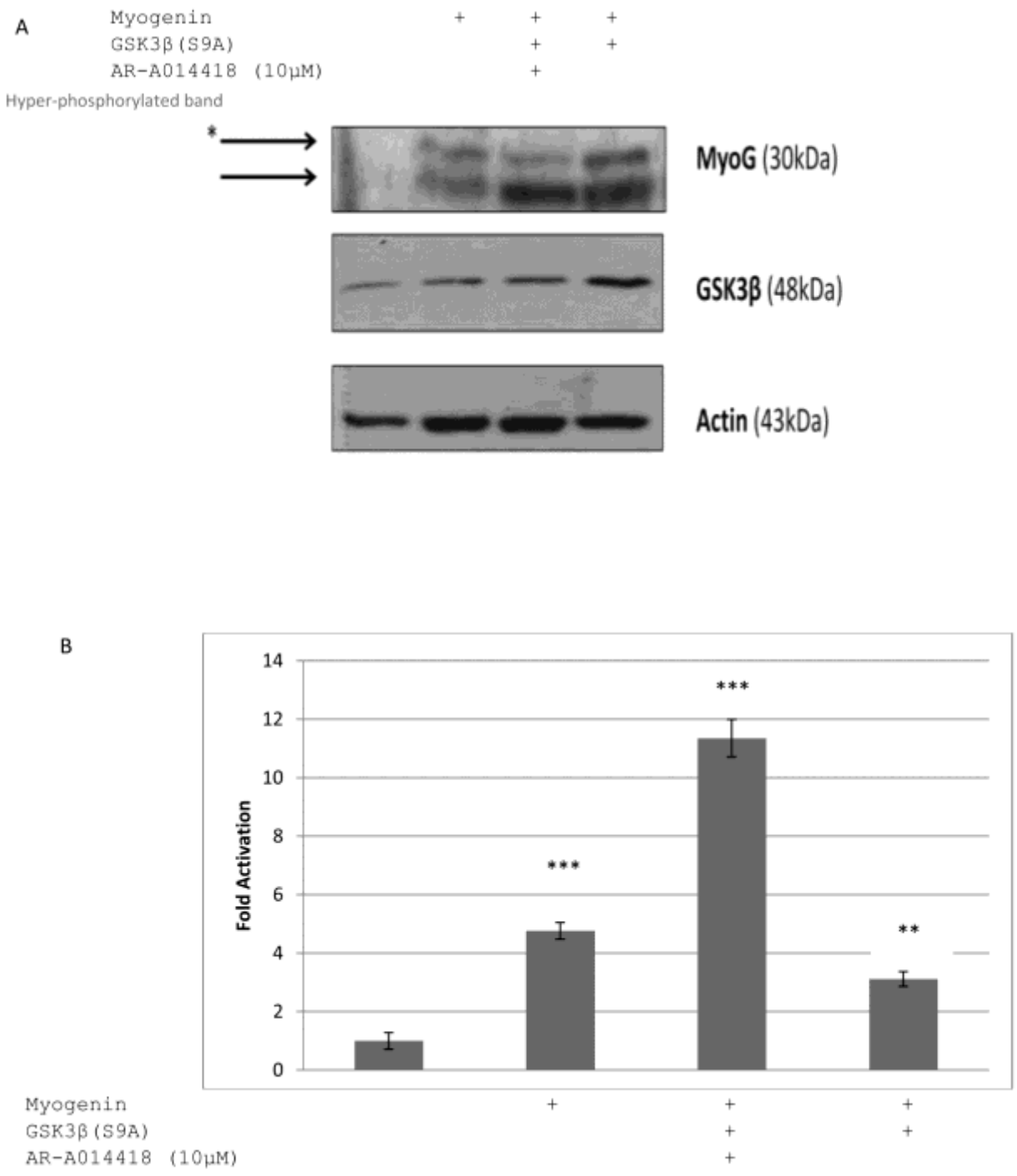


Figure 3.3. GSK3β increases myogenin protein, possibly through phosphorylation and this corresponds with decreased transcriptional activity. (a) Cos7 cells were transiently transfected with or without Myogenin and/or GSK3β(S9A) and then treated for 19h with either 10μM GSK3β

inhibitor or DMSO 24h after transfection as indicated. Protein samples were extracted and western blot analysis revealed an increase in a slower migrating, hyper-phosphorylated myogenin band (lane 4) in the presence of overexpressed HA-GSK3β(S9A) which, was reduced in the presence of GSK3β inhibitor (lane 3). (b) E-box-Luc reporter gene was co-transfected in Cos7 cells using the same conditions that were described above. Overexpressed myogenin significantly enhanced transcriptional activity of the E-box promoter ($p<0.001$) and, this effect was further increased in the presence of GSK3β inhibitor despite overexpression of GSK3β(S9A) ($p<0.001$). Overexpression of GSK3β(S9A) repressed myogenin transcriptional activity ($p<0.05$).

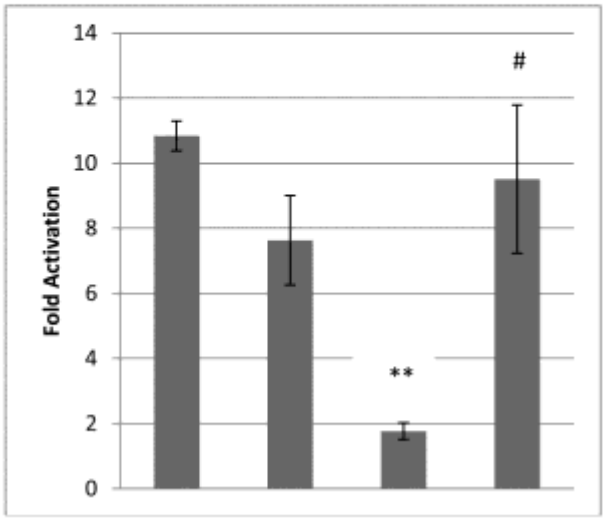
Mutation of a consensus GSK3β phoshoacceptor site on Myogenin (S160/164A) prevents GSK3β mediated repression

By *in silico* analysis, Myogenin contains a highly conserved putative GSK3β consensus phosphoacceptor site (Table 1), which we targeted by neutralizing site-directed mutagenesis. We observed that while wild type Myogenin is sensitive to the repressive effects of constitutively active GSK3β(S9A), Myogenin (S160/164A) was not (Figure 3.4a). Western blot analysis revealed that Myogenin (S160/164A) mutations correspond with a decrease in the low mobility, hyper-phosphorylated upper band (Figure 3.4b, lane 2) and that this effect was not altered by ectopically expressed HA-GSK3β(S9A) Together these data indicate that S160/164A mutations in Myogenin render it insensitive to the repressive effect of GSK3β. GSK3β(S9A) expression resulted in an increase in the low mobility, hyper-phosphorylated form of wild type Myogenin (Figure 3.4b, lane 3) and this corresponded with decreased E-box luciferase activity ($p<0.001$, Figure 3.4a). Although *trans*-activation of the skeletal muscle gene E-box *cis*-element by mutated Myogenin (S160/164A) is marginally less potent than wild type Myogenin ($p<0.05$, Figure 3.4c); it is resistant to inhibition by activated GSK3β ($p<0.001$, Figure 3.4c).

Table 1: GSK3β consensus sequence within Myogenin.

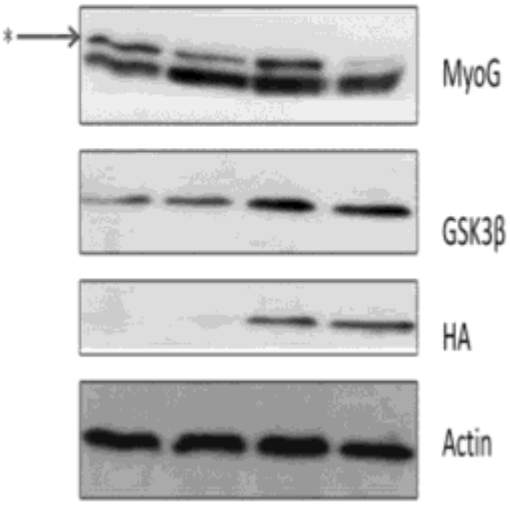
Myogenin Sequence:	Species:
158 VP**S**ECS**S**HSA**S**CSP 171	Human
158 VP**S**ECN**S**HSA**S**CSP 171	Mouse
158 VP**S**ECN**S**HSA**S**CSP 171	Rat

A



Myogenin + + + +
Myogenin (S160/164A) + + + +
HA-GSK3β + + + +

B



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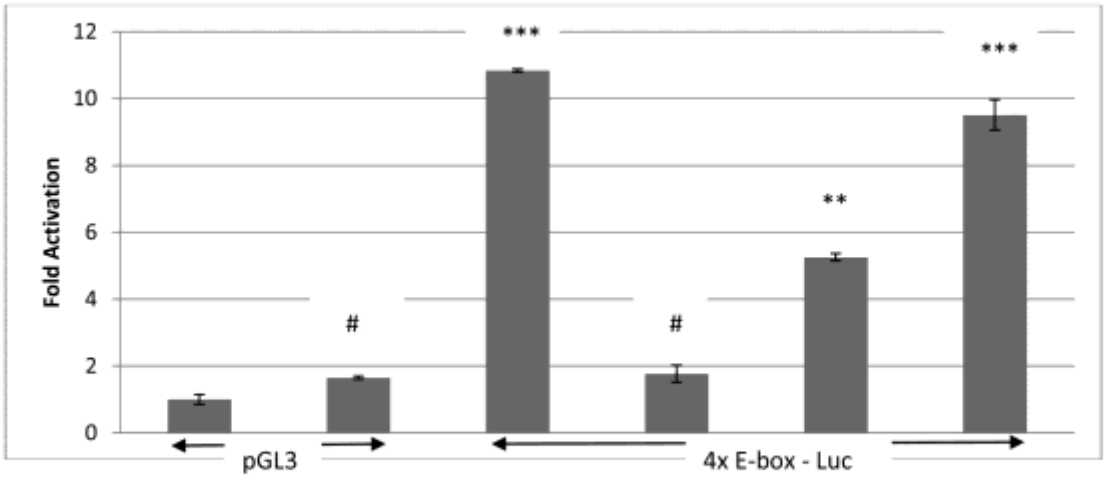


Myogenin + + + +
Myogenin (S160/164A) + + + +
HA-GSK3β + + + +

Figure 3.4. **Myogenin neutralizing phosphomutant (S160/164A) is resistant to GSK3β repression of transcription activity as well as an increased slower migrating, hyper-phosphorylated myogenin band**. (a) 4x E-box-Luc activity was assessed in C2C12 myoblasts that were transfected with either wild-type myogenin or myogenin (S160/164A) and, co-transfected with HA-GSK3β(S9A) or pcDNA3.1 control plasmid as indicated. HA-GSK3β(S9A) repressed myogenin *trans*-activation of the 4x E-box promoter region ($p<0.001$) but had no effect on mutated myogenin (S160/164A) transcriptional activity. (b) Western blot analysis of the same samples revealed a decrease in a slower migrating, hyper-phosphorylated band for overexpressed myogenin (S160/164A, lane 2) with respect to overexpressed wild-type myogenin (lane 1). Co-transfected HA-GSK3β(S9A) caused an increase in the slow migrating, hyper-phosphorylated myogenin band (lane 3) but not with overexpressed mutated myogenin (S160/164A, lane 4). (c) Independent analysis of E-box Luc activity in C2C12 myoblasts with different combinations of overexpressed myogenin, mutated myogenin (S160/164A), HA-GSK3β(S9A) or pcDNA3.1 control plasmid as indicated.

Pax3-foxo1 activation of GSK3β antagonizes muscle creatine kinase promoter activation

To further examine the functional significance of our findings, we used MCK promoter activity, as a key indicator of the activation of myogenic differentiation, in C2C12 myoblasts that were transfected with or without the Pax3-foxo1 oncogene (Figure 3.5a). These data depict that Pax3-foxo1 represses MCK promoter activation in myoblasts that have been co-transfected with Myogenin ($p<0.01$) and this effect is not only abrogated by pharmacological inhibition of GSK3β, but further activated ($p<0.001$, Figure 3.5a). Interestingly, in Pax3-foxo1 expressing, human ARMS derived RH30 cells, ectopically expressed Myogenin had no effect on MCK promoter activity unless it was coupled with pharmacological inhibition of GSK3β using AR-A014418 ($p<0.001$, Figure 3.5b). Conversely, mutated Myogenin (S160/164A) was able to potentiate MCK promoter activity regardless of GSK3β inhibition ($p<0.05$, Figure 3.5b). Taken together, these data provide evidence that S160/164 on Myogenin are likely key targets of GSK3β signaling in alveolar rhabdomyosarcoma resulting in a diminution of the critical E box dependent gene activation that is necessary and sufficient for differentiation.

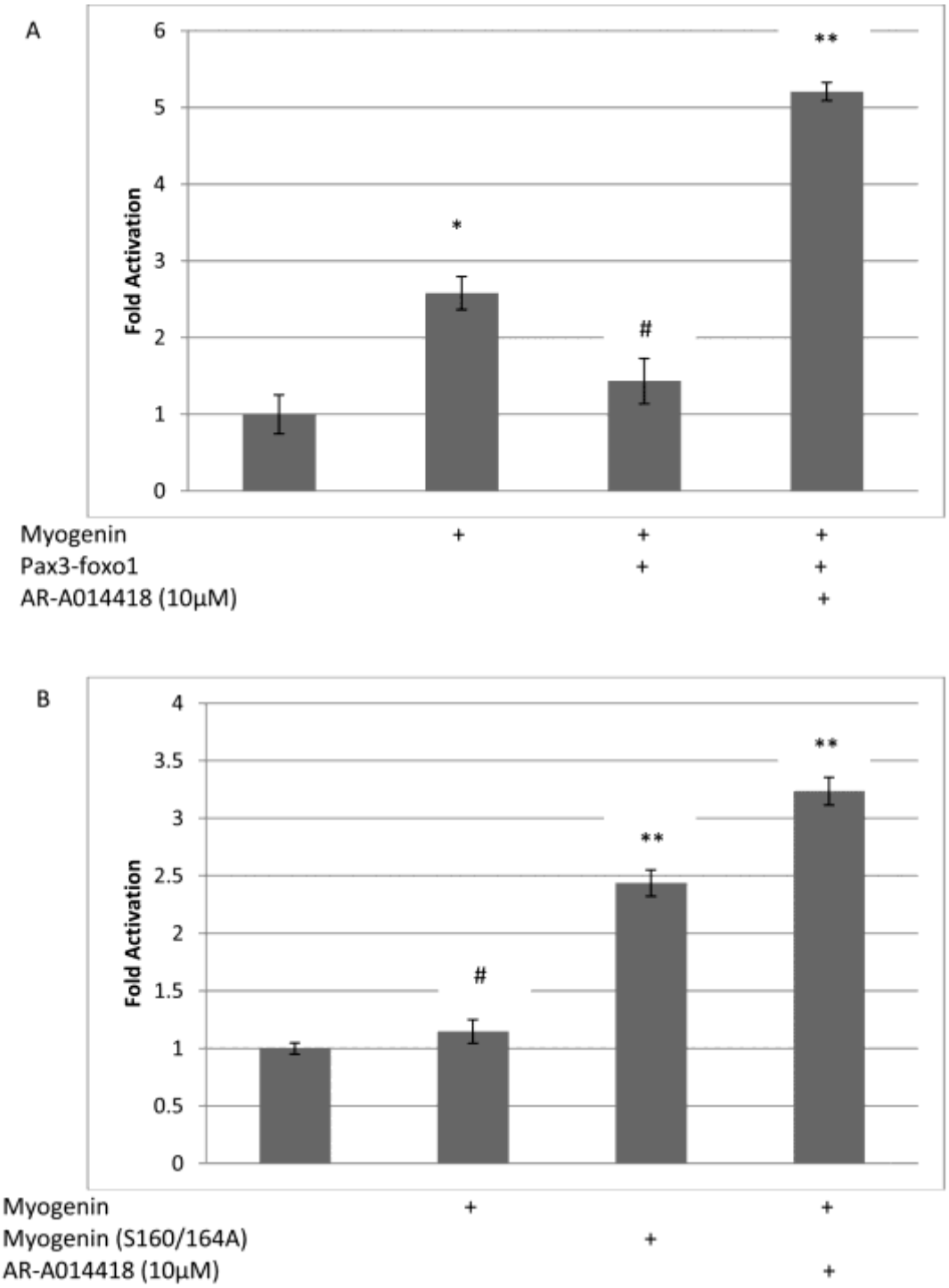


Figure 3.5. **Pharmacological inhibition of GSK3β rescues Pax3-foxo1 repression of myogenin's transcriptional activation of MCK promoter in both C2C12 myoblasts and RH30 human ARMS cells.** (a) MCK-Luc promoter activity was assessed in C2C12 myoblasts that were transfected

with different combinations of myogenin, Pax3-foxo1 and pcDNA3.1 control plasmid as indicated and then treated with either 10μM AR-A014418 or DMSO solvent. Myogenin enhanced MCK-Luc activity as expected ($p<0.001$) and this effect was repressed by co-expression of Pax3-foxo1 ($p<0.01$). Pharmacological inhibition of GSK3β not only reversed the effect of Pax3-foxo1 but resulted in a super-activation ($p<0.001$). (b) To assess the importance of these findings human derived ARMS, RH30 cells were transfected with either myogenin or mutated myogenin(S160/164A) and MCK-Luc promoter activity was assessed. The data shows that wild type myogenin could not *trans*-activate the MCK promoter region unless it was coupled with pharmacological inhibition of GSK3β ($p<0.001$). This was in contrast to mutated myogenin (S160/164A) which could potentiate MCK promoter activity ($p<0.001$) regardless of GSK3β inhibition.

Manipulation of GSK3β and Myogenin activity reduces colony forming properties of ARMS derived, RH30 cells

Colony formation assays were performed as previously described using RH30 cells [35] which can grow in an anchorage independent manner. Equal numbers of RH30 cells that have been transiently transfected with or without Myogenin containing the S160/164A mutations were seeded in growth media with or without 10μM AR-A014418 (GSK3β inhibitor) and allowed to form colonies for 21 days (Figure 3.6). The addition of 10μM AR-A014418 significantly impaired the ability of RH30 cells to form colonies ($p<0.05$) and remarkably reduced the size of the colonies ($p<0.0001$). A similar reduction in colony numbers and size were also evident in RH30 cells that were transfected with Myogenin (S160/164A) mutations (Figures 3.6a, 3.6b). In addition, we confirmed that pharmacological inhibition of GSK3β significantly reduced cell proliferation of Pax3-foxo1 expressing cells (Figure 3.6c). Collectively these findings strongly indicate that GSK3β activity promotes colony formation of RH30 cells, and that this effect is neutralized by expression of Myogenin bearing mutations that render it insensitive to GSK3β.

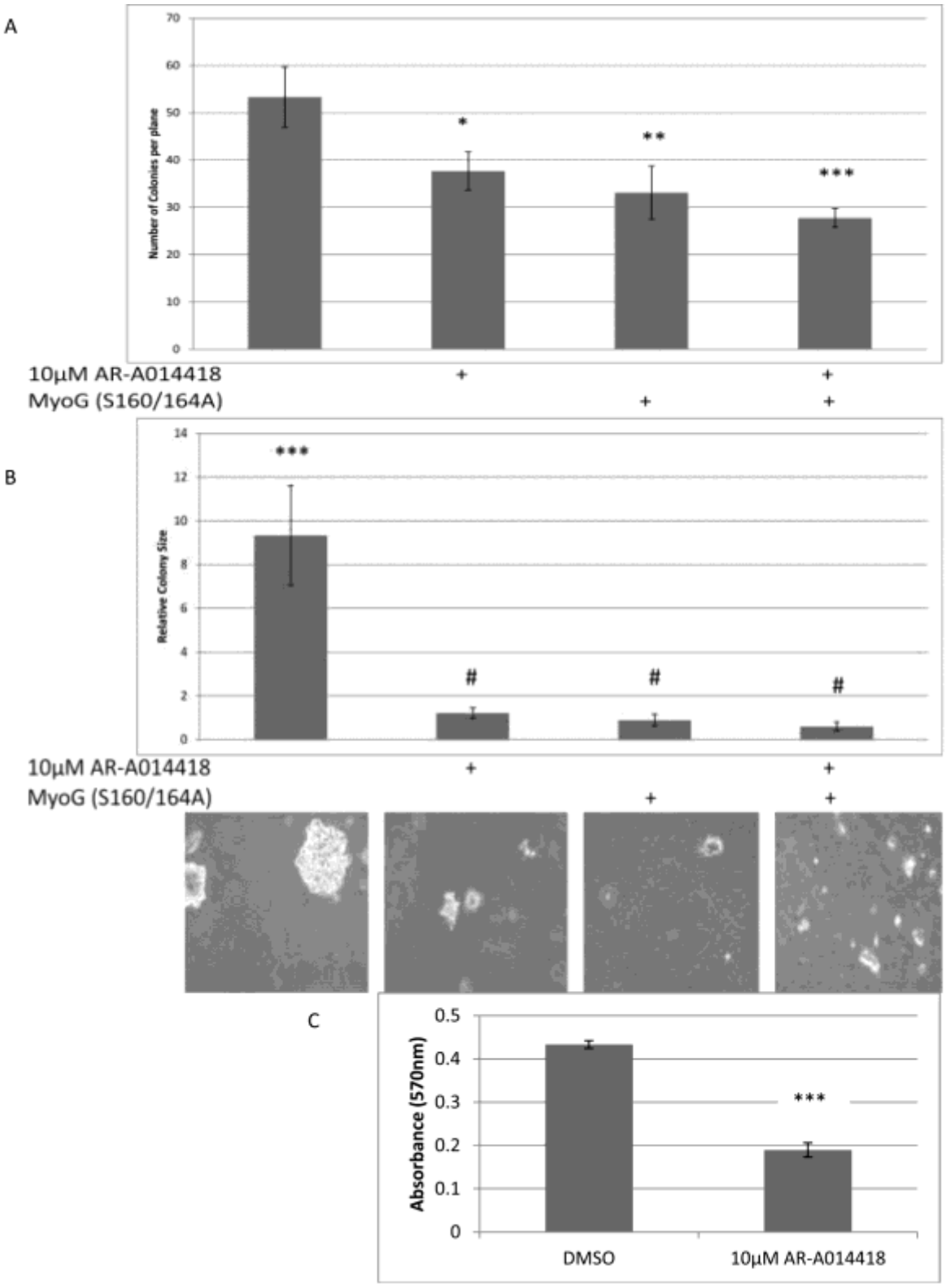
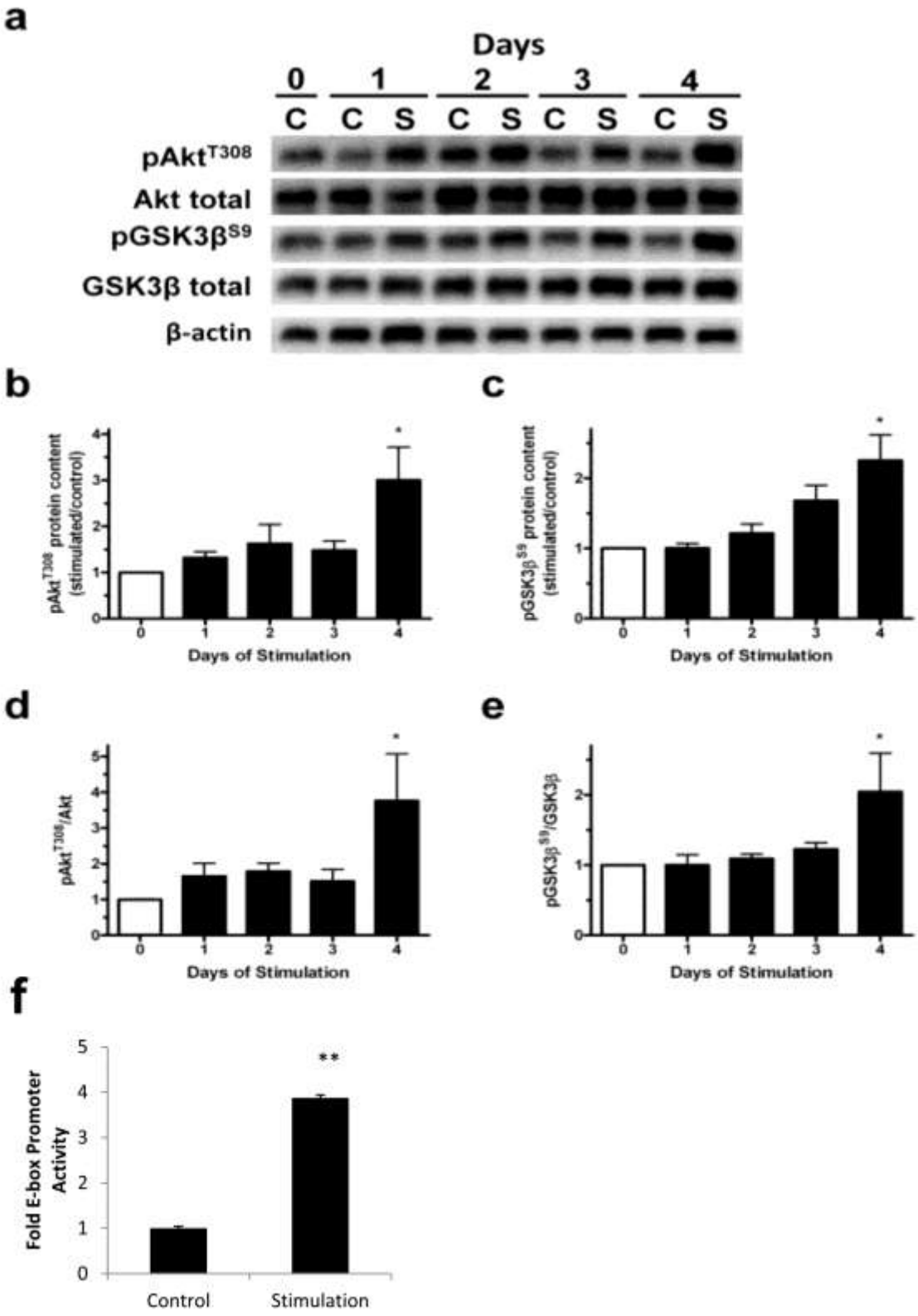


Figure 3.6. **Soft-Agarose Colony Formation and MTT Cell Proliferation Assays:** (a) Equal numbers of RH30 cells were seeded under different conditions as depicted, and allowed to form

colonies for 21 days. On the 22nd day the colonies were stained with 0.005% Crystal Violet overnight. Colonies were counted at different planes (n=10) in 4 independent experiments done in triplicate. The total number of colonies was reduced by (i) 10μM AR-A014418, $p<0.05$ (ii) Transient transfection of Myogenin containing the S160/164A neutralizing mutations, $p<0.01$ and (iii) both, $p<0.001$. Also see supplementary figure for visual representation of the data. (b) We searched for the three largest colonies in each of the 12 plates for each condition and calculated the area using ImageJ software. The data revealed that the control colonies could grow up to 9x bigger than any of the experimental conditions, $p<0.001$. (c) MTT cell proliferation assays were performed in PAX3/FOXO1A expressing cells with and without 10μM AR-A014418 treatment. The experiment revealed that GSK3β inhibition reduces cell proliferation by ~2-fold, $p<0.001$.

Electrical stimulation of ARMS derived, RH30 cells reduces GSK3β activity through Akt

Electrical stimulation of skeletal muscle cells in cell culture has been shown to induce phenotype alterations and differentiation[36]. Given that rhabdomyosarcoma shares properties of the skeletal muscle lineage, we electrically stimulated cultured RH30 cells for 4 hrs/day (5 Hz) for up to 4 days with the idea that it might promote differentiation. Stimulation of these cells resulted in an increase in pAktT308 to levels that were 3.00±0.72 fold higher than those in non-stimulated cells after 4 days of stimulation (Figure 3.7a,b). Concomitantly, pGSKβS9 was also increased 2.25±0.37 fold following 4 days of stimulation (Figure 3.7a,c). These increases in pAktT308 and pGSKβS9 were not a result of increases in total protein (Figure 3.7a) as indicated by the 3.76±1.32 and 2.05±0.55 increases in relative phosphorylation, respectively (Figure 3.7d,e). These changes in kinase activity corresponded with increased E-box promoter activity in stimulated cells compared to controls (Figure 3.7f). Collectively, these data indicate that electrical stimulation suppresses GSK3β activity and correspondingly activates MRF activity supporting our previous findings and also highlighting the possibility of using electrical stimulation as a therapeutic intervention in ARMS patients.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Days of Stimulation
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Days of Stimulation
f
Fold E-box Promoter Activity
**
Control
Stimulation

Figure 3.7. ***In vitro* electrical stimulation of Rh30 cells**. (a) Western blot analysis revealed that electrical stimulation increased PKB/Akt activity and that this corresponded with inhibition of GSK3β at S9. Relative increase of phosphorylation at: (b) Akt at T308 and (c) GSK3β at S9, over time. Graphical depiction of phosphorylated to total amounts of: (d) Akt and (e) GSK3β. (f) E-box promoter activity decreased with electrical stimulation and this also corresponded with inhibition of GSK3β at S9.

3.5 Discussion

ARMS, unlike ERMS, has a well characterized cytogenetic basis in the majority of patients resulting from chromosomal translocations between chromosomes 1 and 13 and also 2 and 13 that result in fusion of the DNA binding domains of either Pax7 or Pax3 with the *trans*activation domain of the Forkhead (FKHR) transcription factor family member Foxo1 [1,2,42]. In view of the well substantiated crucial role of Pax3 and 7 in the development of skeletal muscle [4,5] it is therefore not surprising that the signature of ARMS tumor cells is a muscle like phenotype and the expression of a variety of structural muscle marker genes such as myosin heavy chain and desmin [43]. What is surprising is the sustained expression of MyoD and Myogenin in ARMS [44,45], which are transcription factors that are intimately associated with the terminally differentiated, non- proliferative phenotype of normal myogenic cells, begging the question as to why they cannot exert this effect in ARMS. In particular, the function of Myogenin in the myogenic regulatory hierarchy places it at a pivotal and required step in the terminal commitment of myogenic progenitors to the differentiation program [18,19,47]. Thus, our observations reported here, that Myogenin function in ARMS is repressed by inappropriate sustained signaling by the kinase GSK3β, may be of considerable significance for understanding the etiology of this disease. Moreover, since repression of kinase activity is, in many cases, a tractable pharmacologic approach, we now propose targeting GSK3β activity as a tangible therapeutic strategy for ARMS.

In support of the above, a recent study showed that ARMS-associated Pax3/7-Foxo1 fusion proteins inhibit MyoD target genes [38]. It was also reported that forced MyoD/E-protein dimer expression could not rescue Pax3/7-Foxo1 repression of myogenic factors [38]. Here, we also report that ectopically expressed Pax3-Foxo1 represses the induction of muscle genes, even when MRFs are expressed. We propose that the post-translational repression of Myogenin activity is due to sustained GSK3β activity and, through a cross-talk mechanism, subsequent repression of p38 MAPK as previously described [25]. p38 MAPK and PKB/Akt are both required for activation of MEF2/MyoD transcriptional control and chromatin remodeling events at crucial myogenic loci for the differentiation program [39,40].

In other systems, GSK3β phosphorylation of its protein substrates results in subsequent targeting for proteasomal degradation [26,27]. However, GSK3β does not appear to affect Myogenin protein stability in our experiments since we observe an increase in a slow migrating, hyper-phosphorylated form of Myogenin in response to GSK3β signaling that is not reduced in terms of its level of expression suggesting that proteasomal degradation of Myogenin is not enhanced by GSK3β. Conversely, neutralizing mutations of the GSK3β consensus enhanced Myogenin *trans*-activation of the muscle creatine kinase promoter, and also reduced the tumorigenic properties of ARMS cells (RH30) in a colony formation assay. These findings suggest that GSK3β-mediated inhibition of Myogenin *trans*activation properties impairs Myogenin's ability to promote terminal differentiation in tumorigenic RH30 cells.

Cell cycle control is an essential component of normal growth control and development which goes awry in tumorigenesis. To date several growth-promoting Pax3-Foxo1 target genes have been implicated in RMS such as the IGF-R and c-Met although, while their contribution to proliferation is likely, the extent of their precise involvement in ARMS is still not clear [reviewed in 24]. During normal skeletal myogenesis, up-regulation of a cyclin-dependent kinase inhibitor, p21, stalls myoblasts in the G2/M phase of the cell cycle thus priming them for differentiation by promoting cell cycle exit which is a requirement for subsequent muscle specific gene expression [29]. Consistent with the idea that GSK3β activation may contribute to the oncogenic properties resulting from Pax3-Foxo1 expression in ARMS, we observed that the number of proliferative RH30 cells is approximately halved by pharmacological inhibition of GSK3β. So far, the exact mechanism by which GSK3β regulates cell proliferation in ARMS is unknown. However, GSK3β has recently been shown to activate KLF6 [30] and we recently identified that KLF6 enhances cell proliferation in myogenic cells through a TGFβ/Smad3 dependent pathway [37]. We therefore speculate that Pax3-Foxo1/GSK3β enhancement of cell proliferation may involve KLF6 as a downstream effector since it is also highly expressed in various RMS cell types.

In summary, Myogenin normally activates genes that regulate cell fusion and terminal differentiation of skeletal muscle. In Pax3-Foxo1 expressing ARMS cells, our data indicate that sustained GSK3β activity represses Myogenin function, contributing to the transformed, proliferative phenotype of these cells. Based on this evidence we propose that pharmacologic targeting of GSK3β kinase activity may constitute a tractable therapeutic strategy for ARMS.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKOWLEDGEMENTS

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The following chapter has been published as a research article in the Skeletal Muscle Journal in a slightly modified format (Apr 2013). Contributing authors: MG Dionyssiou, J Salma, M Bevzyuk, S Wales, L Zakharyan, JC McDermott.

The experimental design, figures and data presented in this chapter are mostly of my own efforts with the following exceptions. J Salma made the initial observation that KLF6 was regulated by MEF2 and produced the data in figures 4.1A-B. The chromatin immunoprecipitation assay data presented in Figure 4.2 was produced by S Wales. M Bevzyuk and L Zakharyan assisted me with the execution of the majority of the experiments that I had designed, under my supervision as part of their training. JC McDermott assisted with editing the manuscript. Catherine Chan provided technical support throughout the study.

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

4.1 Abstract

BACKGROUND: Krüppel-like factor 6 (KLF6) has been recently identified as a MEF2D target gene involved in neuronal cell survival. In addition, KLF6 and TGFβ have been shown to regulate each other's expression in non-myogenic cell types. Since MEF2D and TGFβ also fulfill crucial roles in skeletal myogenesis, we wanted to identify whether KLF6 functions in a myogenic context.

METHODS: KLF6 protein expression levels and promoter activity were analyzed using standard cellular and molecular techniques in cell culture.

RESULTS: We found that KLF6 and MEF2D are co-localized in the nuclei of mononucleated but not multinucleated myogenic cells and, that the MEF2 *cis* element is a key component of the KLF6 promoter region. In addition, TGFβ potently enhanced KLF6 protein levels and this effect was repressed by pharmacological inhibition of Smad3. 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 stimulation of myoblast proliferation was reduced in KLF6 depleted cells.

CONCLUSION: 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.

4.2 Introduction

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 located in the C-terminus [1,2]. 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 [3]. Since then, KLF6 has been found to be expressed in most tissues including neuronal, hindgut, heart and limb buds [4] and is localized in the nucleus [5]. 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 [4]. To date, the most well established target gene of KLF6 is TGFβ and its receptors [6] 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 [7]. TGFβ and KLF6 cooperatively regulate a wide range of cellular processes such as cell differentiation, proliferation and epithelial-to-mesenchymal transitions (EMT) [8-13]. Recently KLF6 was identified as a MEF2 target gene that is involved in neuronal cell survival [14]. Since TGFβ and MEF2 are two key regulators of skeletal myogenesis and since KLF6 was identified in the myogenic transcriptome [15], 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 [16]. 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 [17,18]. 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 [19-21)]. 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 [22] and, ultimately result in cell proliferation [23,24]. 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

[25,26]. Cross-talk between these pathways is further supported by Smad7 antagonizing the repressive effects of MEK1 on MyoD [26,27].

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 and, (ii) that MEF2 is recruited to the KLF6 promoter region but is not required for KLF6 activation by TGFβ. Pharmacological inhibition of Smad3 repressed KLF6 expression by TGFβ 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 targeting 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.

4.3 Methods

Plasmids

Expression plasmids for pcDNA3-MEF2D, pCMV β-galactosidase [28,29] and, reporter gene constructs for 3TP-lux [30] MCK-Luc [31] MEF2-Luc [32] have been previously described. KLF6 reporter constructs pRMO6 and pROM6 ΔMEF2 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^{o}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 a 19h incubation.

Immunocytochemistry

C2C12 cells were treated as previously described by Salma and McDermott, 2012 [14] and, incubated overnight with at 4^{o}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).

Co-immunoprecipitation assays

Protein extracts were prepared as described above. Immunoprecipitation 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^{o}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 collected at 5000 rpm for 5 min at 4^{o}C. Cells were lysed using 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 nuclear lysis buffer (50mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS). Chromatin was sheared using a Misonix sonicator to produce 500 bp fragments. Crosslinked sheared chromatin was collected following a 15 minute spin at maximum speed. Twenty percent 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^{o}C with rocking. Protein G Dynabeads (Invitrogen) were blocked with 20 µg salmon sperm DNA in

IP dilution buffer (15 µl beads + 135 µl IP dilution buffer + 20 µg salmon sperm DNA per IP) overnight at 4^{o}C with rocking. 152 µl of pre-blocked beads were incubated with the IP reaction at 4^{o}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^{o}C, and followed with two washes in TE buffer at 4^{o}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 µl of 5 M NaCl (BioShop) at 65^{o}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^{o}C. DNA samples were then purified using a PCR clean up kit (Qiagen).

qPCR

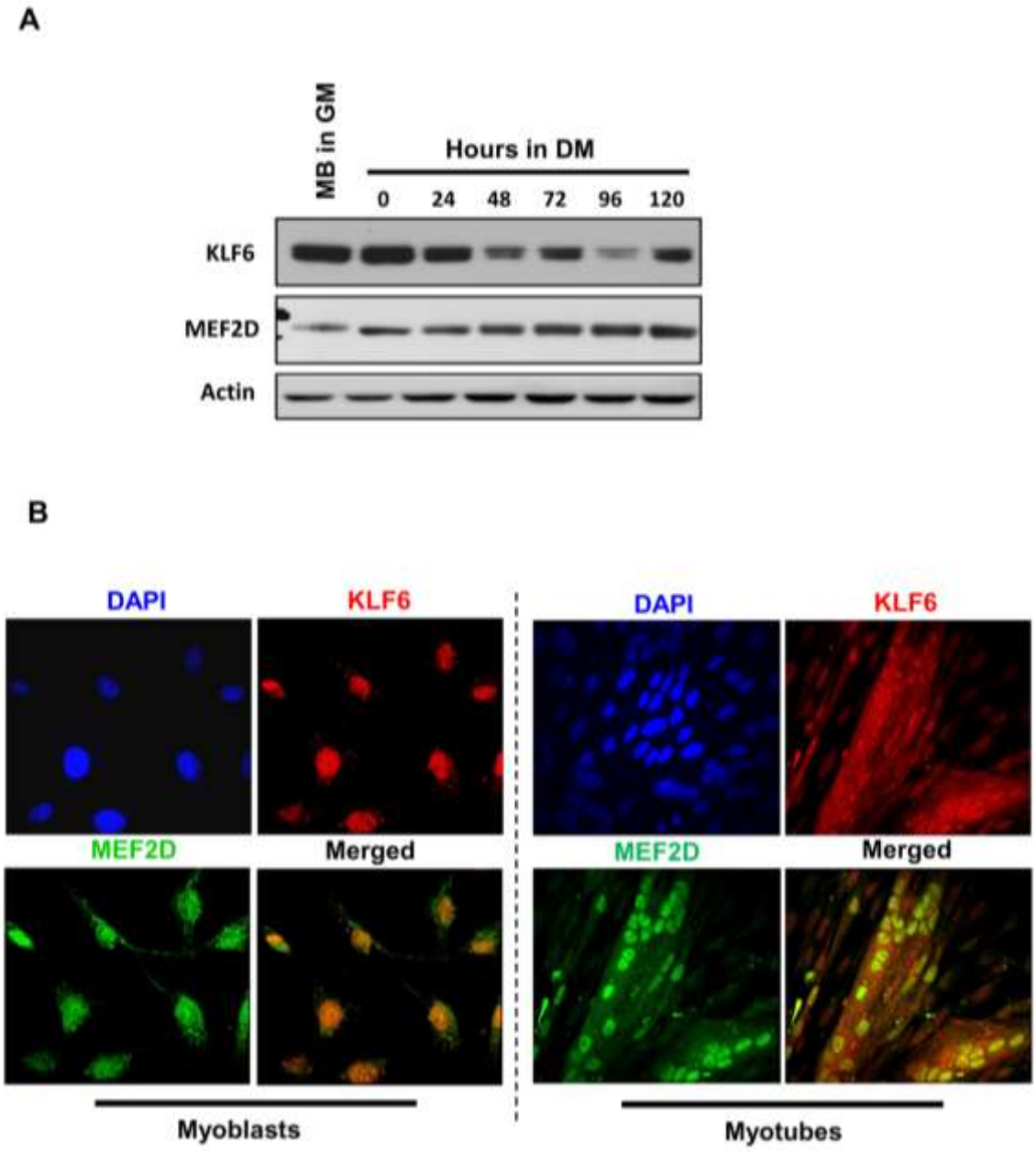
ChIP-qPCR analysis on the KLF6 promoter was done using BioRad Sybr Green as per the user manual with a final primer concentration of 0.5 µM. Antibody used in ChIP: 5 µg αMEF2 (sc-313X; Santa Cruz Biotechnology, Inc.). The equivalent amount of rabbit IgG (12-370, Millipore) was used as a control in each ChIP. Sequences of the primers flanking the ME2 site on the KLF6 promoter were: 5'-CTGCAACGTTGGGCTGTA-3' and 5'-TTGGAAAGACGTCTCACAGG-3'. Each sample was run in triplicate and then analyzed using percent input or fold enrichment.

4.4 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 [15] and has also been shown to be a MEF2D target gene that is involved in the cell survival pathway in primary embryonal hippocampal neurons [14] 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 4.1a and 4.1b). 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 reproducible manner that, at this point is not easily explainable (Fig. 4.1a). 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. 4.1b). Since TGFβ has also been shown to regulate KLF6 expression, we tested the effect of TGFβ on previously characterized KLF6 reporter gene constructs (pROM6-Luc and pROM6-Luc ΔMEF2). Serum was withdrawn 24h after transfection and treatment with 2ng/ml TGFβ for 24h was carried out as indicated in the figure. The data illustrates 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 which requires that the MEF2 *cis* element is intact (Fig. 4.1c).



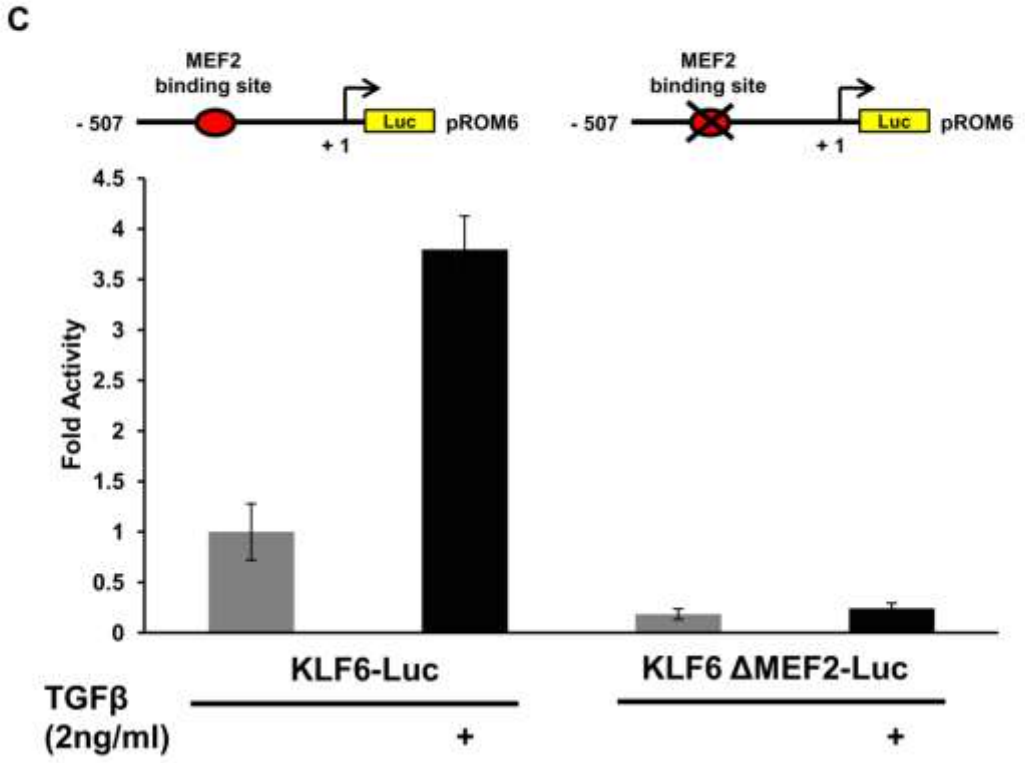


Figure 4.1: (a) **Western blot analysis** reveals that KLF6 and MEF2D are co-expressed in C2C12 myoblasts. Myoblasts were cultured in growth medium (10% serum), followed by serum withdrawal (2%) for 144h and harvested at 24h time intervals. Cells were then lysed and equal amounts of protein (20μg) were 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 [14]. 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 (Fig. 4.2a). 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 analyze this observation, we assessed MEF2 recruitment on the KLF6 promoter with or without TGFβ treatment (Fig. 4.2b). These data indicate that while MEF2 is indeed recruited to the KLF6 promoter in C2C12 myoblasts, there is no change in MEF2 recruitment upon TGFβ treatment compared to the control, implicating a different mechanism for TGFβ activation of KLF6.

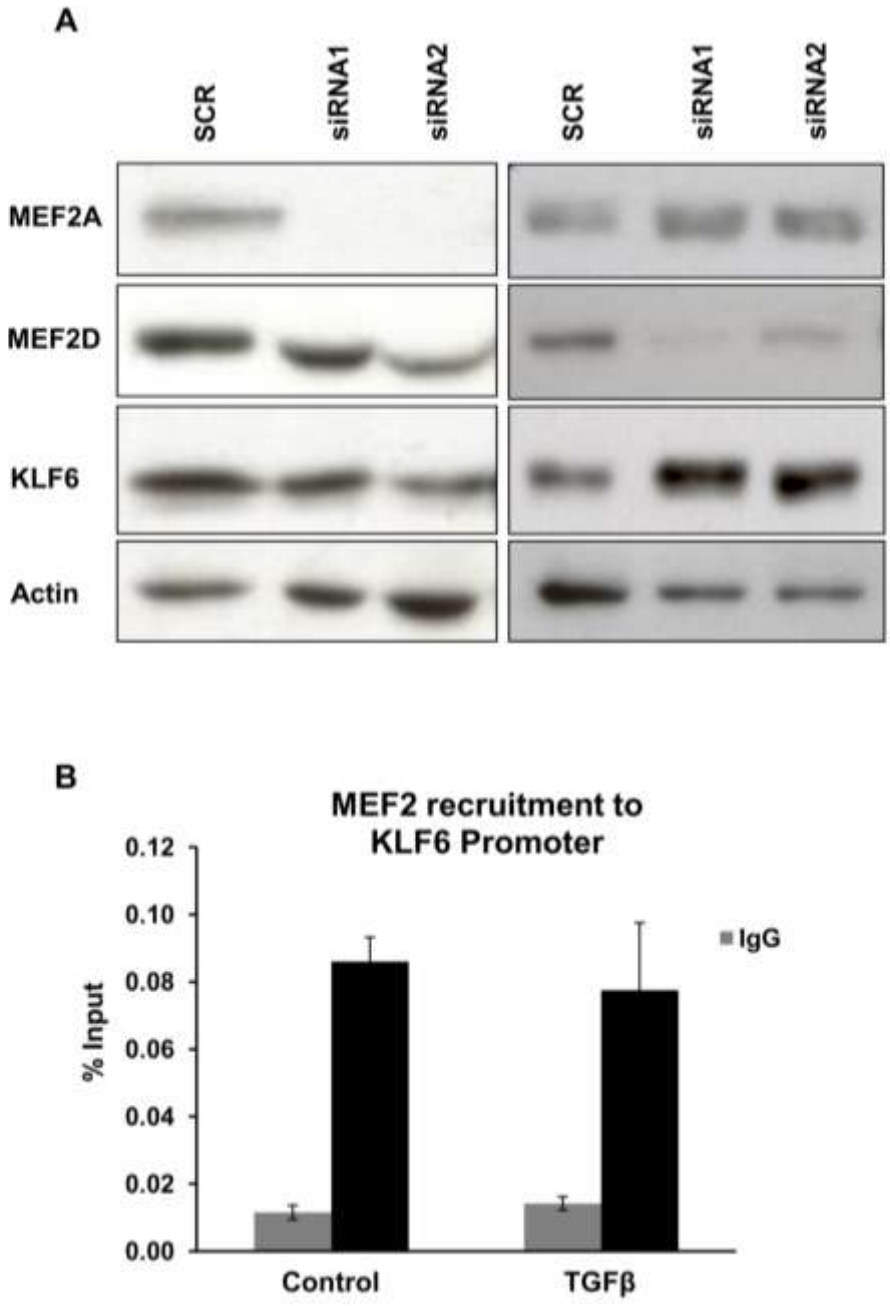


Figure 4.2: (a) **MEF2A/D RNA silencing** reveals that MEF2A/D expression is not required for endogenous KLF6 protein expression. In contrast siMEF2D appears to de-repress endogenous KLF6 protein levels. (b) **Chromatin immunoprecipitation** analysis of MEF2 recruitment onto the KLF6 promoter revealed 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 (Fig. 4.3a). To further investigate the effect of TGFβ on KLF6 we used well documented pharmacological inhibitors of the Smad and ERK1/2 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 (Fig. 4.3b). The data in Fig. 4.3b 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 [33]. 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 Fig. 4.3c, 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 (Fig. 4.3c). Strikingly, pharmacological inhibition of ERK1/2 had no effect on KLF6 levels but instead rescued myotube formation and MyHC expression, thus supporting the idea that TGFβ regulates KLF6 and myogenic differentiation through Smad3 and ERK1/2 distinctively.

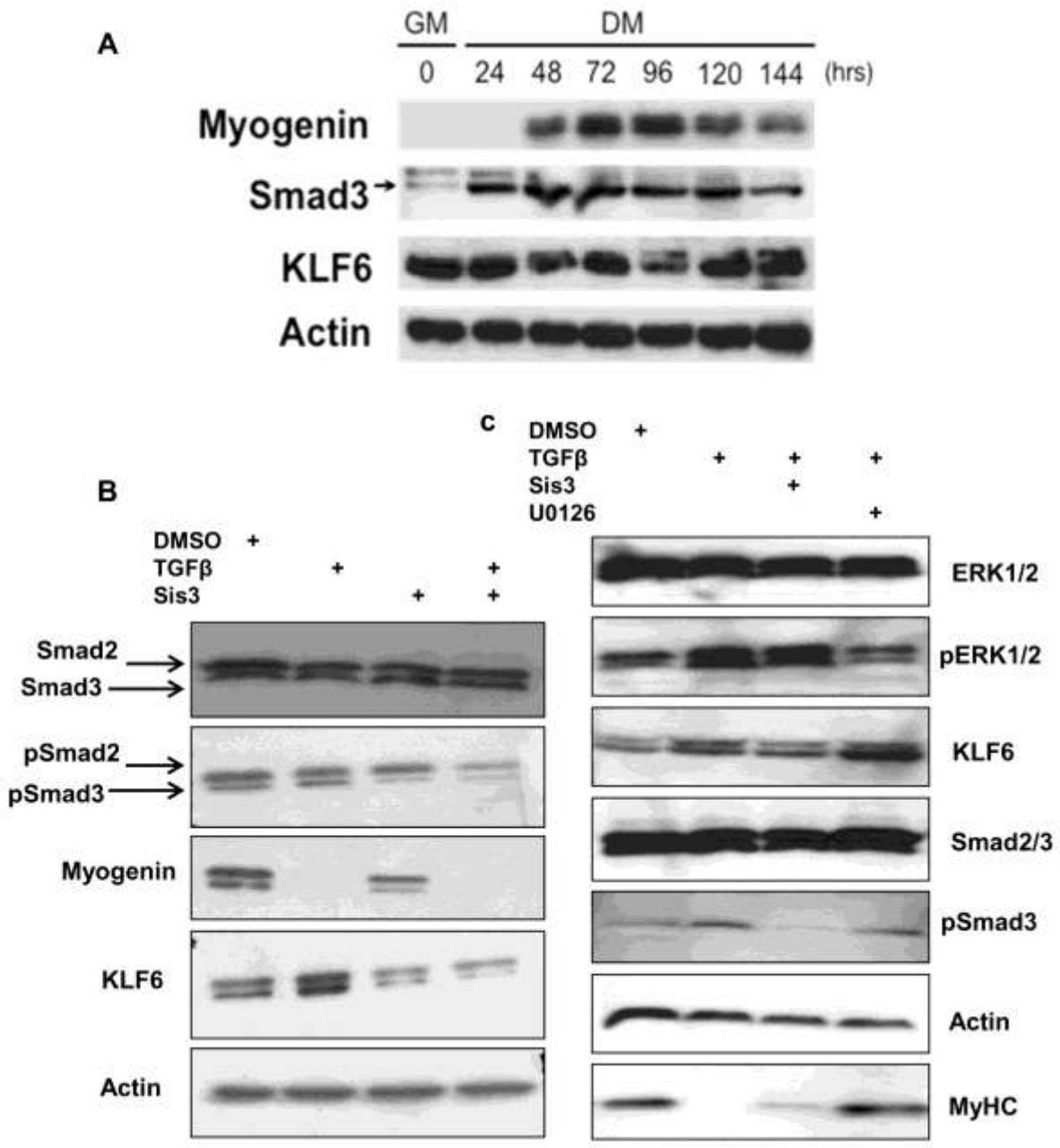


Figure 4.3: (a) **Western blot analysis** revealed that Smad3 and KLF6 are co-expressed in C2C12 myoblasts. Myogenin was used as a protein marker for differentiation and actin was used as a loading control. **Pharmacological manipulation of TGFβ signaling pathway** reveals that TGFβ regulates KLF6 protein 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 depleting KLF6 expression as shown in Fig. 4.4a. Subsequent KLF6 silencing resulted in increased MyoD and myogenin protein expression (fig. 4.4b; upper panel) and this corresponded with a 2.5 fold increase in muscle creatine kinase (MCK) promoter (Fig. 4.4b; lower panel). Furthermore, an MTT cell proliferation assay was performed, and the data showed that at 24h, 2ng/ml TGFβ treatment doubles the number of proliferating cells (Fig. 4.4c). This effect is largely negated following 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 (Fig. 4.4c).

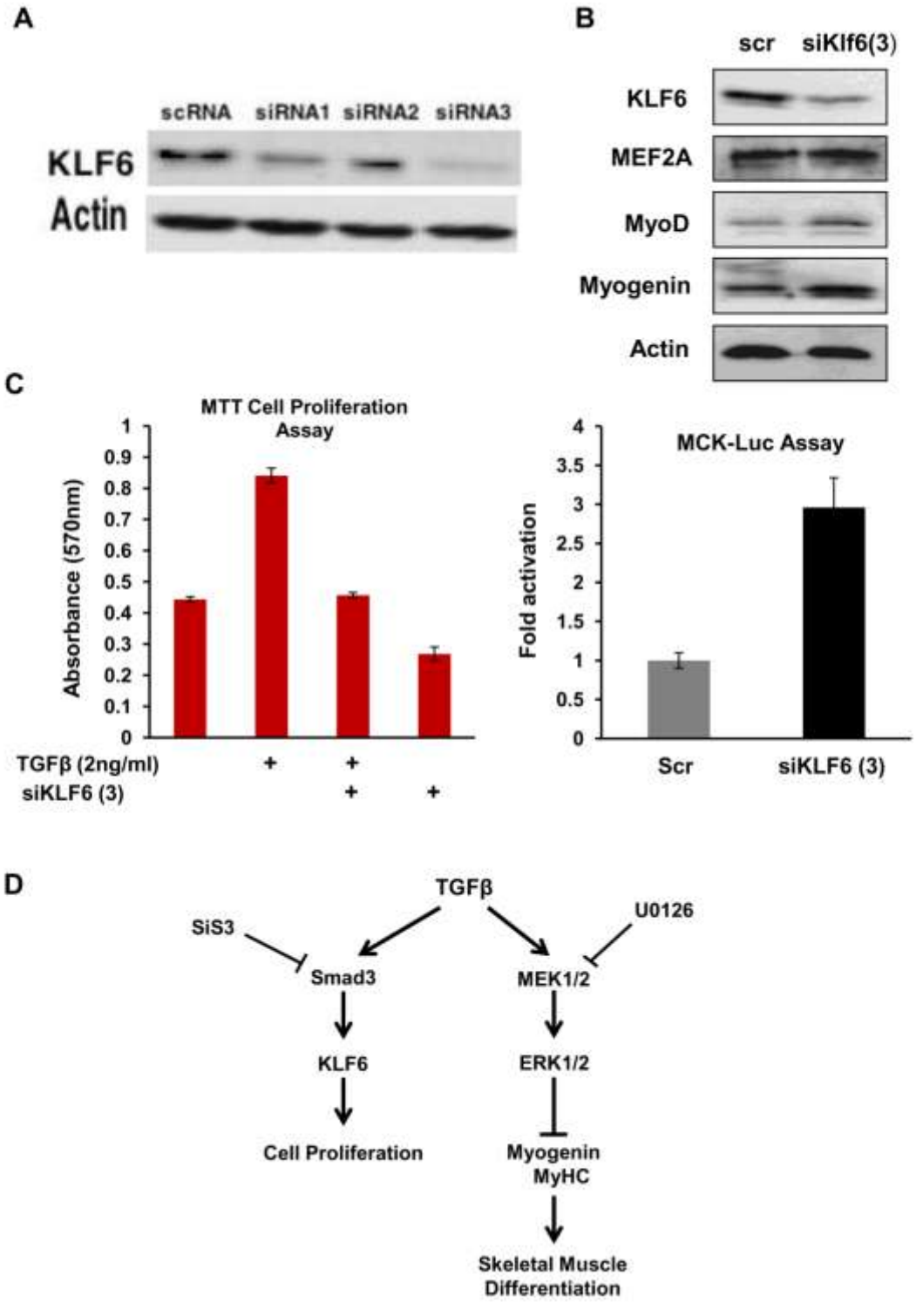

A
scRNA
siRNA1
siRNA2
siRNA3
KLF6
Actin
B
scr
siKlf6(3)
KLF6
MEF2A
MyoD
Myogenin
Actin
C
MTT Cell Proliferation Assay
Absorbance (570nm)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
TGFβ (2ng/ml)
siKLF6 (3)
+
+
+
+
MCK-Luc Assay
Fold activation
4
3.5
3
2.5
2
1.5
1
0.5
0
Scr
siKLF6 (3)
D
TGFβ
SiS3
U0126
Smad3
MEK1/2
KLF6
ERK1/2
Cell Proliferation
Myogenin
MyHC
Skeletal Muscle Differentiation

Figure 4.4: **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.

4.5 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 4.4d. Many myopathies and muscle loss disorders have been linked with increased TGFβ signaling [34] 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 [35].

4.6 References

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Summary

GSK3β signaling suppresses skeletal muscle differentiation, although the precise molecular mechanism has not yet been defined. The studies presented in this report integrate GSK3β and p38 (MAPK) into the signaling network converging on MEF2 transcription factors and downstream skeletal and cardiac target gene expression. Functionally, GSK3 inhibition potentiates MEF2 transcriptional activity and this corresponded with an upward MEF2A band shift that was indicative of phosphorylation. These effects were reversed by pharmacological inhibition of p38 (MAPK) indicating that the effect of GSK3 on MEF2 was p38 dependent. On the other hand, ectopically expressed constitutively active p38/MKK6(EE) could not rescue GSK3β-mediated repression of MEF2. Previous studies have shown that p38 and JNK are negatively regulated by GSK3β via MEKK4 (MAP3K) in COS-7 cells (Abell *et al*., 2007). Based on our data, this did not seem to be the case in C2C12 myoblasts. Instead, repression of p38 was determined to be at the MAPK-level and this could either be by direct phosphorylation (Thornton et al., 2008) or indirectly through activation of phosphatases that can de-phosphorylate MAPKs (Wang *et al*., 2009). Nevertheless our studies show that GSK3 inhibition leads to de-repression of p38 which results in MEF2A phosphorylation and hence *trans*activation of its skeletal and cardiac target genes.

As with skeletal muscle differentiation, several studies have identified GSK3β as a negative regulator of cardiac muscle hypertrophy (Haq *et al*., 2000). In contrast, MEF2 has been shown to mediate stress-dependent pathological cardiac hypertrophy, which is characterized by cardiomyocyte growth, assembly of additional sarcomeres, enhanced contractility and activation of a fetal cardiac gene program (Kim *et al*., 2008). Our data show that GSK3β is a negative regulator of p38/MEF2 signaling and downstream markers of cardiac hypertrophy such as ANF

therefore elevation of GSK3β in the heart could potentially circumvent the chain of events that leads to pathological cardiac remodeling.

The second study which investigated the molecular basis for a lack of myogenic differentiation in Pax3-foxo1a expressing rhabdomyosarcoma cells revealed that GSK3β activity represses myogenesis not only through p38/MEF2 signaling but also by direct phosphorylation and hence inhibition of a key target downstream effector protein, Myogenin. Since Myogenin is a key regulator of cell fusion and hence terminal differentiation as discussed in Chapter 1, the presence of Myogenin in ARMS is extremely paradoxical. Although elevated GSK3β activity has been previously reported in Pax3-foxo1a expressing ARMS (Zeng *et al*., 2010), our studies have provided an answer to its role in maintaining an undifferentiated state in these cells. Neutralizing mutations of the S160/164 GSK3 phosphoacceptor sites resulted in a decrease in a low mobility upper band which coincided with increased Myogenin transcriptional activity. Additionally, colony formation assays revealed that transient transfection of Myogenin (S160/164A) decreased the tumorigenicity of Pax3-foxo1a expressing RH30 cells. Based on our studies, pharmacological inhibition of GSK3 significantly reduced cell proliferation and tumorigenicity *in vitro*. Currently, lithium based drugs are used to treat psychiatric and other brain-related diseases in both adults and children (Nunes *et al*., 2013) and since lithium is known to inhibit GSK3 activity (albeit non-specifically) our studies support the idea of repurposing such pharmacological agents for treating children that suffer from alveolar rhabdomyosarcoma.

Given the opposite roles of MEF2 and GSK3β in the regulation of skeletal myogenesis, and given that MEF2 and GSK3β have both been shown to potentiate KLF6 expression (Salma and McDermott, 2012) and transcriptional activity (Okcu *et al*., 2013) respectively, in different tissue types and cancers, the third study described in Chapter 5 investigated the role of KLF6 in a

myogenic context. For the first time, these studies revealed an anti-myogenic role for KLF6 by enhancing cell proliferation despite having tumor-suppressor properties in other tissue types (Okcu *et al*., 2013). The data presented illustrate that TGFβ/Smad3 signaling and not MEF2 regulates KLF6 protein expression, in myoblasts. Through these studies we were also able to document that TGFβ signaling regulates myogenesis through two distinct pathways: (1) by promoting cell proliferation in a Smad3/KLF6 dependent manner and (2) by inhibiting differentiation through ERK1/2 (MAPK) activation.

Collectively these studies demonstrate cross-talk between various signaling pathways and the importance of proper maintenance of these, in regulating physiological conditions such as proliferation and differentiation. Upsetting the equilibrium of growth factors and other extracellular stimuli that influence intracellular kinase activity can result in regulation of non-canonical substrates and their downstream target genes, which is apparent in alveolar RMS.

Future Direction

Perhaps the most significant finding from these studies is that pharmacological inhibition of GSK3β significantly reduces cell proliferation and tumorigenicity of the alveolar RMS cells, by repressing the p38/MEF2/Myogenin pathway, and possibly also through activation of KLF6. It would be important to confirm enhanced GSK3β activity and Myogenin phosphorylation in human alveolar RMS tissue samples by western blot analysis and RT-PCR. Furthermore, I propose utilizing an existing *in vivo* alveolar RMS mouse model (Gen & Dev., 2006) and then either (1) treating these with GSK3 inhibitor or solvent or (2) surgically insert electrodes into the tumors to test whether or not low voltage stimulates Akt activation and hence repression of GSK3β and tumor progression, as described in Chapter 3 and Figure 3.7.

From a biomolecular aspect, having confirmed that KLF6 promotes cell proliferation in skeletal muscle cells (Chapter 4), and since KLF6 is expressed in RMS (Dionyssiou et al., 2013) and has also already been shown to be activated by direct phosphorylation, by GSK3β (Okcu et al., 2013), I propose confirming this relationship in an RMS model both in vivo and in vitro using the same methods that have already been successfully utilized in identifying Myogenin as a novel GSK3β target (Chapter 3). Since GSK3β is important in cardiac survival, glucose/glycogen metabolism as well as being a key regulator of Wnt signaling and protector against other cancers, it may be unrealistic to suggest that pharmacological inhibition of GSK3 could be used to treat RMS patients. However, if we can better understand the specific effects of GSK3β and identify unique substrates in RMS tissue, then it might be worthwhile for scientists and biomolecular engineers to collaborate on creating new technologies or small molecules that can target these substrates making them resistant to regulation by GSK3β.

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APPENDIX

APPENDIX

Molecular techniques;

Cell Culture

Reagents: 1x Dulbecco's PBS, Versene (0.2g of 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 media (GM) supplemented with 10% DMSO); sterilize the freezing medium by passing through a 0.2um filter), FBS(heat inactivated at 56°C for 30 min), HS (heat inactivated at 56°C for 30 min).

Cell passaging

1. Remove media.
2. Rinse the cell monolayer with 4 ml of Versene.
3. Add 2.0ml of 0.125% Trypsin-EDTA solution to 100mm dish.
4. Remove the Trypsin-EDTA solution.
5. Add 10 ml of GM.
6. Pipette the cells up and down with the GM.
7. Plate cells accordingly.

Inducing Muscle Cell Differentiation

1. At 60-80% confluence, wash cells with PBS and re-feed with 2% HS in DMEM (differentiation medium (DM)).
2. Incubate cells for desired time at 37°C with 5% CO_2

Transfection of Mammalian Cells with DNA

Reagents: 2x HEBS (2.8 M NaCl, 15mM Na_2HPO_4, 50mM HEPES)(adjust pH to 7.15, filter sterilize, store at -20 °C), 2.5 M $CaCl_2$ (filter sterilize, store at -20 °C).

Calcium-phosphate transfection

1. Plate cells day before transfection for 30-50% confluent.
2. Re-feed cell cultures with GM 2-3 h prior to addition of DNA.
3. Label sterile tubes and add 0.5 ml of 2x HEBS to each tube.
4. Prepare DNA-$CaCl_2$ solution as follows, add 25 μg DNA, bring up volume to 450 μl, mix, add 50 μl 2.5 M $CaCl_2$, mix.

5. Add DNA-$CaCl_2$ solution drop-wise to the HEBS.
6. Add DNA mix drop-wise to cell cultures.
7. 16 h after addition of DNA, wash cells with 1XPBS and re-feed with GM.

Luciferase Assay

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

1. Wash adherent cells with 1XPBS.
2. Add 300 µl of lysis buffer per well/dish (35mm).
3. Harvest cells and spin-down cell debris at 15000rmp for 10min.
4. Transfer cell lysate into new tubes.
5. Transfer 30 µl to Luciferase assay tube.

β-Galactosidase Assay

Reagents: ONPG (4 mg/ml in ddH_2O), Z buffer (60 mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$), 1 M Na_2CO_3

1. Prepare reaction mixture (per sample (500 µl Z buffer, 100 µl ONPG, 2.74 µl β-mercaptoethanol)).
2. Incubate tubes at 37 °C until a color change is apparent (yellow).
3. Add 400 µl of 1M Na_2CO_3 to each tube to stop reaction.
4. Measure absorbance of samples at 420 nm.

Protein Extracts

Reagents: ice-cold 1XPBS, 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)**,** 2X SDS sample buffer (BioRad) **(**supplemented with β-mercaptoethanol)

1. Wash cells with ice-cold 1XPBS twice.
2. Scrape cells and transfer to a new tube.
3. Centrifuge at 1500XG for 2min.
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 000XG for 15 min, and transfer supernatant to new tube.

7. Determine protein concentration by Bradford assay.

SDS-PAGE

Reagents: 1.5M Tris pH 8.8, 30% acrylamide mix, 10% SDS, 10% APS, TEMED, Laemmli buffer.

1. Prepare resolving gel and then top with stacking gel.
2. Fill bottom and centre well of mini-gel apparatus with 1X Laemmli buffer.
3. Load samples on a gel.
4. Run a gel at 100-150 V.

Western blotting

1. Transfer protein from a gel to Immobilon-P (Millipore) membrane by wet- transfer at 20 V for 16 hrs.
2. Block membrane with 5 % (w/v) skim milk powder in 1XPBS/TBS (blocking solution).
3. Incubate membrane with primary antibody in blocking solution for 1-16 hrs at 4 °C.
4. Wash membrane with 1XPBS/TBST (3 X 5 min each).
5. Incubate membrane with secondary antibody in blocking solution for 1-2 hrs at room temperature (RT).
6. Wash membrane with PBS/TBST (3 X 5 min each).
7. Apply chemiluminescence reagent, and expose blot to film.

Co-Immunoprecipitation

1. Prepare cell lysates as described in protein extracts section.
2. Dilute protein sample in lysis buffer.
3. To 1 ml of cell lysate (250-1000µg total protein) add 1-5 µg of primary antibody and incubate at 4 °C for 1 h with gently agitation.
4. Add 30-50 µl of Protein G-Agarose, and nutate 16 hrs at 4 °C.
5. Pellet immuno-complex by centrifugation at 1000XG for 30 sec.
6. Wash pellet with 1 ml of lysis buffer.
7. Repeat steps 5 and 6 twice more.
8. Re-suspend pellet in 40 µl of 2 X SDS sample buffer and boil for 3 min, and transfer supernatant to new tube.
9. Sample ready for immuno-blotting.

Immunochemistry

Reagents: Fixative (90% ice-cold methanol), Blocking reagent **(**5 % skim-milk in 1XPBS).

1. Wash cells and fix and permeabilize with 90 % methanol for 10 min at -20°C.
2. Block with 5 % skim-milk in 1XPBS at 37 °C for 30 min.

3. Incubate cells with primary antibody for 1 hr in 5 % skim-milk.
4. Incubate cells with Horseradish peroxidase (HRP)-conjugated secondary antibody, 1 hr in 5 % skim-milk 1XPBS.
5. Wash cells three times with 1XPBS and incubated in developer (0.6 mg/ml DAB, 0.1 % H_2O_2 in 1XPBS).
6. Counter-stain nuclei with haematoxylin.
7. Wash several times in ddH_20.
8. Mounting cells with mounting media and cover-slip

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)

***In vitro* Kinase Assay: Phosphorylation of GST-Myogenin by GSK3β**

GSK3β (Cell Signaling)
GSK3β kinase reaction buffer (10X) 50mM Tris-HCl
10mM $MgCl_2$
2mM DDT
1mM EGTA
0.01% Brij 35 (non-ionic detergent)
pH 7.5
ATP (10mM)
[γ^{32}-P]ATP
Myelin Basic Protein (0.5mg/ml) – positive control
GST – negative control
GST–Myogenin (Abcam)

1. Dilute 10x kinase reaction buffer to 1x

2. Supplement 1x kinase reaction buffer with 200μM ATP and [γ-^{32}P]ATP to a final specific activity of 100-500 μCi/μmol

3. Add 2μl (20ng) of purified activated GSK3β kinase to 3μg of protein

4. Add 10μl of ATP to reaction
5. Incubate for 30 min at 30˚C (water bath)
6. Add 2x sample buffer and boil for 5 min
7. Resolve on a 10% SDS-PAGE gel
8. Coomassie stain gel
9. Dry gel, expose and develop