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Role of Map4k4 in Skeletal Muscle Differentiation: A Dissertation

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ROLE OF MAP4K4 IN SKELTAL MUSCLE DIFFERENTIATION

A Dissertation Presented

By

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Abstract

Skeletal muscle is a complicated and heterogeneous striated muscle tissue that serves critical mechanical and metabolic functions in the organism. The process of generating skeletal muscle, myogenesis, is elaborately coordinated by members of the protein kinase family, which transmit diverse signals initiated by extracellular stimuli to myogenic transcriptional hierarchy in muscle cells. Mitogen-activated protein kinases (MAPKs) including p38 MAPK, c-Jun N terminal kinase (JNK) and extracellular signal-regulated protein kinase (ERK) are components of serine/threonine protein kinase cascades that play important roles in skeletal muscle differentiation. The exploration of MAPK upstream kinases identified mitogen activated protein kinase kinase kinase kinase 4 (MAP4K4), a serine/threonine protein kinase that modulates p38 MAPK, JNK and ERK activities in multiple cell lines. Our lab further discovered that Map4k4 regulates peroxisome proliferator-activated receptor γ (PPARγ) translation in cultured adipocytes through inactivating mammalian target of rapamycin (mTOR), which controls skeletal muscle differentiation and hypotrophy in kinase-dependent and -independent manners. These findings suggest potential involvement of Map4k4 in skeletal myogenesis.

Therefore, for the first part of my thesis, I characterize the role of Map4k4 in skeletal muscle differentiation in cultured muscle cells. Here I show that Map4k4 functions as a myogenic suppressor mainly at the early stage of skeletal myogenesis with a moderate effect on myoblast fusion during late-stage muscle differentiation. In agreement, Map4k4 expression and protein kinase activity are declined with myogenic differentiation. The

inhibitory effect of Map4k4 on skeletal myogenesis requires its kinase activity. Surprisingly, none of the identified Map4k4 downstream effectors including p38 MAPK, JNK and ERK is involved in the Map4k4-mediated myogenic differentiation. Instead, expression of myogenic regulatory factor Myf5, a positive mediator of skeletal muscle differentiation is transiently regulated by Map4k4 to partially control skeletal myogenesis. Mechanisms by which Map4k4 modulates Myf5 amount have yet to be determined.

In the second part of my thesis, I assess the relationship between Map4k4 and IGF-mediated signaling pathways. Although siRNA-mediated silencing of Map4k4 results in markedly enhanced myotube formation that is identical to the IGF-induced muscle hypertrophic phenotype, and Map4k4 regulates IGF/Akt signaling downstream effector mTOR in cultured adipocytes, Map4k4 appears not to be involved in the IGF-mediated ERK1/2 signaling axis and the IGF-mediated Akt signaling axis in C2C12 myoblasts. Furthermore, Map4k4 does not affect endogenous Akt signaling or mTOR activity during C2C12 myogenic differentiation.

The results presented here not only identify Map4k4 as a novel suppressor of skeletal muscle differentiation, but also add to our knowledge of Map4k4 action on multiple signaling pathways in muscle cells during skeletal myogenesis. The effects that Map4k4 exerts on myoblast differentiation, fusion and Myf5 expression implicate Map4k4 as a potential drug target for muscle mass growth, skeletal muscle regeneration and muscular dystrophy.

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List of Frequently Used Abbreviations

Abbreviation	Term
Map4k4	Mitogen-activated protein kinase kinase kinase kinase 4
MRF	Myogenic regulatory factor
MEF2	Myocyte enhancer factor 2
MyHC	Myosin heavy chain
MAPK	Mitogen-activated protein kinase
JNK	c-Jun N terminal kinase
ERK	Extracellular signal-regulated protein kinase
IGF	Insulin-like growth factor
PI3K	Phosphatidylinositol 3-kinase
Akt	Protein kinase B
mTOR	Mammalian target of rapamycin
4EBP1	eIF4E-binding protein-1
eIF4E	Eukaryotic initiation factor 4-E
p70S6K1	p70 ribosomal S6 kinase-1

GSK3	Glycogen synthase kinase 3
FoxO	Forkhead box protein O
MAFbx	Muscle atrophy F-box
MuRF1	Muscle RING finger 1
NFAT	Nuclear factor of activated T cell
RSPO3	R-spondin 3
TNFα	Tumor necrosis factor α
siRNA	Small interfering RNA
Ad	Adenovirus
wt	Wild type
GM	Growth medium
DM	Differentiation medium

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CHAPTER I: Introduction

Myogenesis

Skeletal muscle is a striated muscle tissue with complicated and heterogeneous features that serves multiple critical functions in the organism. Vertebrate skeletal muscle of the trunk and limbs originates from the somites, which are mesodermal structures that are located on either side of the neural tube in vertebrate embryos (201). In response to the signals from distinct environmental cues, somites differentiate and subdivide into two compartments, the dorsal dermomyotome and the ventral sclerotome. Myogenic precursors in the dermomyotome subsequently give rise to myotomes, which are responsible for the formation of the trunk and deep back muscles (130). Meanwhile, some of the cells from the lateral edge of the dermomyotome undergo epithelial-mesenchymal transition and delaminate and migrate to the limb buds, where they give rise to limb musculature following sequential steps including myoblast specification, myocyte differentiation and fusion, and mature myofiber formation (50, 76).

During murine skeletal muscle development, myoblasts are derived from two distinct progenitor populations and contribute to two phases of myogenesis (121). The first wave of mononucleated myocyte fusion into multinucleated myofibers occurs at approximately embryonic day 11 (E11) and is defined as primary or embryonic myogenesis, in which basic muscle patterning occurs. The secondary, or fetal myogenesis that occurs between E14.5 and E17.5 is characterized by fusion of fetal myocytes with each other, or their alignment and fusion with the scaffold-like primary myotubes to form secondary

myofibers (177). At the end of this phase, each myofiber is coated by basal lamina, underneath which some muscle progenitors termed satellite cells are located. Satellite cells normally remain quiescent in adult muscles, but they can be activated upon injury and aid in muscle regeneration. Satellite cell-mediated muscle regeneration is highly similar to developmental myogenesis, as evidenced by common transcription factors and molecular signals that modulate these scenarios (260, 299).

Myogenic transcription factors

Paired-homeobox transcription factors

Myogenesis is elaborately controlled by intrinsic genetic hierarchies of myogenic transcription factors. During mouse muscle development, the precursor cells in the dermomyotome express paired-homeobox transcription factors pax3 and pax7, with preferential expression of pax3 in the dosalmedial and ventrolateral lips, and pax7 in the central region where satellite cells originate (101, 135). Of note, only pax3 is detected in the migrating cells that enter the limb bud. To support this observation, *Splotch* mice with a pax3 loss-of-function mutation fail to develop limb muscles, and no pax3-positive cells are detected in the limb, indicating a lack of progenitor migration to the site (66). Consistently, pax3-deleted mice lose all of their embryonic myofibers (121), further supporting that pax3 is required for normal skeletal muscle development. In contrast, pax7 is dispensable for embryonic or fetal myogenesis because $pax7^{-/-}$ mice do not display skeletal muscle formation defects (243). Instead, a complete absence of satellite cells is observed in the mutant mice (228, 243). However, Hutcheson *et al*. demonstrated

an essential role for pax7 in fetal myogenesis by ablating pax7-expressing cells from mouse embryos (121). Pax7 lineage deletion resulted in the loss of fetal (secondary) myofibers, consistent with the observation that pax7 is expressed in fetal myoblasts (22, 119). Based on these studies, one may propose that pax3 is critical for initial myofiber formation, which then serves as a template for pax7-positive cells to form secondary fibers, and pax7 is required to maintain the satellite cell pool.

Myogenic regulatory factors

Pax3+pax7+ progenitors are mitotically active and cannot differentiate into myotubes (135), suggesting that molecules other than pax3 and pax7 are responsible for myogenic induction and precursor cell differentiation. The discovery of MyoD, a transcription factor that is able to convert mouse pluripotent mesenchymal C3H10T1/2 cells into fusion-capable myoblasts (68) sheds light on the molecular nature of muscle differentiation. Subsequent studies revealed three more transcription factors: Myf5, myogenin and MRF4, which are also able to induce myoblast traits in non-muscle cells (38-39, 83). Characterized by their collective expression in the skeletal muscle lineage, these four transcription factors are termed myogenic regulatory factors (MRFs). MRFs have a conserved basic helix-loop-helix (bHLH) DNA binding domain and relatively variable N-terminal and C-terminal domains to mediate transcriptional activation. The HLH domain also facilitates heterodimerization between MRFs and E-proteins that recognize the E-box consensus sequence CANNTG, which is present in many muscle-specific gene promoters (250).

Myf5 is the first MRF expressed within the dermomyotome on the eighth day of mouse embryonic development, and its expression starts to decrease on E11 (43). In contrast, MyoD is expressed at approximately E10.5, and myogenin transcripts begin to accumulate immediately after MyoD activation (37). Two waves of MRF4 expression have been observed in mouse embryogenesis. The first wave occurs between E9 and E11.5 and the second one starts at E16 and persists through adulthood (210). Genetic studies in mice indicate redundant and differential roles of MRFs in myogenesis. Mice lacking Myf5 or MyoD have no major defects in muscle development. Myf5-null mice have normal skeletal muscle morphology and muscle-specific gene expression, while the appearance of myotome cells is delayed until MyoD is expressed (41). Similarly, myogenesis in Myf5-null mice is fully restored by a MyoD-expressing lineage (97, 105). MyoD deletion results in prolonged and elevated Myf5 expression, which functionally compensates for MyoD and leads to normal skeletal musculature (236). Interestingly, Myf5/MyoD double-null mice are completely absent of skeletal myoblasts or myofibers as well as myogenin expression (237). These observations indicate that Myf5 and MyoD play partially redundant roles in myogenic cell fate determination and myoblast commitment. Myogenin-mutant mice have severe defects in muscle fiber formation with reduced muscle-specific gene expression such as myosin heavy chain and MRF4. However, Myf5 and MyoD expression appears normal, and mononucleated myoblasts are observed in the limbs (110, 191), suggesting that myogenin is essential for committed myoblast differentiation and acts downstream of Myf5 and MyoD. Consistent with its biphasic expression pattern, MRF4 has a dual role in muscle development. Functional

MRF4 presence supports myotome differentiation in the Myf5/MyoD double-null mice (134), indicating a potential myogenic determination role that MRF4 plays as Myf5 and MyoD. $MRF4^{-/-}$ mice have normal skeletal muscle development and demonstrate strong myogenin up-regulation, which may compensate for the absence of MRF4 (304). However, MyoD/MRF4 double mutations result in a severe muscle deficiency that is similar to the myogenin-mutant mice (225). Thus, this upregulation of myogenin expression was insufficient to induce myogenesis, suggesting that MRF4 and MyoD have overlapping functions in myoblast differentiation. Taken together, these studies reveal a hierarchical relationship between MRFs whereby Myf5 locates at the top of the hierarchy and collaborates with MyoD in a redundant fashion to specify myoblasts, while myogenin and MRF4 act genetically downstream to induce myoblast differentiation and muscle-specific gene expression (Fig 1.1).

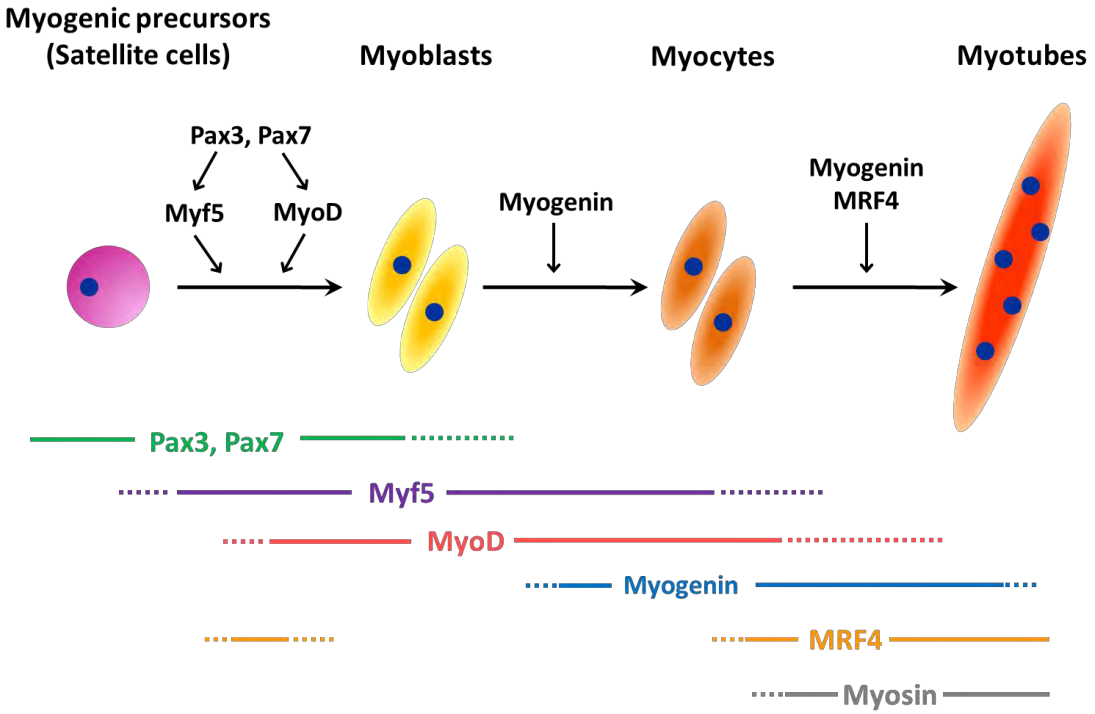

Figure 1.1 **Schematic representation of skeletal myogenesis and transcriptional hierarchy during myogenic process.** Myoblast commitment is marked by the onset of Myf5 and MyoD expression that is regulated by Pax3 and Pax7, which are master regulators of early lineage specification in muscle precursor cells. Myogenin expression further commits myoblasts to the myogenic program. Differentiating myogenin-positive myocytes align and fuse to form nascent myotubes. MRF4 is further required for the mature, multinucleated myotube formation. Myosin is a typical muscle structural protein that marks sarcomeric assembly in myotubes at the late stages of myogenesis. Satellite cell-mediated regenerative myogenesis is highly similar to developmental myogenesis with comparable myogenic process and transcriptional hierarchy.

Interaction of MRFs with transcriptional cofactors

Numerous studies in muscle cell lines and animal models indicate that skeletal myotube formation and myogenic gene expression requires cooperation among MRFs and other molecules. During myogenesis, MRFs function as heterodimers with E proteins (E12, E47 and HEB), which are proteins that also belong to the bHLH transcription factor family and bind to E boxes in many muscle-specific gene promoters (148). In proliferating myoblasts, active Myf5/E protein or MyoD/E protein heterodimers are disrupted by the HLH protein Id (inhibitor of differentiation), which can form complexes with E proteins or MRFs through HLH domain interactions. Id proteins lack the basic DNA binding domain; thus, Id/E or Id/MRF heterodimers fail to bind E boxes in muscle promoters (53, 125). Id protein levels are decreased at differentiation onset (125), allowing for functional Myf5/E protein or MyoD/E protein heterodimer formation, and thus myogenic gene expression.

Full activation of muscle-specific gene expression by MRFs requires their collaboration with myocyte enhancer factor 2 (MEF2) proteins (MEF2A-D), which belong to the MADS (MCM1, agamous, deficiens, SRF) family of transcription factors (25). MEF2 proteins cannot activate muscle-specific genes on their own, but they potentiate the transcriptional activity of MRFs by interacting with the MRF/E protein complexes (181-182). Consistently, the A/T rich sequence for MEF2 binding is often close to the E-box sequences within muscle genes (99). In addition to activating muscle structural genes, MEF2 proteins mediate myogenic bHLH gene expression in a positive feedback mechanism. Upstream signals activate Myf5 and MyoD, which cooperate with MEF2

proteins to induce myogenin expression (127, 298). Myogenin up-regulates MEF2 (63), which not only acts on the myogenin promoter to amplify gene expression (81), but also autoregulates its own promoter (276). Moreover, MRF4 expression requires synergistic function between MEF2 and MRFs such as myogenin (24, 192). All of these auto- and cross-regulatory interactions provide an elaborate transcriptional circuit to modulate myogenic differentiation. During mouse skeletal muscle development, Mef2c is the first member of the MEF2 family to be expressed followed by Mef2a and Mef2d (82). Mef2a or Mef2d homozygous mutant mice display no muscle developmental defects (216-217). However, skeletal muscle-specific Mef2c deletion resulted in disorganized myofibers, disrupted muscle structural gene expression and perinatal lethality, although embryonic and fetal myogenesis appear to be normal (216), indicating that MEF2C is required for the skeletal muscle postnatal maturation, but not early development. Notably, forced active MEF2 expression in mouse skeletal muscle did not trigger premature myogenic differentiation (217), suggesting that MEF2 was not sufficient for this process.

Muscle-specific gene sequences are occupied by nucleosomes that consist of 146 DNA base pairs that are wrapped around a histone octamer in cellular chromatin. The condensed nucleosomal organization prevents access of transcription factors including MRFs and MEF2 proteins to the regulatory regions of these genes, resulting in transcriptional repression. Therefore, chromatin modification and remodeling are required to relax the chromatin and allow for transcription factor access.

One specific chromatin modification is acetylation, which loosens histone-DNA interactions. Several transcriptional co-activators in muscle including p300, a functional homolog of CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF) have intrinsic histone acetyltransferase (HAT) activity; conversely, histone deacetylases (HDACs) repress transcription in skeletal muscle (174). MRFs and MEFs interact with HATs and HDACs. p300 stimulates muscle-specific gene expression by interacting with MyoD to potentiate its transcriptional activity in human and mouse muscle cell lines (220, 239, 300). This enhanced MyoD-mediated gene transcription is not only because of chromatin acetylation and relaxation, but also because of direct acetylation at evolutionarily-conserved lysines on MyoD by p300 and PCAF (80, 215, 240). MyoD mutants that cannot be acetylated display impaired DNA target affinity, transcriptional activity and differentiation potential *in vitro* (215, 240). Mice with knock-in acetylation-deficient MyoD mutations phenocopy $MyoD^{-/-}$ mice and display delayed myogenesis (80). p300 also interacts with MEF2 and acetylates it in skeletal muscle, resulting in enhanced DNA binding ability, transcriptional activity and myogenic differentiation (164, 239). Conversely, HDACs inhibit muscle-specific gene expression and myogenic differentiation by interacting with MyoD and MEF2 (162, 168, 221). Moreover, MEF2C activates HDAC9 expression, providing a negative feedback mechanism to prevent excessive MEF2 activation (104).

In addition to acetylation, chromatin remodeling is modulated by factors that loosen histone-DNA interactions using energy from ATP hydrolysis. The SWI/SNF (switching/sucrose non-fementing) complex is an important chromatin-remodeling

enzyme, which consists of an ATPase subunit brahma-related gene 1 (BRG1) and BRG1-associated factor BAF. Several studies have demonstrated that the SWI/SNF complex plays a crucial role in MyoD and myogenin-mediated muscle gene activation and myogenic differentiation (70, 198-199). On muscle gene regulatory elements, MyoD interacts with SWI/SNF subunit BAF60c, which recruits the catalytic subunit BRG1 to form a functional SWI/SNF complex in differentiating muscle cells, thereby facilitating chromatin remodeling and MyoD-targeted gene expression (90).

Regulation of Myf5 Expression and Activity

Transcriptional Control

A complex interplay of developmental signals from various embryonic tissues accounts for Myf5 activation during skeletal muscle development. The Myf5 transcriptional regulatory region contains a large number of enhancer elements, which span 140 kb upstream of the Myf5 start site (42, 91). Sonic hedgehog (Shh), a signaling molecule that is secreted from the notochord and floor plate, induces Myf5 transcription through a Gli binding site in the epaxial enhancer in somite epaxial muscle progenitors, resulting in muscle cell lineage specification (30, 103). Myf5 is also a direct target of canonical Wnt signaling, in which activated β-catenin binds to the Tcf/Lef sequences immediately proximal to the Myf5 early epaxial enhancer and induces Myf5 expression spatiotemporally (29). Mice that are deficient for Wnt1 and Wnt3a, which are secreted from the dorsal neural tube and activate the Wnt/β-catenin signaling redundantly display decreased Myf5 expression and a lack of a medial dermomyotome compartment (122). In

mouse myogenic C2C12 cell lines, a Wnt/β-catenin signaling activator R-spondin2 (RSPO2) up-regulates Myf5 expression and induces myogenic differentiation through the canonical Wnt pathway (107). Consistently, Myf5 mRNA levels are dramatically reduced in RSPO2-null mouse embryo limbs (107). In contrast to the Shh and Wnt-mediated Myf5 up-regulation, bone morphogenetic protein (BMP), which is expressed in the lateral plate mesoderm, inhibits Myf5 expression in Pax3-expressing dermomyotomal cells (218, 230), and Noggin, a BMP antagonist that is up-regulated by Shh and Wnt (116, 172, 230) enhances Myf5 transcription (230).

Pax3 acts genetically upstream of Myf5 during hypaxial myogenesis (13). Myf5 expression is directly modulated by Pax3 through the limb bud enhancer, which is a 145 bp regulatory element that contains a Pax3 consensus site (13). Pax3 also activates Myf5 expression indirectly through up-regulating doublesex and mab-3 related transcription factor 2 (Dmrt2), which induces Myf5 expression by binding to the early epaxial Myf5 enhancer in the dermomyotome, leading to the myogenic lineage determination (242).

Several independent studies have suggested that other factors are involved in Myf5 transcriptional activation. As mentioned above, muscle gene expression requires HAT-mediated chromatin modification. Roth JF *et al*. demonstrated that p300 protein is essential for Myf5 induction and myogenesis in mouse and embryonic stem (ES) cells (234). In that study, Myf5 expression was severely compromised in mouse embryos lacking p300. p300-null ES cells also failed to induce Myf5 and MyoD expression (234). In the C2C12 cultured myogenic cell line, myoblast Myf5 expression is positively

regulated by the glucocorticoid receptor and AP-1 transcription factors, while protein kinase C (PKC) inhibits Myf5 transcription in these cells (10). In mononucleated muscle reserve cells, which are non-dividing satellite cell-like cells in myotube cultures, activation of calcineurin or its downstream target nuclear factor of activated T cells (NFAT) is necessary and sufficient to induce Myf5 transcription (95). However, the calcineurin- and NFAT-dependent Myf5 up-regulation is not detected in proliferating myoblast cultures, indicating that Myf5 is modulated by different pathways in distinct cell populations (95). This observation is consistent with the in vivo discovery that distinct signals differentially regulate Myf5 expression in various locations in the embryos.

Post-transcriptional Control

Myf5 is post-transcriptionally regulated through translation inhibition by microRNAs. Satellite cells remain quiescent in healthy adult skeletal muscles and express Myf5 and MyoD upon differentiation towards the myogenic lineage during muscle regeneration. The majority of quiescent satellite cells transcribe the Myf5 gene, which remains untranslated because of miRNA-31-dependent sequestration of the transcripts in messenger ribonuleoprotein (mRNP) complexes. Once satellite cells are activated, the mRNP granules are dissociated with reduced miRNA-31 binding to the gene, resulting in Myf5 protein accumulation and thereby myogenic differentiation initiation (62).

Post-translational modifications modulate Myf5 abundance and transcriptional activity. Myf5 undergoes cell cycle-dependent proteolysis in proliferating myoblasts and non-

muscle cells such as *Xenopus* eggs (78, 156). The mitotic stability of Myf5 protein is controlled by its phosphorylation status with a strong correlation detected between Myf5 degradation and its extensive phosphorylation, while hyperphosphorylated Myf5 is stable (78). Multiple kinases such as cyclin-dependent kinase 1 (CDK1) and ERK as well as the phosphatase calcineurin are involved in the phosphorylation-dependent increase in Myf5 stability (78). Myf5 is phosphorylated on serine49 and serine133 by protein kinase CK2 *in vitro* (179). Mutants that cannot be phosphorylated fail to activate E-box-dependent reporter genes and act as dominant repressors of wild-type Myf5 (179), indicating that CK2-mediated Myf5 phosphorylation is essential for its transcriptional activity. One study by Winter B *et al*. revealed that Myf5 activity is suppressed by cAMP-dependent protein kinase (PKA) in the cAMP-induced inhibition of myogenic differentiation (288). Although PKA phosphorylates Myf5 *in vitro*, it does not affect Myf5 DNA binding ability, suggesting a potential mechanism by which phosphorylated Myf5 acts downstream of DNA binding to regulate transcriptional target gene activation (288).

Role of IGF signaling in skeletal muscle differentiation and hypertrophy

Insulin-like growth factor (IGF) signaling regulates many aspects of survival, growth, differentiation and metabolism in a wide range of cell types and mammalian tissues. The pathway is activated by the binding of IGF isoforms IGF1 or IGF2 to the IGF receptor (IGFR), which contains a tyrosine kinase domain that is activated by autophosphorylation upon ligand binding. Two primary signaling pathways have been proposed to be

associated with IGFR activation. One pathway involves the Ras-Raf-MEK-ERK module, which will be described in the following section. Alternatively, activated IGFR provides docking sites for insulin receptor substrate (IRS) proteins that recruit and activate phosphatidylinositol-3-kinase (PI3K) once phosphorylated by IGFR. PI3K phosphorylates membrane phospholipids, generating phosphatidylinositol (3,4)-bisphosphate ($PI(3,4)P_2$) and phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$), which recruits and localizes phosphoinositide-dependent kinase (PDK) and Akt to the inner plasma membrane surface. Co-localization of these two kinases allows PDK to phosphorylate Akt at multiple sites, resulting in Akt activation (150). Akt substrates include glycogen synthase kinase 3β (GSK3β), class O of forkhead box transcription factors (FoxO) and Bcl2-associated agonist of cell death (BAD), which are involved in protein synthesis, protein degradation and apoptosis (171). Akt also stimulates protein synthesis by signaling to mammalian target of rapamycin (mTOR) (147) (Fig 1.2).

Both IGF1 and IGF2 promote myoblast proliferation and differentiation in multiple muscle cell lines (84, 89, 251, 268). Myoblasts begin to actively express IGF2 upon serum withdrawal, and autocrine IGF2 secretion is crucial for myogenic differentiation (89). Forced IGF1 expression in mouse skeletal muscle resulted in muscular hypertrophy and preserved its regenerative capacity during aging (15, 56, 88, 188). Conversely, IGF1 or IGF1 receptor deletion caused dramatic muscle hypoplasia and severe growth retardation, and most of these null mice died perinatally (14, 158, 219). Similarly, skeletal muscle-specific IGF1R deletion or dominant negative IGF1R expression in mice impaired muscle development and regeneration (87, 113, 173).

IGF regulates mitogenesis and myogenesis through distinct signaling pathways. IGF-induced ERK activation is mainly responsible for its mitogenic action, although the IGF-Akt axis may be critical for proliferation as well (112, 273). The myogenic and hypertrophy-promoting action of IGF has been proposed to act mainly through IGF-Akt signaling and IGF-calcineurin signaling (Fig 1.2). In the following sections, these two signaling cascades will be dissected, and the components of each pathway will be discussed for their roles in myogenic differentiation.

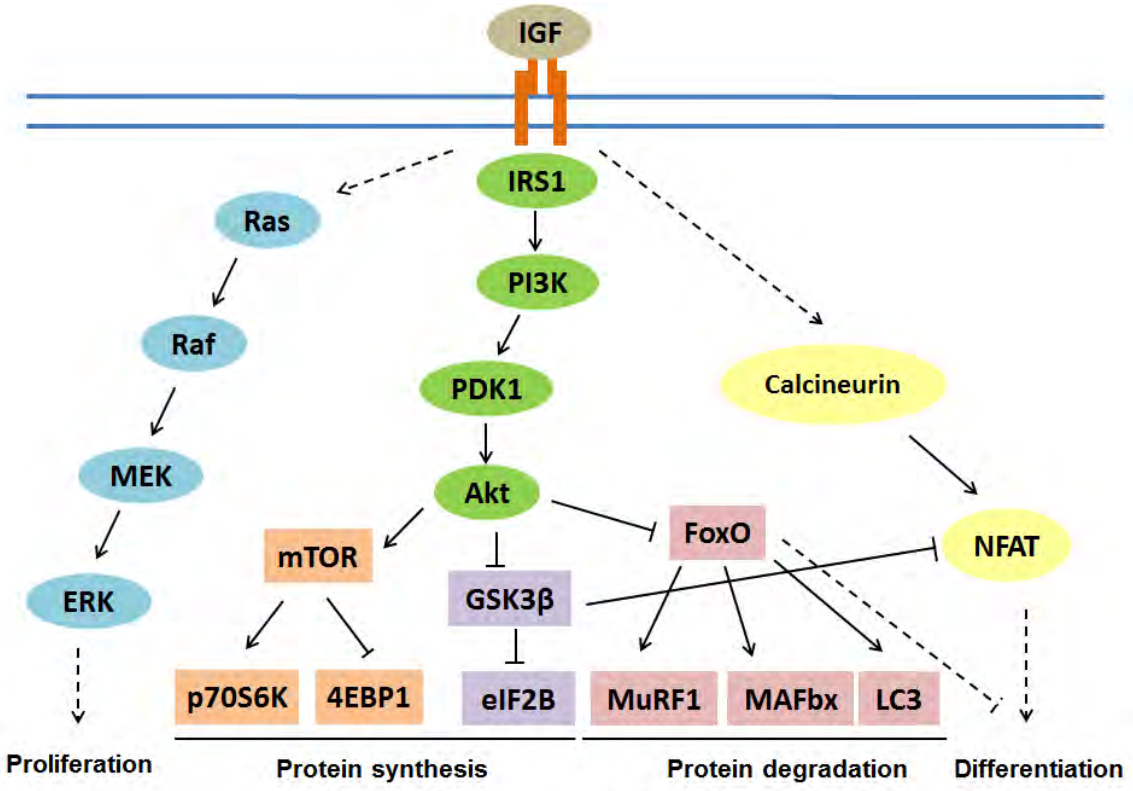

Figure 1.2 **Schematic displaying the main components in IGF-mediated signaling pathways that regulate skeletal muscle growth.** Solid line indicates direct modification and dotted line indicates indirect regulation.

Regulation of skeletal muscle growth by IGF-Akt signaling

IRS, PI3K and PDK1

As mentioned above, receptor-bound IGF signals through IRS and its downstream targets PI3K and PDK1 to activate Akt. IRS1 disruption retards growth in mice (8), while IRS2 deficiency leads to nearly normal growth but severe insulin resistance (289). Numerous studies have identified PI3K as a crucial mediator in myogenesis because IGF-induced myotube formation was prevented by PI3K inhibitor treatment, whereas constitutively active PI3K expression enhanced myogenic differentiation in the absence of IGF1 in muscle cell lines (126, 131, 190, 268). However, skeletal muscle-specific PI3K inactivation by p85 regulatory subunit deletion resulted in normal muscle size (163), as did the muscle-specific PDK1 knockout (185).

Akt

Akt is the key node in the IGF signaling cascade and mediates multiple downstream effectors to regulate skeletal muscle differentiation and hypertrophy. Three Akt isoforms have been identified, of which Akt1 and Akt2 are ubiquitously expressed in various tissues and Akt3 is detected in testis and brain. Evidence from an array of studies suggests that Akt1 and Akt2 play distinct roles during myogenesis. Akt1 levels remain constant during differentiation of several muscle cell lines, whereas Akt2 expression is up-regulated during this process (133, 235, 273). Akt1 and Akt2 are distributed throughout both the cytoplasm and nuclei in proliferating myoblasts. In contrast, Akt2 translocates to myotube nuclei, whereas Akt1 is only detected in the cytoplasm (273).

Consistent with these observations, Akt1 appears dispensable for myogenesis but is required for proliferation (112, 114, 273), while Akt2 promotes myogenic differentiation (114, 133, 258, 273). A positive feedback regulation loop has been reported between Akt2 and MyoD-MEF2. Akt2 activated MyoD-MEF2 transcriptional activity and enhanced myogenin expression during C2C12 differentiation. Meanwhile, MyoD induced Akt2 transcription by binding to multiple E-box sites on the Akt2 promoter (133). Akt2 can also phosphorylate p300 and promote the association of MyoD with p300 and PCAF acetyltransferases, resulting in a conformational change in the chromatin allowing access of MyoD to muscle-specific gene promoters (246). Moreover, prohibitin2/repressor of estrogen activator (PHB2/REA), a transcriptional repressor of myogenesis has been found to interact with Akt2, which down-regulates REA during muscle differentiation (114). Of note, Rotwein's group discovered that selective elimination of Akt1 had no effect on proliferation but inhibited differentiation by impairing MyoD transcriptional activity. However, Akt2-deficient myoblasts differentiated normally and only appeared to have a moderate defect in maturation (235, 286). The selective control of skeletal muscle differentiation by Akt1 has been partially supported by the retarded growth and increased apoptosis phenotype in Akt1 null mice (49, 51). In contrast, Akt2 disruption in micee caused no growth defect but impaired glucose metabolism and insulin resistance (92). In adult mouse skeletal muscle, Akt activation promotes dramatic hypertrophy and prevents denervation-induced atrophy (23, 27, 146), indicating an additional manner by which Akt regulates skeletal muscle growth.

mTOR

One of the best-studied downstream targets of Akt is mTOR, which is a master regulator of cell growth and proliferation. Akt can directly phosphorylate and activate mTOR, or can indirectly activate mTOR by phosphorylating and inhibiting the tuberous sclerosis complex (TSC) protein 1/2 complex. TSC 1/2 acts as a GTPase activating protein (GAP) to suppress the small G protein Ras homolog enriched in brain (Rheb) that activates mTOR signaling. mTOR forms two functionally and structurally distinct multiprotein complexes, the rapamycin-sensitive, Raptor-bound mTOR complex 1 (mTORC1) and the rapamycin-insensitive, Rictor-bound mTOR complex 2 (mTORC2). A major role of mTORC1 is to control protein synthesis through modulating two well-characterized substrates p70 ribosomal S6 kinase 1 (p70S6K1) and eIF4E binding protein 1 (4EBP1). mTORC1 activates p70S6K1, which phosphorylates the small ribosomal subunit S6 to initiate efficient translation of mRNAs that contain 5'-terminal oligopyrimidine tracts. 4EBP1 phosphorylation by mTORC1 releases its inhibitory binding to eIF4E and allows 5'-cap-dependent translation initiation. mTORC2 signals to actin organization through PKC and Rho family GTPases RhoA and Rac1. mTORC2 also phosphorylates and activates Akt (147, 280).

An essential role of mTOR has been implicated for both skeletal muscle differentiation and hypertrophy (85-86, 207-208, 248). Interestingly, the myogenic function of mTOR during early differentiation does not require its kinase activity and involves neither of the downstream effectors p70S6K1 or 4EBP1 (85). Instead, mTOR regulates myogenesis by inducing IGF2 transcription by a kinase-independent mechanism (86). During skeletal myotube maturation, however, mTOR catalytic activity is crucial for the secretion of

unidentified factors that are required for myoblast-myotube fusion (207). IGF1-induced myotube hypertrophy also demands mTOR kinase activity and employs p70S6K1 as a downstream effector (208). A specific role of mTORC2 signaling in myogenic differentiation has been implied in a study where Rictor but not Raptor suppression reduced Akt phosphorylation and prevented C2C12 myoblast fusion (247). However, mouse skeletal muscles lacking Rictor are distinguishable from wild type controls with normal muscle size, while Raptor-deficient muscles progressively develop dystrophy (20). Exacerbated myopathic features were observed in skeletal muscle-specific mTOR depleted mice, probably because mTOR but not Raptor deficiency resulted in reduced dystrophin content, which is a protein complex that is essential for normal muscle structure and function (231). As a downstream mTOR effector, p70S6K1 depletion reduced muscle fiber size with unaltered myonuclei number, suggesting an atrophy phenotype (197).

GSK3β

Another effector that is involved in IGF-induced myogenic differentiation is GSK3β. Once inactivated by Akt phosphorylation at Ser9, GSK3β stimulates myoblast differentiation and fusion and increases myogenic gene expression (205, 232, 271). One factor that is regulated by GSK3β is NFATc3, which translocates into the nucleus and up-regulates myogenic genes in the absence of GSK3β-mediated phosphorylation (205, 272, 274). GSK3β inactivation by inhibitors such as LiCl also enhances NFATc3 transcriptional activity and muscle-specific gene expression without affecting Akt

phosphorylation (205, 271-272), indicating that inhibiting GSK3β activity is sufficient to stimulate muscle differentiation independently of IGF.

Canonical wnt/β-catenin signaling is another pathway that regulates myogenesis in a GSK3β-dependent manner. Under basal conditions, GSK3β and other proteins form a protein complex in which phosphorylated β-catenin is targeted for degradation by the ubiquitin-proteasome pathway. In the presence of wnt signals, the degradation complex is inactivated and GSK3β can no longer phosphorylate β-catenin, resulting in protein accumulation and its subsequent translocation into nucleus. Cytoplasmic β-catenin co-localizes with cadherins at cell-cell contact sites and is essential for myoblast fusion and late stages of myogenic differentiation (124, 143, 186). Nuclear β-catenin binds to TCF/LEF transcription factors and induces expression of target genes such as Myf5 (107, 261). During mouse embryogenesis, canonical wnt/β-catenin signaling is associated with Myf5 expression and dermomyotome formation (29, 122).

GSK3β has been implicated in IGF-induced skeletal muscle hypertrophy (233, 262). Ectopic expression of a dominant negative GSK3β causes dramatic hypertrophy in skeletal myotubes (233), probably by preventing the inhibitory phosphorylation on eukaryotic initiation factor 2B (eIF2B), which promotes translation initiation and protein synthesis (75, 282). A recent study suggested a novel mechanism by which GSK3β promotes muscle growth. GSK3β phosphorylates neublin on the Z bands of myofibrils, thus preventing its interaction with neuronal Wiscott-Aldrich syndrome protein (N-WASP), which is an essential factor for actin assembly. IGF-induced GSK3β inactivation

recruits N-WASP to the unphosphorylated neublin and promotes actin nucleation and actin filament elongation. Because myofiber hypertrophy is associated with enhanced myofibrillogenesis, it is proposed that IGF-Akt signaling regulates muscle maturation and hypertrophy through the GSK3β-neublin-N-WASP pathway (262).

FoxO

FoxO transcription factors are Akt substrates that play roles in both IGF-induced myoblast proliferation and differentiation. Akt phosphorylates FoxO1 at Ser256 in response to IGF1 and prevents its nuclear translocation. In myoblasts, FoxO1 inactivation reduces CDK inhibitor p27 expression, thus enhancing cell proliferation (166). During myogenic differentiation, however, autocrine IGF2 activates Akt, which phosphorylates FoxO and allows differentiation to occur (120), suggesting an inhibitory effect of FoxO in myogenesis. Consistently, FoxO1 loss of function partially rescued Notch-mediated inhibition of myogenic differentiation and increased MyoD expression by down-regulating Hes1 (140), which suppresses myogenesis by inhibiting MyoD transcription (145). Of note, one study by Bois *et al*. suggested that FoxO1a promoted primary myoblast fusion by up-regulating genes involved in cell fusion and extracellular matrix remodeling, indicating a positive role of FoxO1 in myogenesis (28). Interestingly, in this study, FoxO1 regulation appeared to be independent of PI3K/Akt activity, thus providing a potential explanation for the controversies between this study and several others.

In addition to controlling myogenic differentiation, FoxO transcription factors are major modulators of the muscle atrophy program. FoxO1 activates transcription of myostatin,

which is a powerful inducer of muscle atrophy in differentiated myotubes *in vitro* (2). Transgenic mice specifically overexpressing FoxO1 in skeletal muscle have reduced muscle mass and enhanced lysosomal proteinase cathepsin L expression (132). Another member of the FoxO family, FoxO3, functions as a muscle atrophy inducer by acting on two major protein degradation pathways, the proteasomal and autophagic-lysosomal pathways. Evidence for FoxO3-dependent activation of the ubiquitin-proteasome pathway is from the discovery that ubiquitin ligases muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1), which are required for skeletal muscle atrophy (26, 54-55) are transcriptional targets of FoxO3 (238, 252). FoxO3 is essential for up-regulation of autophagy-related genes such as LC3 (169) and is sufficient to induce autophagy in skeletal muscle (306), resulting in protein clearance via the autophagic-lysosomal pathway.

Regulation of skeletal muscle growth by IGF-calcineurin signaling

The calcium-dependent phosphatase calcineurin interacts with IGF signaling to regulate skeletal muscle differentiation and hypertrophy. Calcineurin is a serine/threonine protein phosphatase that is activated by calcium (94). Activated calcineurin dephosphorylates several substrates, including members of the nuclear factor of activated T cell (NFAT) transcription factor family, which translocate to the nucleus and activate tissue-specific genes (153). Conversely, NFATs can be deactivated and trapped in the cytoplasm through phosphorylation by protein kinases such as GSK3β, p38 MAPK and casein kinase (17, 296, 308).

Calcineurin and NFAT control skeletal muscle differentiation and hypertrophy. Forced expression of activated calcineurin enhances myotube formation (73), while calcineurin inhibition attenuates myogenesis with reduced myogenic gene expression (94). Calcineurin activity is also required for overload-induced muscle hypertrophy (79). However, mice that overexpress activated calcineurin in myotubes have normal muscle size compared with wild type controls (193), indicating that calcineurin is insufficient to induce skeletal muscle hypertrophy *in vivo*. There are three NFAT genes NFATc1, NFATc2 and NFATc3 that are expressed at distinct stages of myogenic differentiation (1). NFATc3 is mainly expressed in myoblasts and serves a specialized role in primary (embryonic) myogenesis when myoblasts differentiate and fuse into nascent myotubes (180). NFATc2 regulates myoblast-myotube fusion in secondary (fetal) myogenesis (117) by inducing the secretion of IL4, a fusion-stimulating cytokine that is produced by a subset of muscle cells (118). NFATc1 is usually found in nascent and mature myotubes, where it has been suggested to participate in the hypertrophic myotube formation (189, 245).

The sarcoplasmic reticulum (SR) stores calcium ions and releases them into the cytoplasm through L-type calcium channels, which are also known as dihydropyridine receptors (DHPR) when the muscle cell is stimulated. As a pore-conducting pathway for calcium ions, L-type calcium channels may affect the long-term intracellular calcium signaling (72). An array of studies have demonstrated that IGF activates L-type channels (72) and increases DHPR α1 subunit expression in skeletal muscle cells (229, 281), indicating a potential interaction between IGF and calcium-mediated calcineurin

signaling. Indeed, IGF1 induced skeletal muscle hypertrophy through IGF1-calcineurin-NFATc1 signaling in C2C12 and L6E9 muscle cell lines (189, 245). IGF1 or activated calcineurin induced the expression of transcription factor GATA binding protein 2 (GATA2), which cooperates with hypophosphorylated NFATc1 in myocyte nuclei to activate muscle-specific gene transcription (189). However, it should be noted that a potential inhibitory role of calcineurin signaling in muscle hypertrophy has been proposed by Glass's group, who showed that IGF1 and activated Akt antagonized calcineurin-mediated dephosphorylation and nuclear translocation of NFATc1 in C2C12 cells (233).

Role of MAPK signaling cascades in skeletal myogenesis

Skeletal muscle development is regulated by extracellular stimuli such as growth factors that transmit diverse signals into cells to affect the muscle transcription program. The mitogen-activated protein kinase signaling modules (MAPKs) play critical roles in the process. The MAPK family is categorized into four major groups: c-Jun N terminal kinase (JNK), extracellular signal-regulated protein kinase (ERK), p38 stress activated protein kinase and extracellular signal-regulated protein kinase 5 (ERK5), all of which have been demonstrated to function in mammalian skeletal myogenesis.

JNK signaling and myogenesis

The JNK signaling pathway is activated primarily by environmental stresses, inflammatory cytokines and growth factors. These stimuli trigger activation of MAP3Ks including apoptosis signal-regulating kinase (ASK), mixed-lineage kinase (MLK), MAP/ERK kinase kinase (MEKK) and transforming growth factor β activated kinase 1 (TAK1), which then activate MAP2K isoforms MKK4 and MKK7 that phosphorylate and activate JNK. The activated JNK/SAPKs tanslocate to the nucleus where they regulate the activity of multiple transcription factors that are involved in inflammation, differentiation, survival, apoptosis and cell migration, such as c-Jun, ATF2, Elk1, SMAD4, p53 and NFAT (284). Distinct genes encode for proteins in the JNK family; JNK1 and 2 are expressed in various tissues including skeletal muscle while JNK3 is specifically expressed in the brain, testis and pancreas (294).

The role of the JNK signaling pathway in skeletal myogenesis is quite controversial, which could be simply reflected by inconsistent assessments of JNK activity during myogenic differentiation between distinct studies. Several groups suggested that JNK is activated during C2C12 (64), L6E9 (138) and mouse primary myoblast differentiation (48) while others showed no alteration (48, 291) or a decrease during this process (212). The inhibitory role of JNK pathway in myogenesis has been demonstrated by treating L6 myoblasts with a JNK/p38 activator anisomycin or ectopic expression of upstream kinase MKK7, both of which resulted in impaired myogenin expression and a cytoplasmic redistribution of Myf5 in the cells (176). The JNK pathway also functions negatively in

stimuli-mediated muscle differentiation. Transforming growth factor β (TGFβ) inhibited myogenic differentiation via activation of Rho family proteins Rac1 and Cdc42Hs, which were determined earlier to suppress myogenesis by activating JNK pathway (175-176). TNFα promoted myoblast proliferation and inhibited differentiation through JNK1, but not JNK2 activation-induced expression of leukemia inhibitory factor (LIF), a negative mediator of muscle differentiation (3). By using a JNK interacting protein (JIP)-derived JNK peptide inhibitor, Strle K *et al*. showed that JNK activity is required for TNFα antagonism of insulin-like growth factor 1 (IGF1)-enhanced muscle growth and differentiation (253).

In contrast, basal JNK activity is indispensable for skeletal muscle differentiation. Inhibition of JNK activity by JNK inhibitor II dramatically impaired myotube formation of rat L6E9 muscle cells and induced apoptosis accompanied by increased c-Jun and p53 expression, indicating that JNK is essential for muscle cell survival and differentiation (138). Of note, JNK activity also appears insufficient to regulate myogenesis because forced JNK activation by overexpressing a constitutively active JNKK had no effect on myogenic reporter induction in both C2C12 cells and 10T1/2 fibroblasts that had been converted by myoD (291), which is consistent with the unaltered JNK activity that had been observed in the cells.

ERK signaling and myogenesis

ERK was first discovered as a serine/threonine kinase that phosphorylates microtubule-associated protein 2 (MAP2) in insulin-stimulated adipocytes and thus was named MAP2

kinase (MAP2K) (226). ERK was also known as MAPK, but the name was changed because MAPK stands for mitogen-activated protein kinase, which describes additional kinase groups. Boulton *et al*. redesignated MAPK2 as ERK1 in a DNA cloning study (34) and characterized two ERK1-related kinases as ERK2 and ERK3 (33). ERK1 and ERK2 form central components in ERK signaling cascade, which is responsive to various extracellular stimuli such as hormones, growth factors, chemokines, neurotransmitters and calcium ions to regulate survival, proliferation, differentiation, development, migration and malignant transformation. Despite the involvement of a wide variety of receptors and distinct stimuli, ERK signaling cascades usually include small GTP binding proteins activation such as Ras and Rap1 by guanine-nucleotide exchange factors (GEFs). GTP-bound Ras or Rap1 then activate the MAP3K Raf, which phosphorylates and activates the MAP/ERK kinase (MEK), which phosphorylates ERK1/2 (141).

During skeletal myogenesis, ERK1/2 activity has been described to be either increased (48, 100) or decreased (19, 285) with differentiation. Biphasic ERK1/2 activation was also observed; ERK1/2 kinase activity is high in undifferentiated myoblasts, is decreased with differentiation onset, and increases as differentiation proceeds (291). The potential high activities in myoblasts and myotubes imply a dual role of ERK1/2 in muscle proliferation and differentiation. Evidence from multiple muscle cell lines has demonstrated ERK signaling to be essential for myoblast growth. Serum and growth factors such as basic fibroblast growth factor (bFGF) and IGF1 activate ERK1/2 and stimulate the mitogenic response via an ERK1/2-dependent pathway in myoblasts (59, 129, 178, 285). ERK activity prevents cell cycle withdrawal during G1 (111) and ensures

the G1 to S phase transition during proliferation (129, 178), probably through up-regulation of cyclin D (19, 59) and inhibition of cyclin-dependent kinase inhibitor p21 expression (59).

Because cell cycle exit is required for myogenic differentiation (4), the critical role of ERK1/2 in growth factor-induced proliferation indicates an inhibitory effect on myogenesis. Indeed, the ERK1/2 signaling pathway has been suggested to impair muscle differentiation by reducing p21 transcription (291), inhibiting myoD expression and activation (142, 149, 214, 270), and preventing MEF2 nuclear accumulation (287). However, several studies have controversially revealed that ERK1/2 promotes myogenic differentiation (100, 154). This controversy was solved using a tetracycline-repressible system to overexpress MAPK phosphatase 1 (MKP1), which inactivates ERK1/2 (and p38 MAPK and JNK) by dephosphorylation. In this study, Bennett *et al*. identified ERK1/2 as a suppressor at the early stages of differentiation but an inducer of myoblast fusion and myotube formation at the later stages in C2C12 cells (19). The biphasic requirement for ERK1/2 during myogenesis has been also detected in an independent study where the activity of the ERK1/2 upstream activator MEK was targeted (291). The stimulatory effect of the ERK1/2 cascade in late differentiation and myocyte fusion is likely because of enhanced myoD expression and transcriptional activity (100), increased p21 expression (202, 291) and ERK1/2 substrate ribosomal S6 kinase 2 (RSK2)-induced nuclear factor of activated T cell c4 (NFATc4) activation (52). Of note, several studies have claimed that the ERK signaling module is dispensable for myogenesis because treatment of myoblasts with a MEK1 specific inhibitor did not affect the myotube

formation or rescue the FGF or oncogenic Ras-induced non-myogenic phenotype (64, 93, 285). The controversies among these studies may be explained by the distinct ERK signaling mediators that were targeted, different experimental conditions and methods, and the cell lines that were used.

p38 MAPK signaling and myogenesis

The p38 MAPK signaling pathway was initially identified to be activated by environmental stresses and pro-inflammatory cytokines. Numerous studies have since demonstrated additional functions of this signaling cascade in a wide range of cellular processes including cell cycle arrest, apoptosis, cell growth, cell mobility and differentiation of several cell types like adipocytes, neurons and myoblasts (137). Various extracellular cues such as oxidative stress, cytokines, hormones and growth factors stimulate diverse MAP3Ks including MEKK1-4, TAK1, ASK1/2, DLK, MLK2/3 and TAO1/2/3 that phosphorylate and activate MKK3, MKK4 or MKK6, which in turn phosphorylate and activate the p38 MAPKs. p38 MAPK substrates include transcription factors and protein kinases, several of which are involved in development and differentiation (161, 301).

An array of independent studies has demonstrated that the p38 MAPK signaling pathway is an essential mediator of skeletal myogenesis. P38 MAPK activity increases concomitantly with myogenic differentiation in several muscle cell lines. Deliberate activation of p38 MAPKs by ectopic expression of constitutively active MKK6 or MEKK1 enhanced myotube formation and muscle-specific gene expression in cultured

myoblasts (45, 256, 291), while impaired muscle differentiation was detected in cells after p38 MAPK-specific inhibitor treatment or inhibition of upstream activators such as MKK3 and TAK1 (45, 64, 256, 291-292, 302). During mouse embryonic development, p38 MAPK activity is induced in somites. Inhibition of p38 MAPK signaling using a pharmacological inhibitor attenuated myotomal myogenesis in somite explants and in embryos *in vivo* (69). Interference with the p38 MAPK signaling pathway during *Xenopus Laevis* development also caused myogenic defects, further demonstrating a conserved and crucial function of p38 signaling modules in skeletal muscle differentiation (136). Despite numerous literatures demonstrating the requirement for p38 MAPK activity in myogenesis, an inhibitory function for p38 MAPK signaling in this process has also been reported. In a study by Johnson's group, constitutive MEKK1 activation dramatically inhibited myotube formation in a p38 MAPK-dependent manner in MyoD-converted fibroblasts (203). A later study in C2C12 cells demonstrated that the inhibition of p38 MAPK activity at the late stages of muscle differentiation resulted in up-regulation of muscle-specific genes (256). Likewise, Weston *et al*. detected enhanced muscle formation by repressing p38 MAPK activity in primary limb mesenchyme cultures (283). There are four p38 MAPK isoforms: α, β, γ and δ. The p38α MAPK isoform is absolutely crucial for differentiation of primary myoblasts and cultured muscle cell lines, whereas a discrepancy exists for the role of other p38 MAPK isoforms in myogenic differentiation (151, 212, 277). By using p38 MAPK isoform-specific knockout mice and primary myoblasts that were isolated from them, Munoz-Canoves's group discovered that p38β MAPK and p38δ MAPK are dispensable for myogenesis, and

p38γ MAPK is only required for optimal cell fusion *in vitro,* but not necessary for *in vivo* muscle development (212). However, ectopic expression of p38γ MAPK or its inactive mutant in C2C12 cells enhanced or impaired myotube formation respectively (151), and silencing any of the isoforms with siRNA inhibited C2C12 differentiation (277), indicating that the model systems being used should be taken into consideration.

p38 MAPK regulates myogenic differentiation in multiple ways. First, it triggers cell cycle exit in muscle cell cultures, even in rhabdomyosarcoma cells (222), probably through CDK p21 induction (45, 64, 291, 302) and inhibition of cyclin expression as well as Rb phosphorylation (212-213). Of note, p38α/β MAPK was identified as a molecular switch to activate quiescent satellite cells, implying that p38 MAPK promotes proliferation during muscle regeneration. Second, the p38 pathway regulates the transcriptional activity of MRFs and MEF2 proteins. p38 MAPK stimulates MyoD-dependent gene transcription indirectly (291, 302). One possible mechanism is through MyoD heterodimerization with E47 protein, which is induced by p38 MAPK-mediated E47 phosphorylation on Ser140 (160), resulting in functional MyoD binding to muscle-specific gene promoters (148). However, MEKK1/p38 MAPK signaling may also disrupt MyoD/E47 association by phosphorylating E47 and abrogating myotube formation and muscle specific-gene transcription (203). p38 MAPK signaling may also activate MyoD through p38 MAPK-dependent regulation of MEF2 proteins, which associate with MyoD and potentiate its activity. Zester *et al*. demonstrated that p38 MAPK phosphorylated MEF2C to promote its transcriptional activity and a transcriptionally inactive MEF2C inhibited MyoD-dependent induction of an MCK reporter (302). Further studies have

revealed p38 MAPK mediated-phosphorylation of MEF2A and MEF2D, which enhances the transcriptional activities of the MEF2 proteins (291) and MyoD-induced late gene expression (211). Much evidence argued against the contribution of p38 MAPK-mediated MEF2 phosphorylation to MyoD function stimulation, suggesting that distinct mechanisms control MEF2 transcriptional activity, and that there is functional synergy between MyoD and MEF2 (196, 222, 291). MRF4 is another p38 MAPK substrate; however, its transcriptional activity is negatively regulated by p38 MAPK at the late stages of myogenesis, which results in selective repression of muscle-specific gene expression and inhibition of precocious myoblast fusion (256). p38 MAPK signaling also modulates myogenic differentiation through altering chromatin remodeling on myogenic loci. Puri's group demonstrated that the SWI/SNF (switching/sucrose non-fermenting) chromatin remodeling complex is recruited to the myogenin and MCK promoters in a p38 MAPK pathway-dependent manner, probably through phosphorylation of the SWI/SNF BAF60 subunit by p38 MAPKs (249). This group further demonstrated that TNFα-activated p38α MAPK promoted the interaction between YY1 and PRC2 (polycomb repressive complex 2), resulting in repressive chromatin formation on the Pax7 promoter, the down-regulation of which is required before differentiation occurs (204). One study in MyoD-converted fibroblasts indicated that p38 MAPK activity also facilitates RNA polymerase II recruitment and progression at late muscle-specific promoters, and thus enhances gene expression in a MyoD-mediated feed-forward circuit (211). In addition, the p38 MAPK pathway positively regulated Myf5 expression in *Xenopus* embryos to ensure normal muscle development (136).

Besides modulating myogenic machinery components directly, the p38 MAPK cascade also regulates muscle differentiation indirectly through crosstalk with other myogenic signaling pathways. A study supporting NFκB as an activator of muscle differentiation indicated that p38 MAPK induced NFκB transcriptional activity in C2C12 cells and increased IL6 expression, which is sufficient to promote myogenic differentiation (12). The p38 MAPK and ERK signaling pathways have also been suggested to antagonize each other because p38 MAPK activity inhibition enhanced ERK1/2 activation and vice versa in L6E9 muscle cells (139). This interaction could explain the opposite roles of p38 MAPK and ERK1/2 in cell cycle withdrawal and myoblast differentiation. Another interesting relationship between p38 MAPK and JNK pathways has been revealed in one study that demonstrated that continuous proliferation was caused by sustained JNK activation in primary p38α MAPK-deficient myoblasts (212). Furthermore, the p38 MAPK and PI3K/Akt/mTOR signaling pathways may crosstalk, which was suggested by the discovery that inhibiting any p38 MAPK or the mTOR kinase suppressed the activity of the other (64). It is further demonstrated in two independent studies that Akt2 is regulated by the p38 MAPK pathway at the protein and mRNA levels during C2C12 differentiation (44, 98). However, one study indicated that PI3K acted upstream of MKK6 to mediate p38 MAPK activation (98), while another reported reciprocal communication between the pathways (44).

ERK5 signaling and myogenesis

ERK5, also known as big MAP kinase 1 (BMK1), is the last identified MAPK member to date. ERK5 is activated by oxidative and osmotic stresses, certain inflammatory cytokines and various growth factors. The MAP3Ks MEKK2 and MEKK3 phosphorylate and activate MEK5, the sole upstream MAP2K that activates ERK5. A number of transcription factors are ERK5 substrates, including MEF2, c-Myc, c-Fos and Elk4. The conventional N-terminal kinase domain and the unique C-terminal transactivation domain of ERK5 enable it to maximally activate these transcription factors (195).

ERK5 is abundant in skeletal muscle and heart (152, 307). Both ERK5 and MEK5 null mice displayed defective limb bud development and are embryonic lethal (227, 279, 295). Dinev *et al*. first identified ERK5 as a positive regulator in myogenesis *in vitro* (77). In the study, ERK5 was activated upon differentiation induction in C2C12 cells. Selective ERK5 pathway activation enhanced MEF2C transactivation, myogenic gene expression and myotube formation, while ERK5 inhibition by antisense RNA resulted in a differentiation-defective phenotype, indicating that ERK5 is both required and sufficient for myogenesis. MEK5 and ERK5 have been also demonstrated to be essential mediators of the pro-myogenic action of IGFII, which activates ERK5 and promotes its nuclear translocation (46). A recent study revealed a critical role for ERK5 in muscle cell fusion, suggesting a MEK5-ERK5-Sp1-Klf2/4 signaling module that is essential for the fusion process (259). However, no alteration of p21, MyoD or MEF2 expression was observed in the study, inconsistent with previous observations (46, 77).

Map4k4

Mitogen activated protein kinase kinase kinase kinase (Map4k4) is a serine/threonine protein kinase that belongs to the germinal center kinase GCK-IV group of *Saccharomyces cerevisiae* sterile 20 protein (Ste20) kinases (74). Based on the location of kinase domains, Ste20 kinases are divided into two families, p21-activated kinases (PAK) and germinal center kinases (GCK), which are further categorized into two and eight subfamilies, respectively, according to their distinct structural features in the kinase domains and non-catalytic regions (65). Map4k4 contains a NH2-terminal kinase domain and a COOH-terminal regulatory domain, a feature of Ste20 kinases in the GCK family.

Map4k4 was first identified as a mammalian serine/threonine kinase that interacts with the SH3 domain of the receptor tyrosine kinase adaptor protein Nck and was termed Nck-interacting kinase (NIK) (254). Transient overexpression of Map4k4 (NIK) specifically activated the SAPK/JNK signaling pathway by interacting with MEKK1 through the regulatory domains of both proteins. The human Map4k4 ortholog, known as hepatocyte progenitor kinase-like/germinal center kinase-like kinase (HGK), was demonstrated later to activate a TAK1, MKK4/7, JNK cellular signaling cascade in 293T cells (297). Other studies have also suggested Map4k4 and its orthologs misshapen (msn) in *Drosophila melanogaster* and mig-15 in *Caenorabditis elegans*as as upstream activators of the JNK signaling pathway in various cell types and animal models to regulate morphogenesis, development and tumor growth (18, 157, 165, 206, 255).

Unlike Msn, which stimulates drosophila embryonic dorsal closure through a JNK signaling module (255), mouse Map4k4 plays an essential role in somite formation and presomitic mesoderm differentiation into dermomyotome in a JNK-independent manner. This was made evident by undisturbed mesodermal and somite development in JNK1- and JNK2-deficient mice (293). In this study, presomitic mesodermal cells in Map4k4 null embryos were observed to fail to migrate away from primitive streak, and it was further demonstrated by another study in which Map4k4 has been shown to act upstream of p38 MAPK to decrease E-cadherin protein expression and prevent its inhibitory effect in mesoderm migration during gastrulation (309). Further studies have identified Map4k4 as a promigratory kinase (58) and a modulator of cellular transformation, invasion and adhesion (106, 290). Consistently, its expression is associated with worse prognosis in various cancers (109, 155, 223).

A genetic screen revealed that Map4k4 positively regulates antigen-mediated T-cell responses and is involved in tumor necrosis factor α (TNFα) promoter activation in Jurkat cells (167). Silencing Map4k4 in macrophages *in vitro* attenuated lipopolysaccharide (LPS)-induced TNFα expression independently of MAP kinase and NFκB signaling pathways (7), which have been demonstrated to regulate TNFα production in macrophages (61, 267). More interestingly, Map4k4 suppression in macrophages *in vivo* protected mice from LPS-induced lethality by inhibiting TNFα and interleukin-1β (IL-1β) expression (7), suggesting that Map4k4 mediates cytokine production in immune cells.

A role for Map4k4 in metabolic regulation of cell function and insulin action has been discovered and investigated in recent years. Increased Map4k4 expression was observed in abdominal subcutaneous tissue from obese human individuals, correlating with a decreased number of preadipocytes that can undergo differentiation (123). Common variations in Map4k4 are associated with insulin resistance and pancreatic β cell dysfunction in humans (241), indicating a potential role for Map4k4 in diabetes pathogenesis. In an RNAi-based screen for regulators of adipocyte function, Map4k4 was discovered to inhibit peroxisome proliferator-activated receptor γ (PPARγ) and glucose transporter isoform 4 (GLUT4) expression, adipogenesis and insulin-stimulated glucose transport (263). The Map4k4-mediated PPARγ suppression was later elucidated to be translational downstream of the mammalian target of rapamycin (mTOR) signaling pathway (102). Furthermore, TNFα signaling, which down-regulates GLUT4 expression, is impaired upon Map4k4 silencing in cultured adipocytes, indicating that Map4k4 is required for optimal TNFα action (263). Interestingly, TNFα selectively stimulates Map4k4 expression by activating transcription factors c-Jun and ATF2 through TNFα receptor 1 (TNFR1) signaling cascades (266).

Map4k4 also functions in muscle to blunt insulin sensitivity. Map4k4 gene silencing in primary human skeletal muscle cells prevented TNFα-induced insulin resistance by impeding excessive activation of JNK and ERK signaling pathways as well as IRS-1 phosphorylation (36). In rat pancreatic β cells, TNFα inhibits glucose-stimulated insulin secretion by decreasing IRS-2 expression and glucose-induced phosphorylation of Akt, AS160, the insulin receptor and ERK. In these cells, Map4k4 activated p70S6K, JNK,

p38 MAPK, and NF-κB. Map4k4 depletion rescued the β cells from the detrimental effects of TNFα on insulin secretion and signaling as well as p70S6K and JNK activation, while p38 and NF-κB phosphorylation was unaffected (35).

Specific Aims

An array of studies has identified Map4k4 as an upstream regulator of members of MAPK family including p38 MAPK, JNK and ERK in multiple cell lines. Furthermore, Map4k4 was discovered to regulate PPARγ translation through modulating mTOR/4EBP1 signaling pathway in cultured adipocytes. Both MAPKs and mTOR have been demonstrated to play important roles in skeletal muscle differentiation. mTOR is also a component of IGF/Akt signaling, which promotes skeletal muscle development and hypertrophy though regulating multiple downstream effectors including mTOR, GSK3β and FoxO. Given the regulatory interactions between Map4k4 and MAPKs as well as mTOR, and the involvement of the latter proteins in skeletal myogenesis, I aimed to test whether Map4k4 regulates skeletal muscle differentiation as its identified downstream effectors do. To address this RNAi and adenovirus-mediated overexpression strategy were employed for an *in vitro* study in murine muscle cell line C2C12. The specific aims of this study were:

(1) To determine the role of Map4k4 in skeletal muscle differentiation.

(2) To identify the signaling pathways that Map4k4 may participate in to regulate the myogenic process.

CHAPTER II: Identification of Map4k4 as a novel suppressor of skeletal muscle

Disclaimer:

All experiments were performed by the author except for Figure 2.1A and Figure 2.3B which were done in collaboration with Shinya U. Amano. Figure 2.1C was done in collaboration with Rachel J. Roth Flach. Anil Chawla generated the construct for Myf5 overexpression. Dr. Diane L. Barber provided the adenoviruses expressing GFP, native Map4k4 and kinase-inactive Map4k4 mutant.

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Abstract

Myoblast differentiation into mature myotubes is a critical step in the development and repair of human skeletal muscle. Here we show that siRNA-based silencing of the Ste20-like mitogen-activated protein 4 kinase 4 (Map4k4) in C2C12 myoblasts markedly enhances expression of myogenic differentiation genes, myoblast fusion and myotube diameter. In contrast, adenovirus-mediated expression of native Map4k4 in C2C12 cells attenuates each of these processes, indicating Map4k4 is a negative regulator of myogenic differentiation and hypertrophy. Expressing a Map4k4 kinase-inactive mutant enhances myotube formation, suggesting kinase activity of Map4k4 is essential for its inhibition of muscle differentiation. Map4k4 regulation of myogenesis is unlikely to be mediated by classic MAPK signaling pathways because no significant difference in phosphorylation of ERK, p38 or JNK is observed in Map4k4-silenced cells. Furthermore, silencing of these other MAPKs does not result in a hypertrophic myotube phenotype as does Map4k4 depletion. Uniquely, Map4k4 silencing up-regulates the expression of myogenic regulatory factor Myf5, depletion of which inhibits myogenesis. Furthermore, Myf5 is required for enhancement of myotube formation in Map4k4-silenced cells, while Myf5 overexpression rescues Map4k4-mediated inhibition of myogenic differentiation. These results demonstrate that Map4k4 is a novel suppressor of skeletal muscle differentiation, acting through a Myf5-dependent mechanism.

Introduction

Skeletal muscle differentiation is a highly coordinated multistep process in which mononucleated myoblasts first withdraw from the cell cycle in response to extracelluar cues, differentiate into post-mitotic myocytes (early differentiation), and subsequently fuse into multinucleated myotubes (late differentiation) which finally bundle to form mature muscle fibers (terminal differentiation). This process is elaborately controlled by activation of Myf5, MyoD, myogenin and MRF4, four myogenic regulatory factors (MRFs) belonging to a family of basic helix-loop-helix transcription factors. During myogenesis, MRFs are activated and operate in concert with other transcriptional regulators such as myocyte enhancer factor 2 (MEF2) in a coordinated manner to regulate the transcription of muscle-specific genes including myosin heavy chain (MyHC) and muscle creatine kinase (MCK) (40, 181, 200). Previous studies have confirmed Myf5 and MyoD as muscle determination factors that are mainly expressed in undifferentiated myoblasts and differentiating myocytes, while myogenin is activated in early differentiation (21). Mrf4 has been shown to be transiently expressed during somitogenesis and later fiber maturation (115), playing roles in myogenic lineage commitment (134) as well as myoblast fusion and differentiation (256-257).

Mitogen-activated protein kinases (MAPKs) are components of serine/threonine protein kinase cascades that respond to extracellular stimuli and regulate essential cellular functions such as proliferation (305), differentiation (5-6, 32) and apoptosis (275). The MAPK family is categorized into three main groups: p38 stress activated protein kinase,

c-Jun N terminal kinase (JNK) and extracellular signal-regulated protein kinase (ERK) (128), all of which have been demonstrated to be involved in mammalian skeletal myogenesis (141, 161). The exploration of new upstream kinases that modulate the downstream effector MAPKs identified mitogen activated protein kinase kinase kinase kinase (MAP4K4), a serine/threonine protein kinase that belongs to the germinal center kinase GCK-IV group of *Saccharomyces cerevisiae* sterile 20 protein (Ste20) kinases (65). Map4k4 may activate the JNK signaling pathway in some cell types and mediate cancer cell proliferation, apoptosis and motility (58, 157). It was found to play an essential role in development (293) and it has been shown to be critical for mesoderm migration during gastrulation by acting upstream of p38 MAPK (309). In an RNAi-based screen for regulators of adipocyte function, we discovered that Map4k4 down-regulates expression of peroxisome proliferator-activated receptor γ (PPARγ), a transcription factor that is essential for adipocyte differentiation and function (263). Cell size, insulin-mediated glucose transport and triglyceride content were found to be significantly increased upon Map4k4 silencing in cultured adipocytes (102), indicating that Map4k4 is a negative regulator of insulin-stimulated lipogenesis and adipose hypertrophy.

More recently it was found that Map4k4 also functions in muscle to enhance insulin sensitivity (7). Map4k4 gene silencing in primary human skeletal muscle cells prevented tumor necrosis factor α (TNFα)-induced insulin resistance (36). However, the role of Map4k4 in skeletal myogenesis has not been addressed. In the present study we used C2C12 murine myoblasts to investigate the function of Map4k4 on myogenic processes.

We demonstrate that Map4k4 acts upstream of Myf5 as a negative regulator of skeletal muscle differentiation.

Materials and Methods

Molecular biology. Mouse Myf5 clone (accession number: NM_008656) was purchased from Open Biosystems. The coding regions of Myf5 gene was PCR-amplified and cloned into pCMV plasmid carrying three N-terminal hemaggutinin (HA) tags (pCMV-3HA) to create a N-terminally HA-tagged Myf5 construct. Primers used to amplify PCR fragments of Myf5 coding region were as follows: 5' fragment: CGATCGCCACGCGTATCTCGAGCTATGGACATGACGGACGGCTGCCAGTTCT CCCCT; 3' fragment: GCGTACGGATCCGTCGACTCATAATA CGTGATAGATAAGTCTGG AGCTGGAGGGTCC.

Cell culture and transfection. Mouse C2C12 myoblasts (American Type Culture Collection) were cultured in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO2. To induce differentiation, 95% confluent cells were placed in differentiation medium (DM) consisting of DMEM with 2% horse serum. Multinucleated myotubes were evident after 3 days of differentiation. For siRNA transfection, C2C12 myoblasts cultured in growth medium were transfected with 50 pmol siRNA using Lipofectatmine RNAiMAX (Invitrogen) according to the manufacturer's instruction for reverse transfection. Twenty-four hours later, cells were switched to DM and cultured for the indicated times before harvesting. To transfect siRNA in

differentiated myotubes, siRNA/endoporter complexes were used as described previously (265). Briefly, 50 pmol of siRNA was incubated with 2.5 nmol of endoporter (Gene Tools) in phosphate buffered saline (PBS) for 15 minutes and added to cells. All of the siRNA was purchased from Dharmacon (Lafayette, CO). For plasmid transfection, GM-cultured C2C12 myoblasts were transfected with 2 μg of plasmids using Lipofectatmine 2000 (Invitrogen) according to the manufacturer's instruction for reverse transfection. Twenty-four hours later, cells were switched to DM and cultured for the indicated times before harvesting.

Adenovirus infection. C2C12 myoblasts were grown until 90% confluence and were then infected with GFP control virus, Map4k4 wild type virus or Map4k4 D152N virus at the dose of 10^4 virus particles per cell for 18 hours in GM before differentiation. 72 hours post-differentiation, cells were fixed for immunofluorescence or harvested for western blotting. All the adenoviruses were gifts from Dr. Diane L. Barber (Department of Cell and Tissue Biology, University of California, San Francisco, CA)

Myotube analysis. Myotube nuclei were counted in approximately 100 randomly chosen MyHC-positive cells containing three or more nuclei. Myotubes were categorized into three groups (3-6 nuclei, 7-15 nuclei and more than 15 nuclei per myotube) and were expressed as a percentage of total myotube number. The fusion index was calculated as the ratio of nuclei in MyHC-positive myotubes to the total number of nuclei in the field in five random fields. To analyze myotube diameter, five fields were chosen randomly, and

three myotubes were measured per field. The average diameter per myotube was calculated as the mean of three measurements taken along the long axis of the myotube.

Isolation of mouse satellite cells. Satellite cells were isolated from 6-8 week old C57B6/J mice by FACS sorting as described previously (209)**.** Briefly, skeletal muscles were digested with collagenase B (10 mg/ml, Roche) and dispase II (2.4 U/ml, Roche), filtered through 250, 100 and 40 μm nylon meshes successively. The resulting mononuclear cells were incubated with anti-mouse Integrin α7 clone 3C12 (MBL) and biotin anti-mouse CD34 (eBioscience) antibodies for 20 minutes and then stained with hoechst 33258 (sigma), PE anti-mouse CD11b (eBiosciences), PE anti-mouse CD45 (eBiosciences), PE anti-mouse Ly-6A-E (Sca1, BD Bioscience), PE anti-rat CD31 (BD Bioscience), streptavidin-APC-Cy7 (BD Bioscience) antibodies. Integrin $\alpha7^{+}CD34^{+}CD31^{-}CD11b^{-}CD45^{-}Sca1^{-}$ cells were considered as satellite cells and sorted by BD FACS Aria II.

Western blotting. Cells were solubilized with ice-cold lysis buffer (20 mM HEPES, pH 7.2, 100 mM NaCl, 1mM EDTA, 100 mM PMSF, 0.01% Triton X-100, 1% SDS and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific)) and protein concentrations were assessed by BCA assay (Thermo Scientific). Equal amounts of protein were loaded on 8.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The following antibodies were used: anti-Map4k4 (Bethyl), anti-Myf5 (sc-20, Santa Cruz), anti-MyoD (BD biosciences), anti-Mef2C (Cell Signaling), myogenin F5D (Developmental Studies Hybridoma Bank (DSHB), University of Iowa), sarcomeric

myosin heavy chain (MHC) MF20 (DSHB, University of Iowa), anti-phospho-p38 (Cell Signaling), anti-total p38α (Cell Signaling), anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Cell Signaling), anti-p44/42 MAPK (ERK1/2) (Santa Cruz), anti-phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling) and anti-SAPK/ JNK (Cell Signaling).

Immunofluorescence microscopy. Cells grown on glass coverslips were fixed with 4% formaldehyde and blocked in PBS containing 2% goat serum (Invitrogen), 1% bovine serum albumin (Sigma), 0.1% Tween 20 and 0.05% Triton X-100 (American Bioanalytical) for 1 hour at room temperature. The cells were then incubated with MF20 mAb against MHC (1:40, DSHB) for 2.5 hours and subsequently with Alexa 488 or Alexa 594-conjugated secondary antibody (1:200, Invitrogen) for 1 hour at room temperature. Cells were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Images were obtained using a Zeiss Axiovert 200 inverted microscope equipped with a Zeiss AxioCam HR CCD camera.

EdU incorporation test. C2C12 cells were were incubated for 1 hour with 10 uM EdU (Invitrogen) before harvesting. Cells were washed once with 1% BSA and 0.09% NaN3 (FACS buffer) before fixation with Fixation/permeabilization buffer (eBioscience) and permeabilized with permeabilization buffer (eBioscience). EdU was chemically conjugated to Alexa 405 fluorophore according to the instructions of the manufacturer (Invitrogen). Sample data were acquired on a BD LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Creatine kinase activity assay. Cells were lysed in ice-cold lysis buffer (20 mM HEPES, pH 7.2, 100 mM NaCl, 1mM EDTA, 100 mM PMSF, 0.01% Triton X-100 and protease and phosphatase inhibitor cocktail). Lysates were centrifuged at 14000 × g for 10 min at 4°C and the supernatants were used immediately for creatine kinase (CK) activity assay. CK activity was measured using a spectrophotometric-based kit (Stanbio Laboratory, Boerne, TX, USA) according to the manufacturer's instructions. Specific CK activity was calculated by normalizing to total protein content.

Map4k4 kinase activity assay. Cells were solubilized with ice-cold lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% NP-40, 0.1% deoxcholic acid and protease and phosphatase inhibitor cocktail). Cell lysates were immunoprecipitated with anti-Map4k4 antibody (Bethyl). Myelin basic protein (MBP) (1 μg) and 10 μCi of [γ-^{32}P]-ATP were added into the immunoprecipitates and incubated for 30 min at 30°C in kinase buffer (20 mM HEPES, 10 nM $MgCl_2$, 1 mM DTT and protease and phosphatase inhibitor cocktail). Samples were separated by 12% SDS-PAGE and visualized by autoradiography. Map4k4 kinase activities were determined by normalizing the radioactivity of ^{32}P-labeled MBP to the amount of immunoprecipitated Map4k4 protein as detected by Western blot.

Isolation of RNA and Real Time PCR. RNA isolation was performed according to the Trizol Reagent Protocol (Invitrogen). cDNA was synthesized from 1.5 μg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules CA) according to the manufacturer's instructions. For real time PCR, synthesized cDNA and iQ SYBR Green

Supermix were run on the MyIQ Realtime PCR System (Bio-Rad) with following primer pairs: Map4k4 5'-CATCTCCAGGGAAATCCTCAGG-3', Map4k4 5'-TTCTGTAGTCGTAAGTGGCGTCTG-3'; Myf5 5'-TATGAAGGCTCCTGTATCCC-3', Myf5 5'-ACGTGCTCCTCATCGTCTG-3'. 36B4 was used as an internal loading control. Relative gene expression was determined using the ΔCt method (159).

Statistics. The statistical significance of the differences in the means of experimental groups was determined by two-tailed student's *t* test using Microsoft EXCEL. The data were presented as the means ± SEM. A p value of <0.05 was considered significant.

Results

Map4k4 expression and protein kinase activity are decreased during skeletal muscle differentiation.

To determine whether Map4k4 may play a role in muscle differentiation, we first examined Map4k4 expression in primary mouse satellite cells and mature muscle fibers. Adult satellite cells are considered to be progenitor cells of somitic origin in skeletal muscle development (101). Real-Time PCR (RT-PCR) analysis revealed a 4-fold decrease in Map4k4 expression in mouse quadriceps compared with purified satellite cells isolated by fluorescence-activated cell sorting from the same mice (Fig 2.1A). We also investigated Map4k4 expression during differentiation of C2C12 cells, a well-established cell line that is derived from mouse satellite cells and faithfully mimics skeletal muscle differentiation process *in vitro*. A reduction of Map4k4 protein levels was detected during C2C12 myogenic differentiation by Western blot, concomitant with the

increased expression of differentiation markers myogenin and myosin heavy chain (MyHC; Fig 2.1B). Furthermore, we measured Map4k4 protein kinase activity during C2C12 differentiation, determining it was highest in confluent myoblasts and decreased dramatically after 24 hours of differentiation (Fig 2.1C). These results indicated that Map4k4 is dynamically regulated during skeletal muscle differentiation, suggesting a potential role for Map4k4 in muscle development.

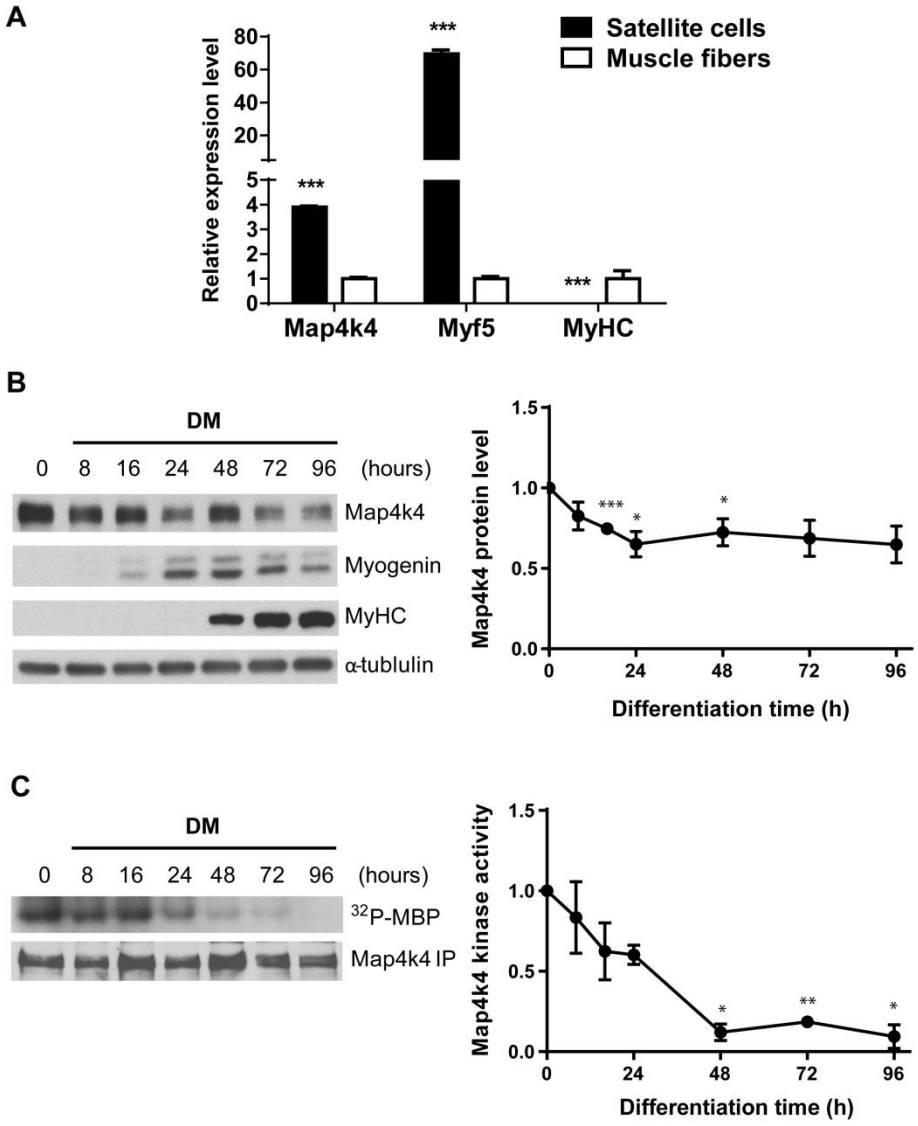


Figure 2.1 **Expression and kinase catalytic activity of Map4k4 during skeletal muscle differentiation**. (A) RT-PCR analysis of Map4k4 expression in isolated mouse satellite cells vs. quadriceps. Data represent mean ± SEM from three independent experiments. (B) Expression of Map4k4 during C2C12 myogenic differentiation. Densitometry was representative of mean ± SEM from four independent experiments. (C) C2C12 myoblasts were incubated in differentiation medium (DM) for the indicated times. Map4k4 kinase activity at each time point was assessed by kinase assay and immunoblotting. Results represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Map4k4 silencing promotes skeletal muscle differentiation.

To explore the function of Map4k4 in myogenic differentiation, we used siRNA directed against Map4k4 to deplete the protein kinase in C2C12 myoblasts and monitored morphological differences during cell differentiation. Map4k4 silencing resulted in significant sustained reduction of Map4k4 protein throughout differentiation and formation of larger myotubes after 48 hours in differentiation medium (DM) (Fig. 2.2A). Enhanced muscle cell fusion was observed in Map4k4-silenced cells on differentiation day 3, as there was a shift toward myotubes containing more nuclei per myotube (Fig. 2.2B) and an increased fusion index (Fig. 2.2C). Map4k4 silencing also resulted in a 70% increase of cell diameter in day 3 myotubes (Fig 2.2D), likely due to enhanced myoblast fusion. Similar nuclei numbers in random microscopic fields were detected in Map4k4-silenced myoblasts and myocytes (Fig 2.3A), excluding the possibility that the hyper-nucleated myotubes with increased size resulted from an increased number of undifferentiated myoblasts available for the fusion process.

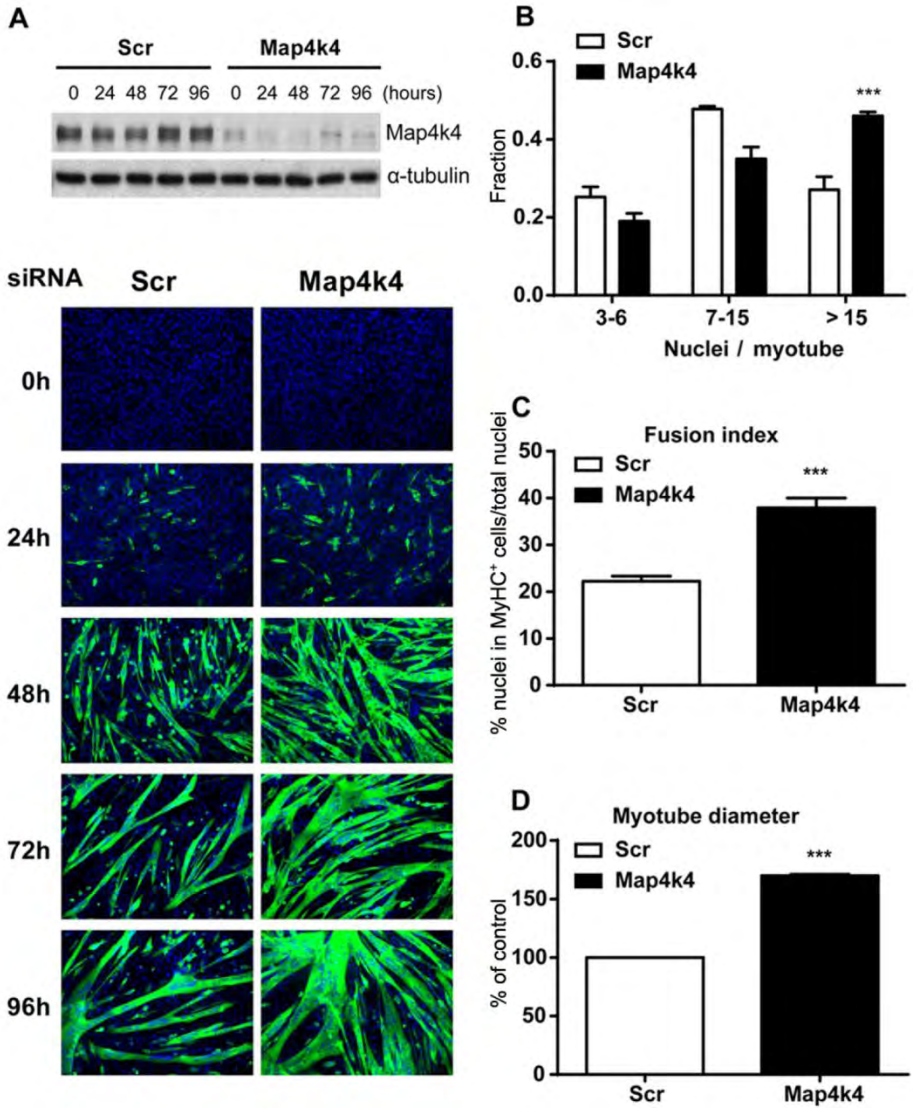


Figure 2.2 **Map4k4 silencing promotes myotube formation in C2C12 cells**. C2C12 myoblasts were transfected with scrambled siRNA or siRNA against Map4k4. 24 h later the cells were transferred DM for the indicated times. (A) Top panel, knockdown efficiency of Map4k4 was determined by immunoblot. Bottom panels, cells were fixed and immunostained for MyHC and myoblast differentiation was observed by fluorescence microscopy (green, MyHC; blue, DAPI, 100×). Data is representative of at least three independent experiments. (B) Fraction of myotubes with the indicated number of nuclei were quantified in 100 randomly chosen myotubes after 72 h in DM. (C) The fusion index for day 3 myotubes was calculated from the ratio of nuclei number in MyHC-positive myotubes versus the total number of nuclei in one field in five random microscopic fields. (D) Myotube diameters were measured in day 3 myotubes. Results represent mean ± SEM from three independent experiments. *** $p < 0.001$.

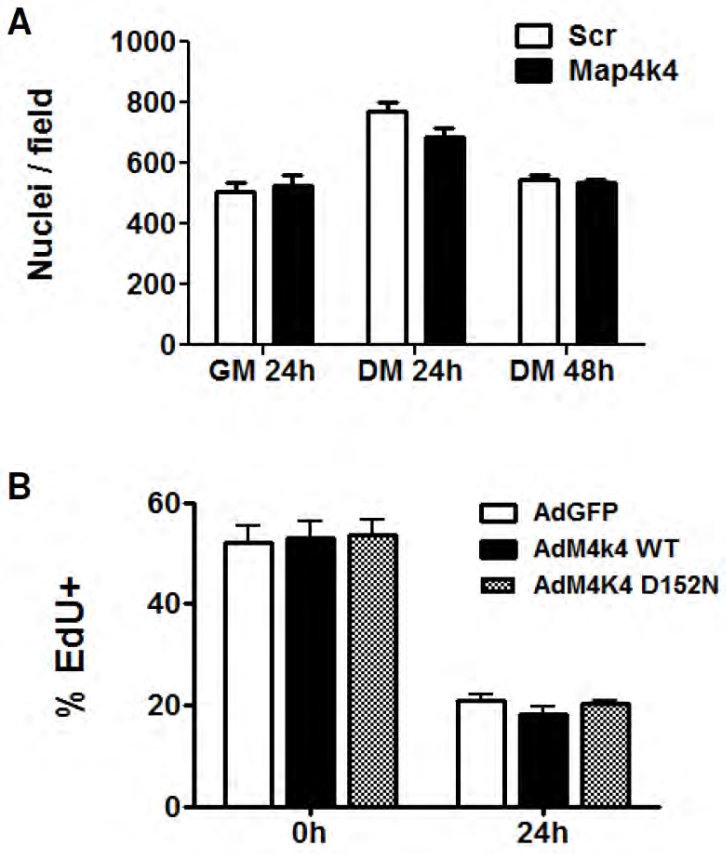


Figure 2.3 **Map4k4 silencing has no effect on C2C12 myoblast proliferation.** (A) C2C12 myoblasts were transfected with scrambled or Map4k4 siRNA. 24 hours later, the cells were cultured for additional 24 hours in GM or transferred to DM. Nuclei were counted after 24 hours in GM or 24 hours and 48 hours in DM. The values were obtained after counting at least 6 microscopic fields in duplicate samples. (B) C2C12 myoblasts were infected with adenoviruses expressing GFP, wild type Map4k4 or Map4k4 kinase-inactive mutant D152N for 24 hours. Factions of GFP-positive cells in S phase in GM (0 h) and in DM for 24 hours were analyzed by FACS after staining with EdU. Data represent mean ± SEM for triplicates in two independent experiments.

We further studied the differentiation program by examining the expression of muscle differentiation markers. No significant changes in MyoD protein levels were detected in Map4k4-silenced cells compared with scrambled siRNA-transfected controls during differentiation (Fig 2.4, A and B). However, significant transient increases in myogenin and Mef2C expression were detected in Map4k4-silenced cells at 16 hours and 48 hours of differentiation respectively (Fig 2.4, A and B). MyHC expression starts in a population of mononuclear myoblasts and rapidly increases with myoblast fusion during late myogenesis ((194) and Fig 2.4A). Map4k4 silencing enhanced MyHC expression during late C2C12 differentiation, although the increase was only significant at 48 hours of differentiation with trends toward increased expression at later time points (Fig 2.4, A and B). MCK activity, a later marker of skeletal muscle cell differentiation, was increased by 45% in Map4k4-silenced cells at day 3 of differentiation. These results suggest that silencing of Map4k4 enhances myotube formation and promotes skeletal myogenic differentiation.

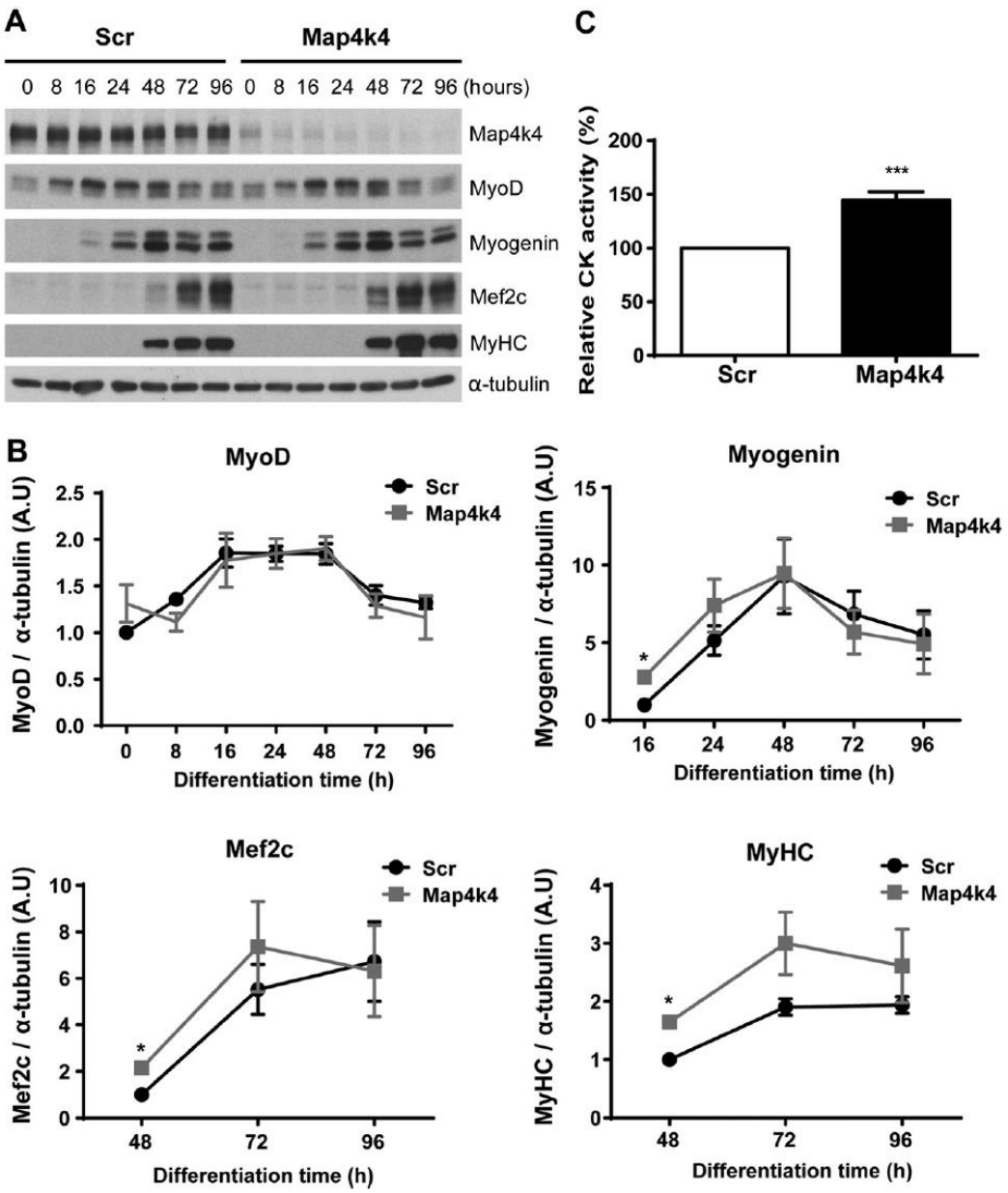


Figure 2.4 **Map4k4 silencing enhances C2C12 myogenic differentiation.** C2C12 myoblasts were transfected with scrambled or Map4k4 siRNA. Cells were transferred to DM 24 h after transfection and differentiated for the indicated times. (A) Expression of myogenic differentiation proteins was assayed by immunoblotting with the indicated antibodies. Data is representative of three independent experiments. (B) Densitometric analysis from western blot in (A). (C) Creatine kinase (CK) activities of transfected cells were measured after 3 days in DM. Data represent mean ± SEM from three independent experiments. * $p < 0.05$, *** $p < 0.001$.

Inhibition of myogenic differentiation by Map4k4 requires its kinase activity.

Since suppression of Map4k4 expression enhanced skeletal muscle differentiation, we hypothesized that Map4k4 overexpression would have the opposite effect. To test this, adenoviruses expressing GFP control (AdGFP) or wildtype (wt) Map4k4 (AdMap4k4 wt) (16) were used to infect C2C12 myoblasts for 18 hours prior to differentiation. Overexpression of wt Map4k4 impeded MyHC-positive myotube formation (Fig 2.5A) and myoblast fusion (Fig 2.5B) within 72 hours of serum deprivation. Western blot analysis confirmed that the expression of Mef2c and late myogenic differentiation marker gene MyHC was inhibited in wt Map4k4-overexpressing cells (Fig 2.5C). We also assessed the effect of a Map4k4 kinase-inactive mutant on myogenic differentiation. C2C12 myoblasts were infected with adenoviruses expressing Map4k4 D152N, a kinase-inactive mutant of Map4k4 (AdMap4k4 D152N) (16) and were induced to differentiate into myotubes for 72 hours. Interestingly, Map4k4 D152N overexpression caused the formation of larger myotubes and a substantial increase in myoblast fusion (Fig 2.5, A and B), similar to the results of the Map4k4 knockdown experiments (Fig 2.5, A and C). An increase in Mef2c and MyHC expression was also observed in Map4k4 D152N-overexpressing cells (Fig 2.5C). These data suggest that the Map4k4 kinase-inactive mutant functions as a dominant-negative inhibitor, possibly by competing with the functional endogenous Map4k4 in C2C12 cells, and that Map4k4 kinase activity is required to repress skeletal muscle differentiation. Furthermore, Map4k4 does not regulate myogenic differentiation through affecting myoblast proliferation, because no change in the percentage of EdU-positive cells was observed in wt or kinase-inactive

Map4k4-overexpressing C2C12 cells (Fig 2.3B), consistent with the unaltered nuclei numbers in myoblasts and myocytes upon Map4k4 silencing (Fig 2.3A).

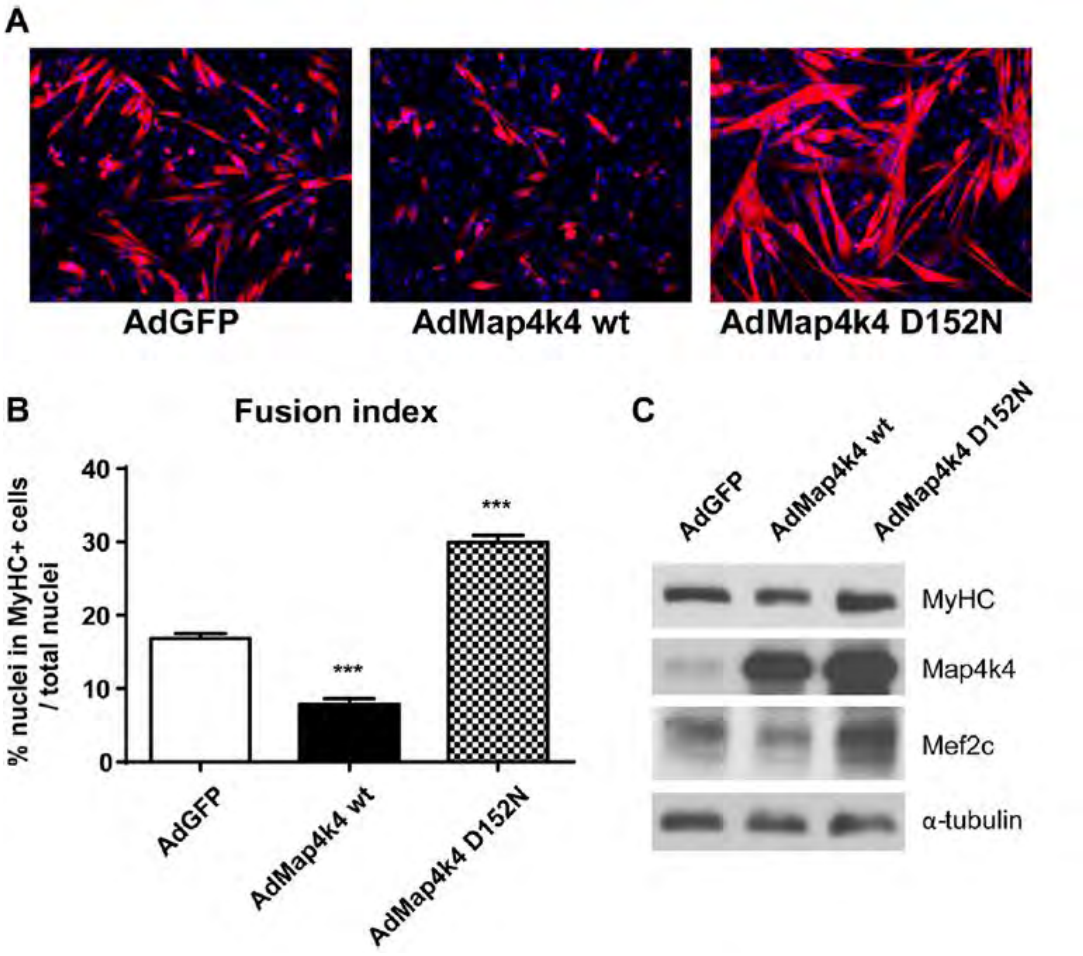
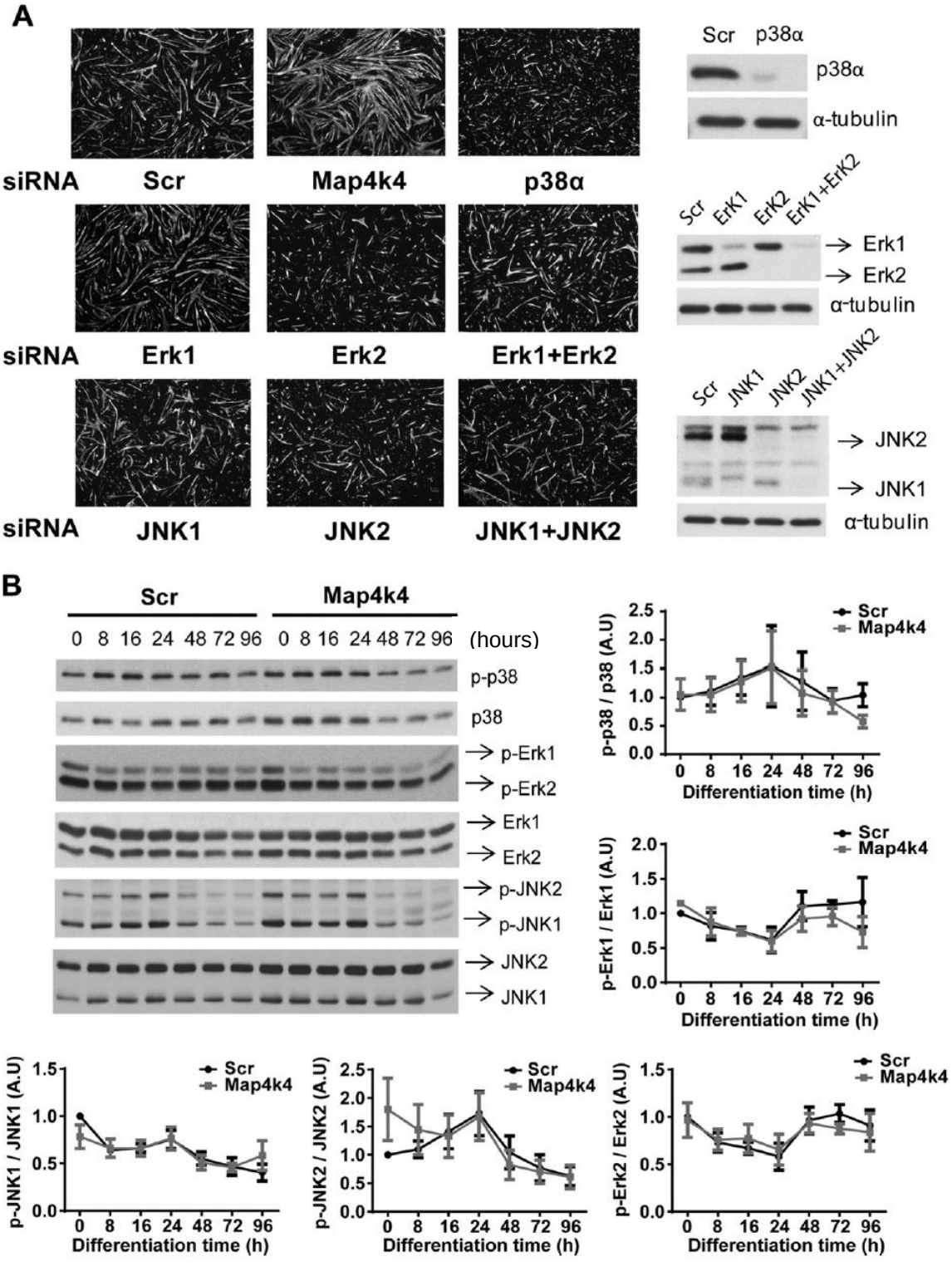


Figure 2.5 **Map4k4 kinase activity is required for its inhibition of C2C12 myogenic differentiation.** C2C12 myoblasts were infected with adenoviruses expressing GFP, wild type Map4k4 or Map4k4 kinase-inactive mutant D152N and differentiated for 72h. (A) Cells were immunostained with anti-MyHC antibody. Images were photographed by fluorescence microscopy (red, MyHC; blue, DAPI, 100×). (B) Quantitative analysis of myogenic conversion scored by fusion index. Data represent mean ± SEM from two independent experiments. *** $p < 0.001$. (C) Immunoblot analysis of Map4k4 and myogenic markers. Data is representative of three independent experiments.

Map4k4 does not regulate myogenic differentiation through canonical MAPK signaling pathways.

In other systems Map4k4 has been described as an upstream effector in JNK, ERK and p38 signaling pathways. These pathways are also reportedly involved in skeletal muscle differentiation, thus it seemed possible that Map4k4 regulates myogenic differentiation through these canonical MAPK pathways. To assess this hypothesis, we used siRNA to suppress MAPK expression separately or in combination in C2C12 myoblasts and monitored myogenic differentiation by visualizing MyHC-positive myotube formation. We posited that if Map4k4 functions upstream in the respective signaling pathway to regulate myogenesis, then silencing of the downstream effectors would result in a similar phenotype as Map4k4 silencing. However, depletion of p38α abolished myogenic differentiation because few p38α-silenced cells fused into multinuclear myotubes (Fig 2.6A), consistent with the conclusion derived from previous studies that p38α is critical for skeletal myogenesis (64, 291, 302). Other reports have shown that basal JNK activity is essential for regulation of skeletal muscle differentiation and inhibition of JNK activation inhibited myogenesis by inducing myoblast apoptosis (138). We suppressed JNK1 expression in myoblasts and observed a minimal effect of JNK1 silencing on myotube formation under our experimental conditions. However, JNK2 or JNK1/2 silencing in combination inhibited myogenic differentiation, as shown by reduced myotube formation (Fig 2.6A). ERK1/2 is essential for myoblast proliferation, and is inhibitory to early differentiation, but is also required for myocyte fusion. Inhibition of ERK activity early in myogenesis promotes differentiation, whereas later inhibition

impedes differentiation (291). In our study, ERK1 silencing in C2C12 myoblasts promoted myotube formation while knockdown of ERK2 resulted in the formation of smaller myotubes. Myotubes differentiated from ERK1 and ERK2 double knock down myoblasts had modestly decreased size compared to the ones differentiated from the scrambled siRNA transfected control (Fig 2.6A). These results revealed that Map4k4 functions differently on myogenic differentiation than the canonical MAPK pathways. This conclusion was further confirmed by measurement of phosphorylation levels of the MAPKs during differentiation. No significant changes in p38α, ERK1/2 or JNK1/2 phosphorylation were observed in Map4k4-silenced cells (Fig 2.6B), indicating that Map4k4 failed to regulate their activities during myogenic differentiation.


A
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p-Erk2 / Erk2 (A.U)
Differentiation time (h)

Figure 2.6 **Map4k4 silencing does not promote skeletal muscle differentiation through canonical MAPK signaling pathways.** C2C12 myoblasts were transfected with scrambled siRNA or siRNA against Map4k4, p38α, JNK1, JNK2, JNK1+JNK2, ERK1, ERK2 or ERK1+ERK2. Cells were transferred to DM 24 h after differentiation and incubated for 72h. (A) Left panels, myoblast differentiation was observed by immunostaining for MyHC expression (50×). Right panels, knockdown efficiency of the MAP kinases was determined by western blot. (B) C2C12 myoblasts were transfected with scrambled or Map4k4 siRNA. Cells were transferred to DM 24 h after transfection and incubated for the indicated times. Lysates were immunoblotted with the indicated antibodies. Densitometric analysis represents mean ± SEM from three independent experiments.

Map4k4 functions mainly at the early stage of myogenic differentiation.

To investigate the stages of myogenic differentiation in which Map4k4 functions, C2C12 cells were transfected with scrambled siRNA or siRNA targeting Map4k4 at multiple stages of differentiation and for variable periods of time (Fig 2.7A, right panels), and myotube formation was assessed on day 4 after initiation of differentiation by measuring the fusion index. In these experiments 90% of Map4k4 proteins were depleted in myotubes at this day 4 point in response to transfections of Map4k4 siRNA at the different time points shown (Fig 2.7B). Map4k4 silencing in myoblasts (day -1) provoked the most robust myotube formation (Fig 2.7A, upper left panels), as the fusion index in Map4k4-silenced cells was 60% higher than in the control cells on day 4 (Fig 2.7C). Map4k4 depletion at day 1 in myocytes that are about to enter the late stage of differentiation still resulted in larger myotubes and increased myoblast fusion compared to the control cells. However, the promotion of myotube formation was less than that resulted from Map4k4 silencing at earlier stages in myoblasts differentiation (Fig 2.7, A, middle left panels and C). When siRNA against Map4k4 was transfected into day 2 myotubes, coincident with onset of terminal differentiation, the myotubes showed even smaller changes in size or fusion compared to the results obtained from Map4k4 suppression in myoblasts and day 1 myocytes (Fig 2.7, A, lower left panels and C). These results indicate that Map4k4 functions in multiple stages of muscle differentiation, but the enhanced myotube formation observed in Map4k4-depleted cells at later stages of differentiation mainly results from a role that Map4k4 plays at the onset of myogenic differentiation. That Map4k4 apparently plays an early role in the process is also

consistent with the higher Map4k4 kinase activity at the early stage of muscle differentiation (Fig 2.1B).

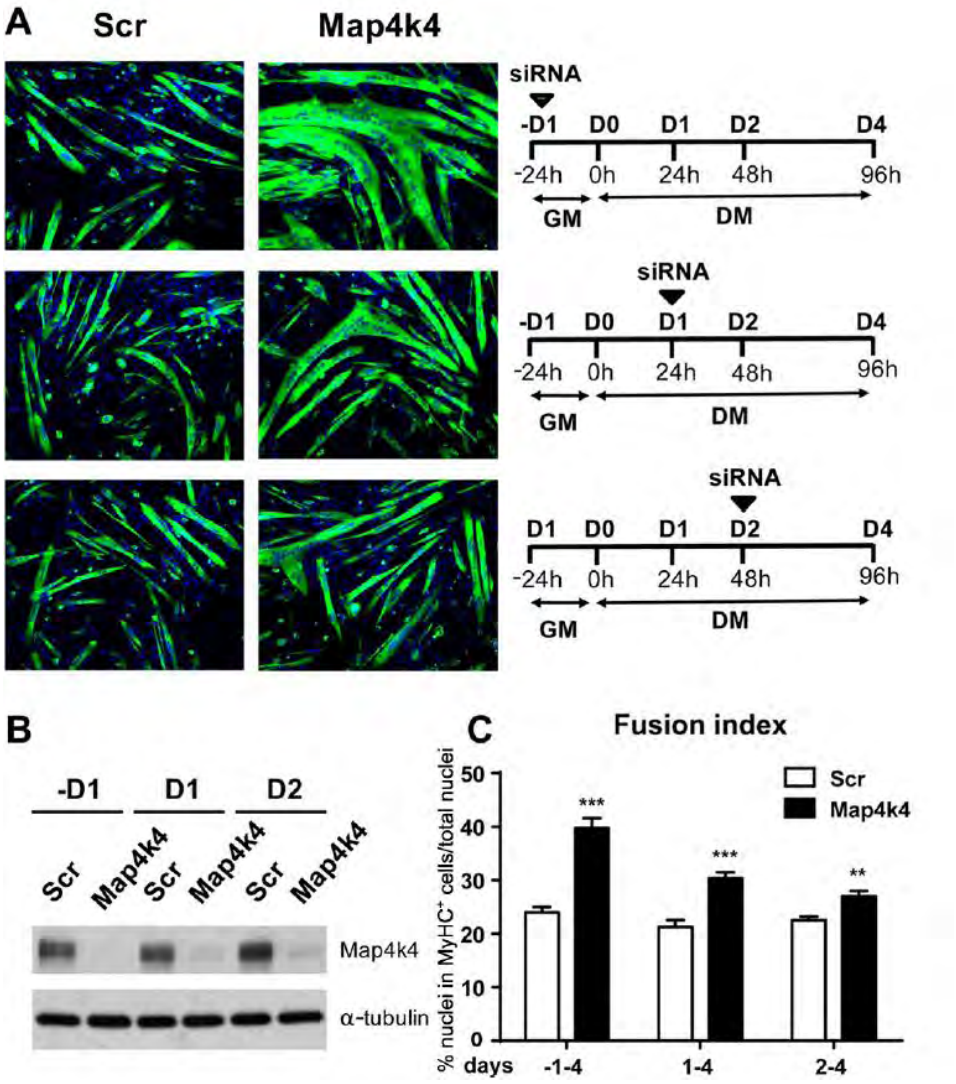

Figure 2.7 **Map4k4 functions mainly at the early stage of myogenic differentiation.** C2C12 myoblasts were transfected with scrambled or Map4k4 siRNA at different stages of differentiation for the indicated time periods. (A) Left panels, cells were fixed and immunostained for MyHC (green) after 4 days in DM (100×). Right panels, schematics of the time course of siRNA application. (B) Knockdown efficiency of Map4k4 was determined by immunoblot. (C) The fusion index was calculated by dividing the number of nuclei in MHC-positive cells by the total number of nuclei in that field. Data represent mean ± SEM from two independent experiments. ** p <0.01, *** p < 0.001.

Map4k4 regulates myogenic differentiation in a Myf5-dependent manner.

Among the four myogenic regulatory factors, Myf5 and MyoD regulate the early stage of skeletal muscle differentiation. Because no change in MyoD expression was detected in Map4k4-silenced cells during differentiation (Fig 2.4, A and B), we examined expression Myf5 expression by RT-PCR and Western blot. In cells treated with scrambled siRNA, Myf5 expression increased in early differentiation, peaked at 24 hours, and then decreased (Fig 2.8, A and B). Map4k4 silencing increased Myf5 mRNA transcripts by 1.4-fold within 16 hours of myogenic differentiation (Fig 2.8A). More dramatically, a three-fold increase in Myf5 protein levels was detected in Map4k4-depleted undifferentiated myoblasts and myocytes at the early stage of differentiation (Fig 2.8B).

To determine whether the increase in Myf5 protein levels is essential for the enhanced myogenic differentiation that is found after Map4k4 depletion, we performed double knockdown experiments to suppress Map4k4 and Myf5 expression simultaneously in C2C12 myoblasts, and examined differentiation by microscopic analysis and Western blot. As expected, Map4k4 knockdown promoted myogenic differentiation and Myf5 expression (Fig 2.9). In contrast, Myf5 silencing impeded myogenic differentiation as shown by reduced myotube formation (Fig 2.9A), decreased myoblast fusion (Fig 2.9B) and lower expression of myogenin, Mef2C and MyHC during differentiation (Fig 2.9C). Importantly, when compared with Map4k4 suppression alone, smaller myotubes with less fusion and myogenic differentiation factor expression were observed when Map4k4 and Myf5 were silenced simultaneously (Fig 2.9), indicating that reduced levels of Myf5 expression partially inhibit the effects of Map4k4 silencing on myogenic differentiation.

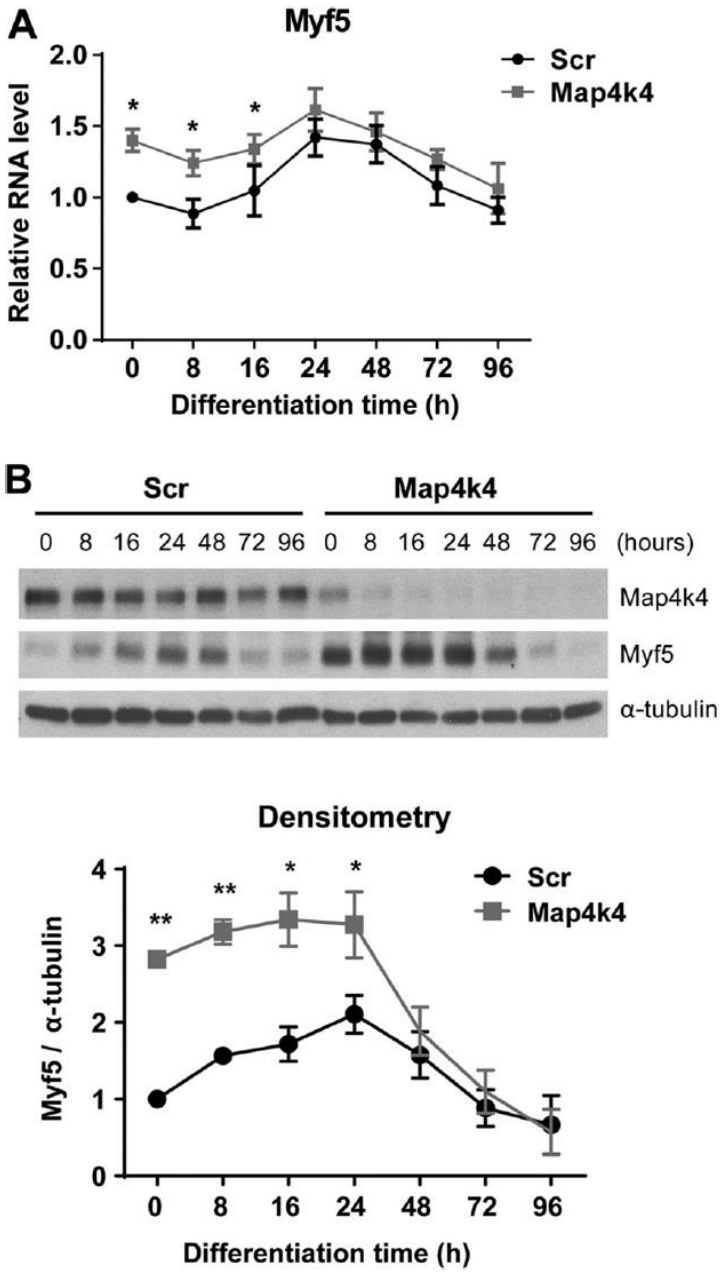

Figure 2.8 **Map4k4 silencing increases Myf5 expression.** C2C12 myoblasts were transfected with scrambled or Map4k4 siRNA and transferred to DM for the indicated times 24 h post transfection. (A) Myf5 mRNA level was determined by Real-Time PCR. (B) Upper panel, Myf5 protein level was determined by immunoblot. Lower panel, densitometry of Myf5 as normalized to α-tubulin. Data is represented as the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

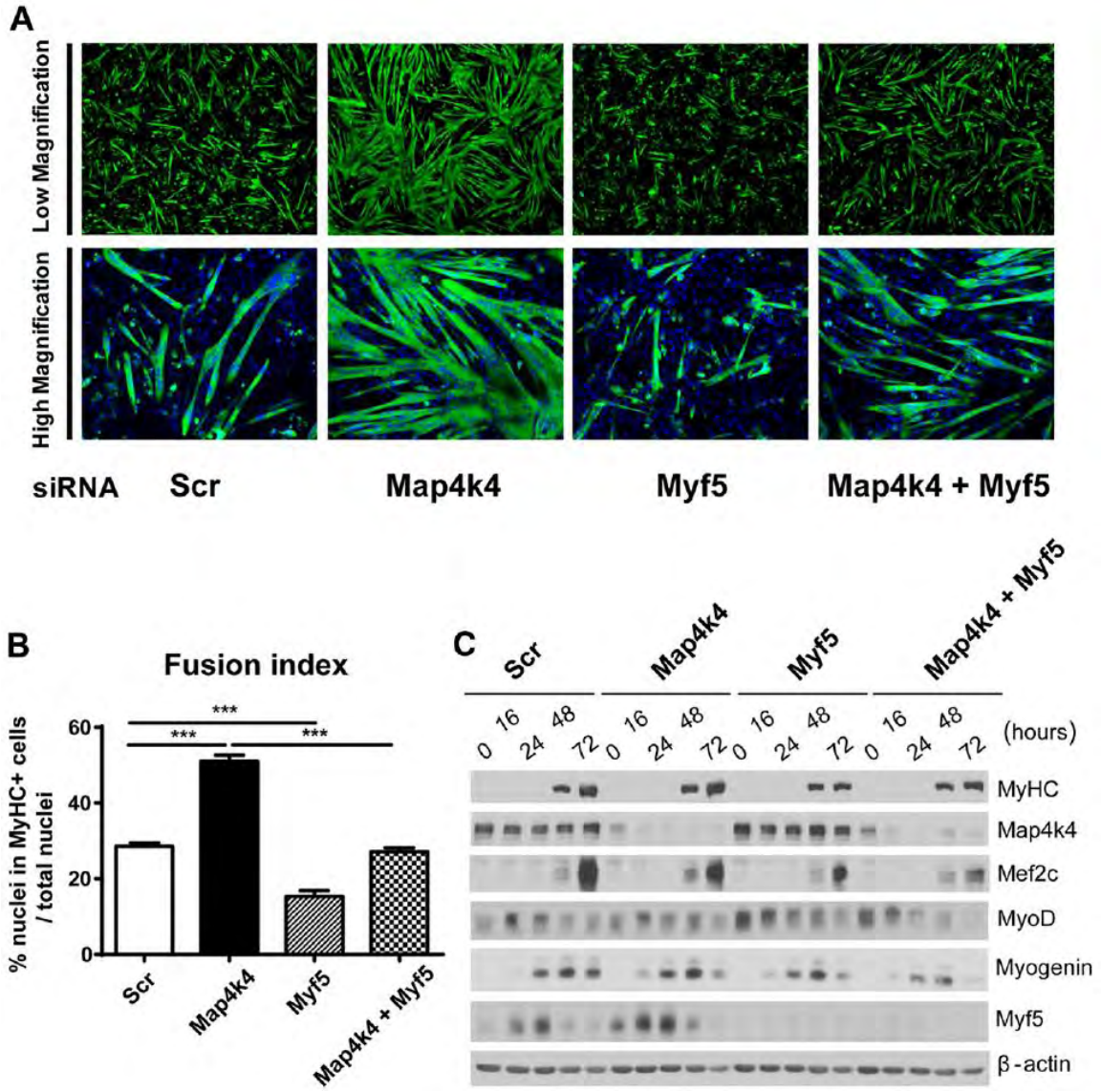

Figure 2.9 **Suppression of Myf5 impairs Map4k4 silencing-enhanced myogenic differentiation.** C2C12 myoblasts were transfected with scrambled siRNA, siRNA against Map4k4, Myf5 or a combination of both. (A) Cells were fixed and immunostained for MyHC (green) at day 3 after differentiation and myogenic conversion was observed by fluorescence microscopy (50× and 100×). (B) The fusion index was calculated for transfected cells after 3 days in DM. Results represent mean ± SEM from three independent experiments. *** $p < 0.001$. (C) Immunoblot of myogenic differentiation proteins. Data is representative of three independent experiments.

To further demonstrate that Myf5 is a downstream effector of Map4k4 signaling in the regulation of skeletal muscle differentiation, we overexpressed Map4k4 and Myf5 simultaneously in C2C12 myoblasts, and examined muscle differentiation by microscopy and immunoblotting. Expression of native Map4k4 protein kinase inhibited C2C12 myogenic differentiation while Myf5 expression robustly enhanced myotube formation (Fig 2.10, A and B) and late differentiation marker expression (Fig 2.10C). Interestingly, Myf5 expression significantly reversed the inhibitory effect of Map4k4 expression in myogenic differentiation (Fig 2.10). Based on these results, we conclude that Map4k4 regulates skeletal myogenesis at least partially by regulating Myf5 expression.

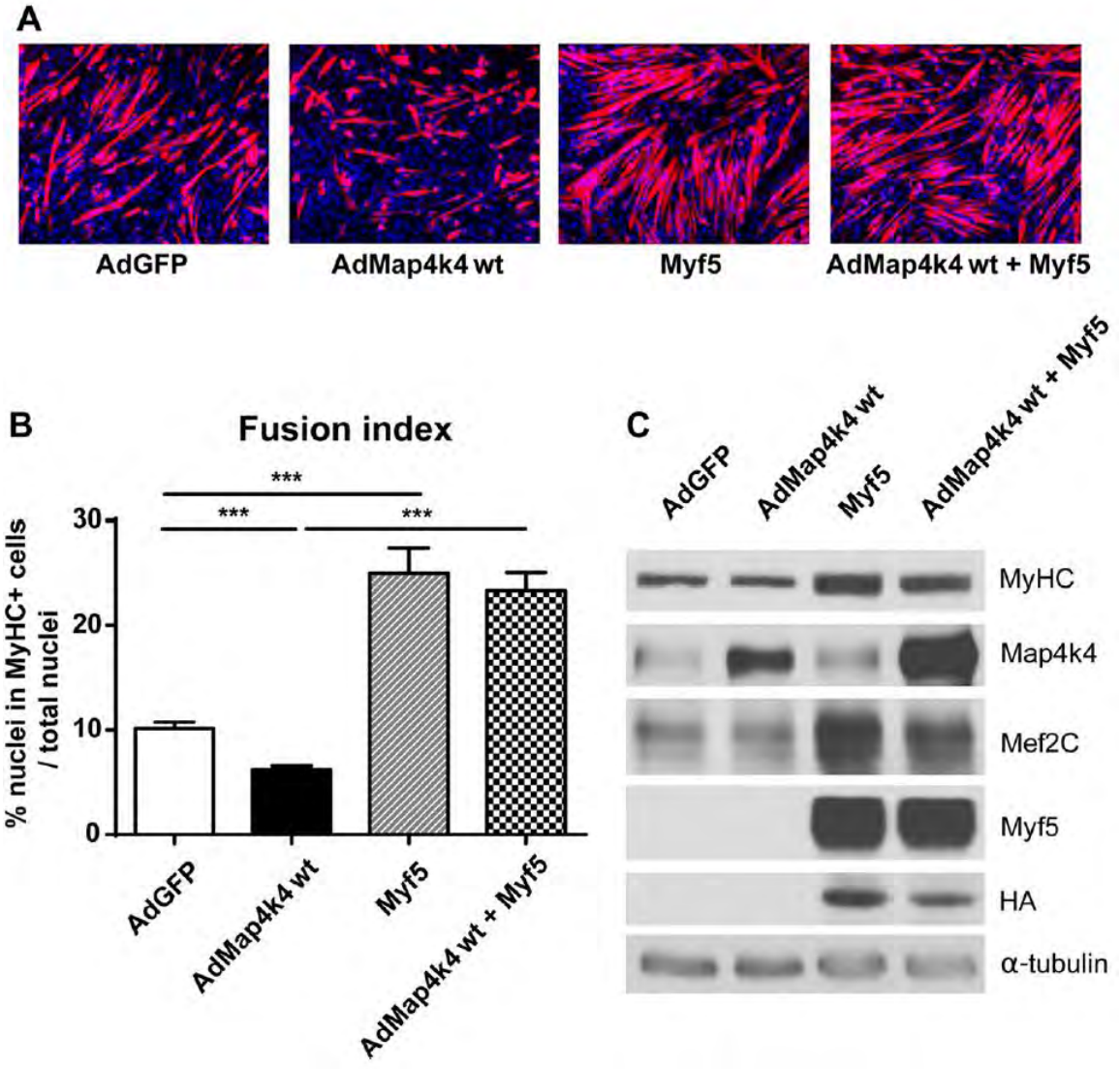

Figure 2.10 **Myf5 expression reverses the impaired myogenic differentiation caused by Map4k4.** C2C12 myoblasts were transfected with empty vector or a construct expressing Myf5. Six h later, the cells were infected with adenoviruses expressing GFP or Map4k4. (A) Cells were fixed and immunostained for MyHC (red) at day 3 after differentiation and myogenic conversion was observed by fluorescence microscopy (100×). (B) The fusion index was calculated for transfected cells after 3 days in DM. Results represent mean ± SEM from three independent experiments. *** $p < 0.001$. (C) Immunoblot of myogenic differentiation proteins. Data is representative of three independent experiments.

Discussion

In this study we demonstrate that Map4k4 negatively regulates C2C12 myogenic differentiation through a Myf5-dependent mechanism. RNAi-mediated gene specific silencing of Map4k4 expression or expression of a Map4k4 kinase-inactive mutant enhances the differentiation of C2C12 myoblasts (Fig 2.2, 2.4 and 2.5), while Map4k4 expression inhibits this process (Fig 2.5). Map4k4 suppression results in a significant increase in Myf5 expression at the early stage of differentiation (Fig 2.8). Furthermore, silencing of Myf5 inhibits C2C12 myoblast differentiation and also suppresses the ability of Map4k4 silencing to enhance myotube formation (Fig 2.9). In contrast, Myf5 expression promotes myotube formation and reverses the Map4k4-mediated myogenic differentiation inhibition (Fig 2.10), suggesting that Map4k4 regulates C2C12 myogenesis at least in part through Myf5.

We found that Map4k4 does not likely mediate myogenic differentiation via activation of the canonical MAPK signaling pathways, although it has been reported that Map4k4 can act upstream of p38 MAPK, JNK and ERK. In contrast to the dramatic enhancement of myotube formation upon Map4k4 silencing, a slight increase of myotube formation was observed in ERK1-silenced cells, while suppression of other MAPKs resulted in either no morphological difference or smaller myotubes. In addition, no change in levels of phosphorylated MAPKs were observed upon Map4k4 knockdown, which further suggests that the Map4k4-mediated increase in myogenesis is independent of MAPKs (Fig 2.6). These findings implicating signaling pathways distinct from canonical MAP

kinase cascades are consistent with our studies of Map4k4 signaling in other cellular contexts. We have previously shown that Map4k4 functions to reduce lipopolysaccharide (LPS)-induced TNFα expression in macrophages in a MAP kinase-independent manner. In that study, silencing of macrophage Map4k4 had no effect on the phosphorylation of p38 MAPK, ERK1/2, JNK1/2 or their substrates ATF2 or c-Jun in response to LPS (7). Map4k4 has also been suggested to regulate lipogenesis in cultured adipocytes independently of JNK signaling pathway, because JNK kinase activity remained unaltered upon either Map4k4 silencing or overexpression as determined by anti-phosphorylated JNK or c-Jun antibodies and protein kinase assay (Laura Danai, unpublished data). These findings strongly suggest that Map4k4 may regulate cellular processes independently of MAPK signaling pathways. The discrepancy on the relationship between Map4k4 and MAPKs between our studies and others' may result from differences in cell types, animal models and experimental methods that have been used. In fact, JNK activity regulation by Map4k4 could only be observed when both kinases were overexpressed in 293T cells, as it has been shown in several other studies (254, 297), but not when the endogenous kinases were examined in these cells (Laura Danai, unpublished data). Of note, we have not examined whether Map4k4 may regulate other p38 MAPK isoforms (β, γ and δ), which have been demonstrated to be necessary for C2C12 myogenic differentiation (151, 277). Further investigations are required to test this possibility.

In the current study, Myf5 expression was enhanced at the early stage of myogenic differentiation in Map4k4-depleted myocytes (Fig 2.8). We further found that Myf5 is

critical for myogenic differentiation because siRNA-mediated Myf5 suppression significantly inhibited C2C12 myoblast differentiation (Fig 2.9), consistent with the observations reported in a recently published study (224). Furthermore, we found that silencing of Myf5 reduced the enhanced myotube formation that ensued upon Map4k4 suppression to the same level as the scrambled siRNA control (Fig 2.9), indicating that Map4k4 regulates myogenic differentiation in a Myf5-dependent manner. The marked rescue by Myf5 overexpression of myogenic differentiation inhibition because of Map4k4 in myoblasts demonstrates that Map4k4 regulates skeletal muscle differentiation by acting upstream of Myf5. Other regulators may also be involved in the regulation of myogenesis by Map4k4, such as proteins related to myoblast fusion that occurs in late differentiation. This concept is supported by the observation that Map4k4 suppression during late differentiation still significantly increased myotube formation (Fig 2.7, middle panels).

In human skeletal muscle, Map4K4 silencing protected against TNFα-mediated insulin resistance by preventing activation of JNK1/2 and ERK1/2 (36). TNFα has been demonstrated to inhibit skeletal muscle differentiation in several studies (3, 48, 57). Therefore, Map4k4 may mediate in part the inhibitory effect of TNFα on C2C12 differentiation. When C2C12 myoblasts were differentiated with DM containing 5ng/ml of TNFα for 3 days, we observed a marked inhibition of myotube formation, dramatically reduced myogenic gene MyHC expression (~10 fold) and increased phosphorylation of JNK1/2. However, silencing Map4k4 in TNFα-treated cells only marginally rescued the inhibitory effect of TNFα on C2C12 differentiation as demonstrated by a slight increase

in myotube formation and MyHC expression (~1.9 fold), with no significant alteration in JNK1/2 phosphorylation (Fig A.1). The fact that the silencing was not complete in these studies makes data interpretation difficult, and cells from Map4k4 knockout mice will be required to address whether Map4k4 plays a role in TNFα-mediated inhibition of myogenic differentiation.

The negative effect of Map4k4 on myoblast fusion suggests a potential involvement of Map4k4 in skeletal muscle regeneration. Muscle regeneration is a rapid and extensive self-renewal process relying on the presence of satellite cells, a population of quiescent, mononucleated stem cells that are resident in adult skeletal muscle (47, 264). Upon work overload or injury, satellite cells are activated, then proliferate and differentiate into myoblasts that either fuse to each other to create new myofibers, or fuse to existing damaged myofibers for repair. The fusion process in regeneration shares similar features to muscle cell fusion in myogenic differentiation. As Map4k4 is abundantly present in satellite cells (Fig 2.1) and it is able to regulate myoblast fusion in C2C12 differentiation, it may also mediate the fusion process in muscle regeneration. Moreover, we showed here that Map4k4 silencing in C2C12 myoblasts substantially increased Myf5 expression during early myogenic differentiation (Fig 2.8). In muscle regeneration, satellite cell activation is associated with Myf5 up-regulation (60) and Myf5 null mice had a significant delay in the regenerative process (96). These studies support Map4k4 as a potential regulator in skeletal muscle regeneration by regulating Myf5 expression and fusion in satellite cells.

In summary, the results of this study reveal a novel role for Map4k4 in skeletal myogensis and identify Myf5 as a protein that is regulated by Map4k4 to mediate myogenic differentiation. Given the effects that Map4k4 exerts on fusion and Myf5 expression, Map4k4 inhibition may enhance the regenerative capacity of damaged muscles in trauma and degenerative diseases such as muscular dystrophies. The hypertrophic myotube formation induced by Map4k4 suppression also suggests that Map4k4 may be an attractive therapeutic target for the treatment of cachexia or sarcopenia. Further investigation into other Map4k4 effectors that are involved in myogenesis, especially muscle-specific Map4k4 substrates, will be necessary to fully understand the role of Map4k4 as a new signaling node in muscle development and function.

Chapter III: Map4k4 does not regulate IGF-mediated signaling pathways in C2C12 muscle cells

Contributions: All experiments and data analysis were performed by me.

Abstract

IGF signaling has profound effects on skeletal muscle size by promoting myoblast proliferation, differentiation and myofiber protein synthesis. siRNA-mediated silencing of Map4k4, a novel suppressor of myogenic differentiation, results in markedly enhanced myotube formation that appears identical to the hypertrophic phenotype that is caused by IGF signaling activation. Here we show that Map4k4 silencing does not alter IGF1-induced effector phosphorylation including Akt, p70S6K, S6, 4EBP1 and ERK1/2 in C2C12 myoblasts. Map4k4 suppression neither appears to affect IGF/Akt signaling pathway during C2C12 myogenic differentiation, as demonstrated by unchanged phosphorylation of Akt and its substrates GSK3 and FoxO1 as well as mTOR substrates p70S6K and 4EBP1. Furthermore, no change in the expression of FoxO1 targets MAFbx and MURF1 was observed in Map4k4-silenced C2C12 cells during differentiation. These results demonstrate that Map4k4 is not involved in IGF signaling-mediated skeletal muscle differentiation and hypertrophy.

Introduction

Skeletal muscle is a dynamic tissue that can alter its size as an adaption to various environmental stimuli such as nutrients, hormones and exercise. Increased muscle mass (i.e. muscle hypertrophy) is characterized by elevated myonuclei number and myofiber protein content, which are mediated by molecular signaling pathways that are involved in myoblast proliferation, differentiation and protein metabolism. Insulin-like growth factor (IGF) is a potent modulator of skeletal muscle mass. Forced IGF1 expression in mouse

skeletal muscle resulted in increased myoblast proliferation, protein synthesis and significant myofiber hypertrophy (15, 56, 88, 188), while IGF1 or IGF1 receptor deletion caused dramatic muscle hypoplasia and severe growth retardation (14, 158, 219). An array of studies in multiple muscle cell lines also identified IGF1 and IGF2 as important inducers of myoblast proliferation and differentiation *in vitro* (84, 89, 251, 268).

Signaling through the IGF receptor stimulates several different downstream intracellular signaling cascades including ERK1/2 and Akt signaling. IGF induced-ERK activation is mainly responsible for mitogenesis (59, 178), although the IGF/Akt axis is critical for proliferation as well (178, 273). Akt activation promotes myogenic differentiation and hypertrophy by targeting three major downstream kinases: mTOR, GSK3β and FoxO. Akt-mediated mTOR activation stimulates protein synthesis via p70S6K1 activation and 4EBP1 inhibition. P70S6K1 phosphorylates the small ribosomal subunit S6 to initiate efficient mRNA translation and phosphorylated 4EBP1 releases its inhibitory binding to eIF4E, further allowing for the translation initiation. GSK3β phosphorylation by Akt inactivates this kinase, therefore abolishing the inhibitory effects of GSK3β on muscle differentiation and protein synthesis. Similarly, Akt-mediated phosphorylation of FoxO, a negative regulator of myogenesis, prevents its nuclear translocation, resulting in enhanced muscle differentiation and down-regulation of two atrophy-inducing, muscle-specific ubiquitin ligases MAFbx and MuRF1, which are FoxO1 transcription targets (171).

Map4k4 is a serine/threonine protein kinase that belongs to the germinal center kinase GCK-IV group of *Saccharomyces cerevisiae* sterile 20 protein kinases (74). In an RNAi-based screen for regulators of adipocyte function, Map4k4 was discovered to inhibit PPARγ expression in cultured adipocytes (263). A following study suggested that the PPARγ suppression by Map4k4 was at the translational level involving mTOR signaling (102). Map4k4 silencing activated mTOR, and increased phosphorylation of its substrate 4EBP1 but not p70S6K. 4EBP1 phosphorylation releases its inhibitory binding to eIF4E and allows 5'-cap-dependent translation initiation, resulting in increased PPARγ protein levels and global protein synthesis rates in Map4k4-silenced adipocytes (102).

We have recently identified Map4k4 as a novel suppressor of skeletal muscle differentiation (278). Map4k4 suppression by RNAi technology or kinase-inactive Map4k4 overexpression in C2C12 myoblasts dramatically enhanced myotubes formation with increased myonulcei number and myotube size (Fig 2.2 and 2.5, (278)), which phenocopied IGF1 or IGF2-induced myotube hypertrophy (251, 268). The Map4k4 silencing-stimulated myotube formation was partially because of elevated Myf5 expression, which was required for the enhancement of myotube formation in Map4k4-silenced cells and was sufficient to rescue Map4k4-mediated inhibition of myogenic differentiation (Fig 2.8, 2.9 and 2.10, (278)). However, other mechanisms may contribute to Map4k4-mediated suppression of muscle differentiation because Myf5 silencing only partially impaired the Map4k4 depletion-induced myotube formation.

We hypothesized that Map4k4 might regulate muscle differentiation and hypertrophy through an IGF-mediated signaling pathway because of the high similarity between the muscle phenotypes caused by Map4k4 silencing and IGF induction. We were particularly interested in a potential role for Map4k4 in mTOR regulation because it is a downstream effector of IGF-Akt signaling in muscle cells and because Map4k4 suppressed the mTOR/4EBP1 signaling cascade in cultured adipocytes (102).

Materials and Methods

Cell culture and transfection. Mouse C2C12 myoblasts (American Type Culture Collection) were cultured in growth media (GM) consisting of Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO2. To induce differentiation, 95% confluent cells were placed in differentiation media (DM) consisting of DMEM with 2% horse serum. For siRNA transfection, C2C12 myoblasts cultured in growth media were transfected with 50 pmol siRNA using Lipofectatmine RNAiMAX (Invitrogen) according to the manufacturer's instructions for reverse transfection. Twenty-four hours later, cells were switched to DM and cultured for the indicated times before harvesting.

Western blotting. Cells were solubilized with ice-cold lysis buffer (20 mM HEPES, pH 7.2, 100 mM NaCl, 1mM EDTA, 100 mM PMSF, 0.01% Triton X-100, 1% SDS and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific)), and protein concentrations were assessed by BCA assay (Thermo Scientific). Equal amounts of protein were loaded onto 8.5% SDS-polyacrylamide gels and transferred to nitrocellulose

membranes. The following antibodies were used: anti-Map4k4 (Bethyl), anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Cell Signaling), anti-p44/42 MAPK (ERK1/2) (Santa Cruz), anti-phospho-Akt (Ser473) (Cell Signaling), anti-Akt (Cell Signaling), anti-phospho-GSK3α/β (Ser21/9) (Cell Signaling), anti-GSK3α/β (Cell Signaling), anti-phospho-FoxO1 (Ser256) (Cell Signaling), anti-FoxO1 (Cell Signaling), anti-phospho-p70S6K (Thr389) (Cell Signaling), anti-p70S6K (Cell Signaling), anti-phospho-S6 ribosomal protein (Ser240/244) (Cell Signaling), anti-S6 ribosomal protein (Cell Signaling), anti-phospho-4EBP1 (Thr37/46) (Cell Signaling) and anti-4EBP1 (Cell Signaling) antibodies.

Isolation of RNA and Real Time PCR. RNA isolation was performed according to the Trizol Reagent Protocol (Invitrogen). cDNA was synthesized from 1.5 μg total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules CA) according to the manufacturer's instructions. For real time PCR, synthesized cDNA and iQ SYBR Green Supermix were run on the MyiQ Real-Time PCR System (Bio-Rad) with following primer pairs: MAFbx forward 5'-ATGCACACTGGTGCAGAGAG-3', MAFbx reverse 5'-TGTAAGCACACACTGCAGGTC-3'; MuRF1 forward 5'-ACGAGAAGAAGACTCGAGC-3', MuRF1 reverse 5'-CTTGGCACTTGAGAGGAA-3'. 36B4 was used as an internal loading control. Relative gene expression was determined using the ΔCt method.

Results

Map4k4 silencing does not alter IGF1-induced ERK1/2 phosphorylation in C2C12 myoblasts.

IGF1 promotes myoblast proliferation by activating the ERK1/2 signaling pathway (59, 178). To investigate whether Map4k4 regulates the IGF1-ERK1/2 signaling axis, we suppressed Map4k4 expression using siRNA in C2C12 myoblasts and examined IGF1-induced ERK1/2 phosphorylation at multiple time points. IGF1 transiently activated ERK1/2 as indicated by the increased ERK1/2 phosphorylation at Ser202/204 within 15 minutes of IGF1 treatment (Fig 3.1). However, no significant alteration in ERK1/2 phosphorylation was observed in Map4k4-silenced myoblasts compared with the scrambled siRNA controls (Fig 3.1), suggesting that Map4k4 is not involved in IGF1-induced ERK1/2 activation.

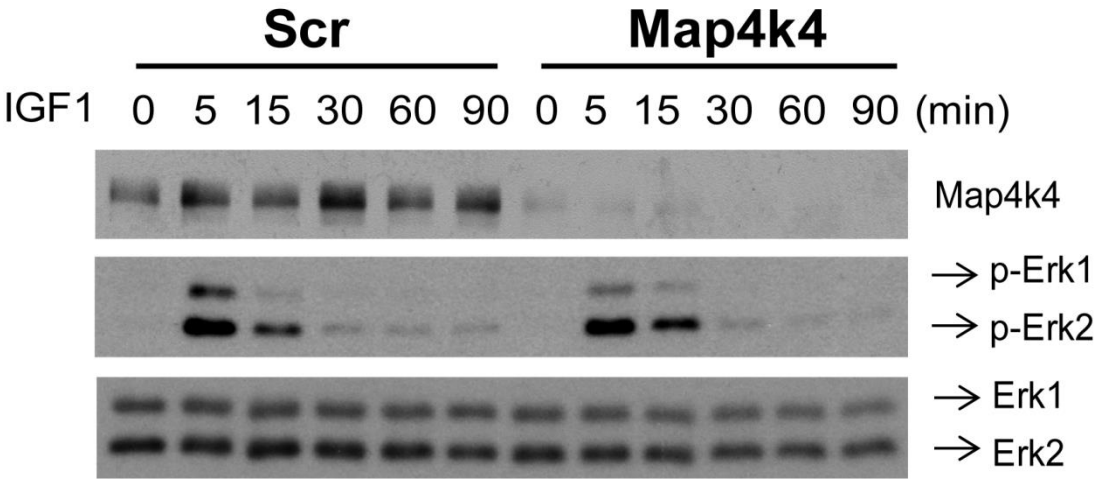

Figure 3.1 **Map4k4 silencing does not alter IGF1-induced ERK1/2 phosphorylation in C2C12 myoblasts.** C2C12 myoblasts were transfected with scrambled siRNA or siRNA against Map4k4. 24 h later, the cells were starved for 2 h and then treated with IGF1 (100ng/ml) for the indicated times. ERK1/2 phosphorylation was assessed by Western blotting using the indicated antibodies. A representative Western blot is shown.

Map4k4 silencing does not affect IGF1/Akt/mTOR signaling in C2C12 myoblasts.

In addition to ERK1/2, IGF1 also activates Akt signaling in myoblasts. Downstream of Akt, several signaling branches are activated by IGF including the mTOR signaling pathway. Because Map4k4 selectively inhibited mTOR activity and 4EBP1 phosphorylation in adipocytes, we aimed to determine whether Map4k4 modulates IGF1/Akt/mTOR signaling in muscle cells. To test this, we used Map4k4 siRNA to deplete its expression in C2C12 myoblasts and monitored the phosphorylation of downstream IGF1/Akt/mTOR signaling pathway effectors at multiple time points after IGF1 treatment. In the scrambled siRNA control cells, IGF1 activated Akt as early as 5 minutes after stimulation, and this activation lasted for at least 90 minutes (Fig 3.2). mTOR substrate p70S6K phosphorylation was induced 5 minutes after IGF1 treatment and began to decrease after 60 minutes, which was similar to the p70S6K substrate S6 ribosomal protein, which peaked at 30 minutes in IGF1-treated cells (Fig 3.2). 4EBP1 phosphorylation was stimulated at 5 minutes, peaked at 30 minutes and began to decrease slightly after that (Fig 3.2). The time-dependent and sequential phosphorylation of Akt and downstream mTOR effectors indicated that IGF1 sufficiently activated Akt/mTOR signaling in C2C12 myoblasts and the time window that we chose was appropriate to study the role of Map4k4 in the signaling cascade. Upon Map4k4 depletion, the phosphorylation status of each effector remained similar compared with the scrambled control, implying that Map4k4 does not regulate the IGF/Akt/mTOR signaling pathway in C2C12 myoblasts.

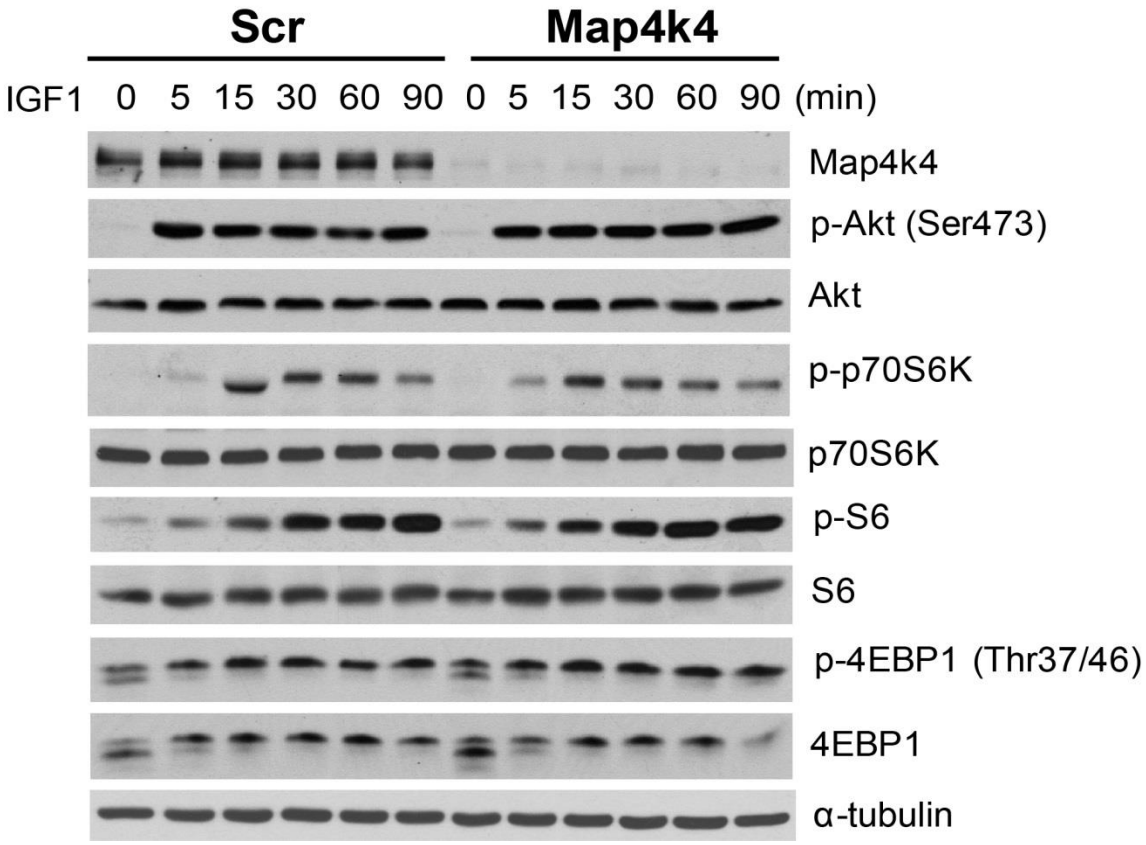

Figure 3.2 **Map4k4 silencing has no significant effect on the IGF/Akt/mTOR signaling pathway in C2C12 myoblasts.** C2C12 myoblasts were transfected with scrambled or Map4k4 siRNA. 24 h later, the cells were starved for 2 h and then treated with IGF1 (100ng/ml) for the indicated times. IGF/Akt/mTOR signaling pathway activation was assayed by Western blotting using the indicated antibodies. The data is representative of four independent experiments.

Map4k4 silencing has no impact on Akt or its downstream effector activation during C2C12 differentiation.

Myoblasts begin to express IGF2 upon serum withdrawal and continuously produce IGF2 with muscle differentiation *in vitro* (89). Several studies have demonstrated that myogenic differentiation is mediated through endogenous Akt activation that is induced by autocrine/paracrine IGF2 (89, 183). To assess whether Map4k4 regulates myogenesis through endogenous Akt signaling, C2C12 myoblasts were transfected with scrambled or Map4k4-targeting siRNA and Akt signaling pathway component phosphorylation was examined by Western blotting at multiple time points during muscle differentiation. Akt signaling was relatively active in the myoblasts, as demonstrated by the high Akt phosphorylation levels as were the downstream effectors including GSK3, FoxO1 and mTOR substrates p70S6K and 4EBP1 (Fig 3.3A). Serum withdrawal, a crucial step to induce myogenic differentiation, dramatically inactivated Akt signaling, which was rebounded gradually with C2C12 differentiation (Fig 3.3A). Map4k4 silencing did not result in any significant changes in the phosphorylation of Akt or its downstream effectors (Fig 3.3A). The expression of MAFbx and MURF1, two targets of the transcription factor FoxO1 were not altered in the Map4k4-silenced muscle cells compared with scrambled siRNA-treated controls (Fig 3.3B), suggesting that FoxO1 transcriptional activity did not change upon Map4k4 suppression. These findings indicate that Map4k4 does not modulate myogenic differentiation through the endogenous Akt signaling pathway.

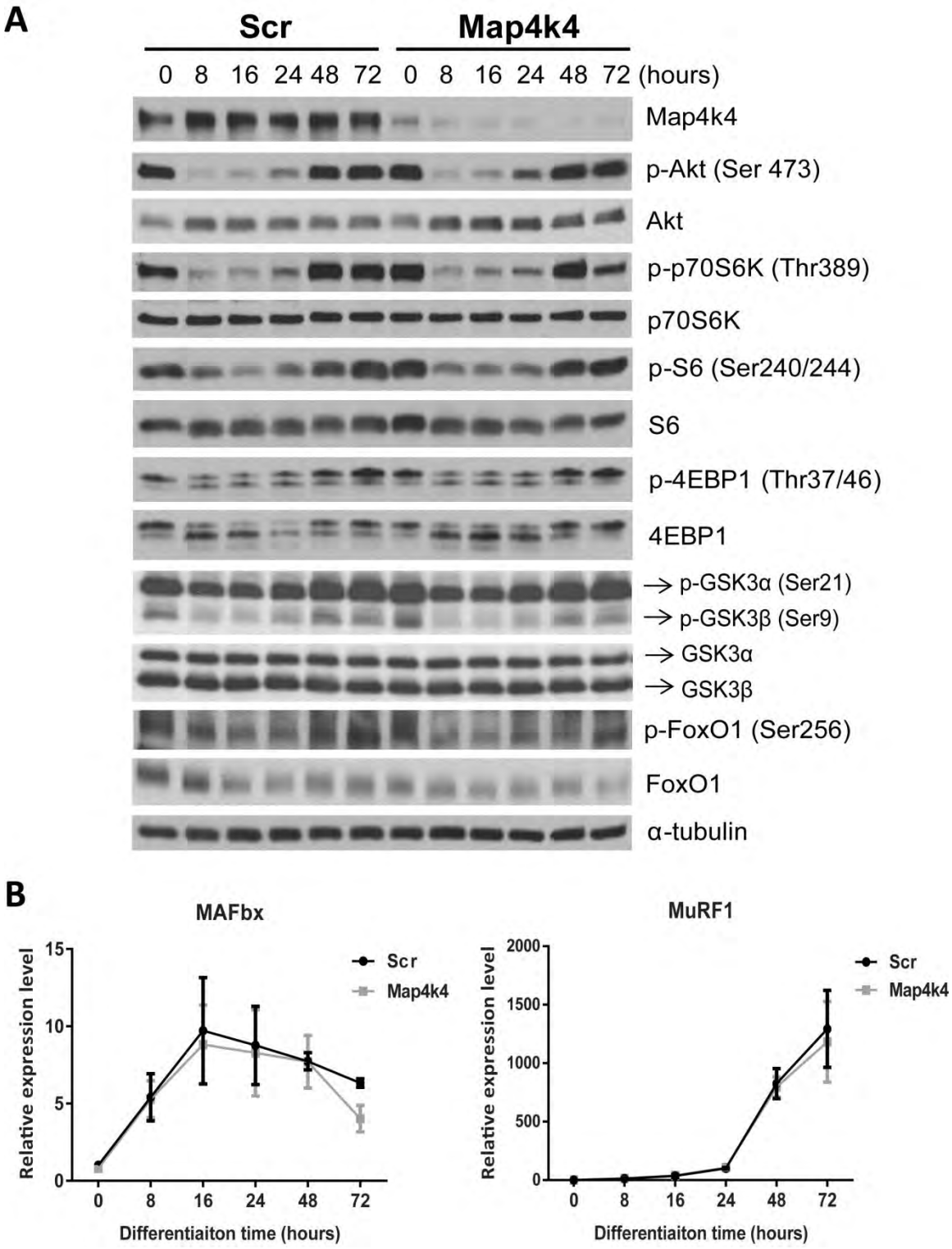
A
Scr
Map4k4
0 8 16 24 48 72 0 8 16 24 48 72 (hours)
Map4k4
p-Akt (Ser 473)
Akt
p-p70S6K (Thr389)
p70S6K
p-S6 (Ser240/244)
S6
p-4EBP1 (Thr37/46)
4EBP1
→ p-GSK3α (Ser21)
→ p-GSK3β (Ser9)
→ GSK3α
→ GSK3β
p-FoxO1 (Ser256)
FoxO1
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Figure 3.3 **Map4k4 silencing has no impact on activation of Akt or its downstream effectors during C2C12 differentiation**. C2C12 myoblasts were transfected with scrambled or Map4k4 siRNA. 24 h later, the cells were transferred to DM for the indicated times. (A) Activation of Akt signaling during muscle differentiation was determined by immunoblotting with the indicated antibodies. The data is representative of at least three independent experiments. (B) MAFbx and MuRF1 expression was assessed by real-time RT-PCR. The data represent the mean ± SEM from three independent experiments.

Discussion

In a previous study, we identified Map4k4 as a novel suppressor of skeletal muscle differentiation (Chapter 2 and (278)). Map4k4 silencing in C2C12 myoblasts resulted in a dramatic enhancement of myotube formation that is reminiscent to the hypertrophic myotubes caused by IGF, which is a potent inducer of myoblast proliferation, myogenic differentiation and muscle hypertrophy both *in vitro* and *in vivo*. In the current study, we discovered that Map4k4 appeared not to be involved in two IGF signaling branches, the IGF/ERK1/2 signaling axis and the IGF/Akt signaling axis. No significant alterations in IGF-induced phosphorylation of ERK1/2, Akt or its downstream effectors were detected in Map4k4-silenced cells compared with the scrambled controls (Fig 3.1 and 3.2). Endogenous Akt signaling activity was also unchanged during myogenic differentiation in Map4k4-depleted muscle cells (Fig 3.3).

Previous studies have demonstrated that IGF induces myoblast proliferation through activating both ERK1/2 and Akt signaling pathways. The failure to detect ERK1/2 and Akt phosphorylation upon Map4k4 suppression (Fig 3.1 and Fig 3.2) is consistent with unchanged EdU incorporation into kinase-inactive Map4k4-overexpressing C2C12 cells as demonstrated in Fig 2.3, further suggesting that Map4k4 does not play a role in myoblast proliferation.

mTOR signaling is a key signaling branch downstream of Akt. Map4k4 silencing in cultured adipocytes promoted PPARγ translation and global protein synthesis via mTOR signaling pathway activation. However, Map4k4 silencing in C2C12 myoblasts had no

detectable effects on the mTOR signaling pathway during myogenic differentiation, as determined by unaltered phosphorylation of mTOR downstream effectors p70S6K and 4EBP1 (Fig 3.2). Moreover, Map4k4 suppression did not enhance IGF/Akt/mTOR signaling in the IGF1-treated C2C12 myoblasts compared with the scrambled siRNA controls (Fig 3.3). These results indicate that Map4k4 may not target the canonical mTOR signaling pathway during skeletal muscle differentiation. Notably, the myogenic function of mTOR during early differentiation has been demonstrated to be kinase-independent and involves neither of the downstream effectors p70S6K1 or 4EBP1 (85). Instead, mTOR induces IGF2 transcription in a kinase-independent mechanism and thus activates Akt signaling indirectly (86). We have preliminary data showing that Map4k4 silencing resulted in a trend to increased IGF2 mRNA expression during C2C12 differentiation, implying that Map4k4 may target mTOR without altering its kinase activity to regulate IGF2 expression and thereby myogenesis. However, this hypothesis contradicts with the lack of alteration in IGF/Akt signaling upon Map4k4 silencing during C2C12 differentiation (Fig 3.3). One possibility is that the observed IGF2 up-regulation is only at the transcriptional level. Further investigation will be required to determine whether IGF2 protein content is altered in the Map4k4-silenced muscle cells.

GSK3β phosphorylates and inactivates the eukaryotic initiation factor eIF2B, a guanine nucleotide exchange factor that converts the inactive GDP-bound eukaryotic initiation factor 2 (eIF2) to the active GTP-bound eIF2, therefore impairing mRNA translation and global protein synthesis (282). Insulin stimulation induces dephosphorylation of the eIF2B inhibitory site by phosphorylating and inactivating GSK3β at Ser9 in a PI3K-

dependent manner, indicating that insulin (or IGF) stimulates translation through PI3K/Akt/GSK3β/eIF2B/eIF2 signaling independently of mTOR (282). In the present study, we did not detect any changes in GSK3β phosphorylation upon Map4k4 silencing in C2C12 muscle cells, implying that Map4k4 would not regulate protein synthesis via the IGF/Akt/GSK/eIF2B/eIF2 pathway. A similar result was obtained in Map4k4-deficient mouse embryonic fibroblasts; in these cells, GSK3α/β phosphorylation remained unchanged from wild-type controls (Laura Danai, unpublished data). However, evidence from co-immunoprecipitation experiments suggested a potential interaction between Map4k4 and one of the eIF2 regulators in cultured adipocytes (Adilson Guilherme, unpublished data), therefore Map4k4 may modulate the regulator activity by phosphorylation and thus affecting eIF2 activity in a GSK3β-independent manner. Map4k4 silencing-induced muscle hypertrophic phenotype may result from eIF2 activation-induced increases in protein accumulation. To test this hypothesis, we would need to determine whether Map4k4 regulates global protein synthesis in skeletal muscle. Further investigations are required to confirm the interaction between Map4k4 and the eIF2 regulator in muscle cells and determine the functional effects of the interaction.

In the present study, we have provided evidence that Map4k4 does not regulate muscle differentiation by interfering with the IGF-induced ERK1/2 or Akt signaling pathways. However, the calcineurin/NFAT signaling pathway is also downstream of the IGF receptor has not been assessed. Numerous studies have revealed the positive roles of calcineurin and its target NFATs in skeletal muscle differentiation and hypertrophy (73,

79, 94, 180). An interesting future study is to determine whether Map4k4 targets the calcineurin signaling to regulate skeletal myogenesis.

CHAPTER IV: Discussion

We have identified Map4k4 as a novel suppressor of skeletal myogenesis. Map4k4-mediated differentiation inhibition is not through the MAP kinase signaling pathways or the IGF-activated Akt signaling cascades. Instead, Map4k4 regulates Myf5 expression, which is the first MRF that is expressed during skeletal muscle development and marks the commitment of the muscle lineage. When Map4k4 was silenced in C2C12 myoblasts, the enhanced myotube formation was correlated with a transient increase in Myf5 expression at the early stage of differentiation (Fig 2.2 and 2.8).

Map4k4 and MAPKs

Role of Myf5 in skeletal muscle differentiation

It is unclear whether Myf5 up-regulation is a primary cause of the promoted myogenic differentiation because data from different studies are controversial. Early studies demonstrated that Myf5 induced myogenic conversion of non-muscle cells (9, 11, 39). Ectopic Myf5 expression in embryonic C3H10T1/2 fibroblasts resulted in multinucleated syncytia formation and muscle-specific gene expression (39) as well as a rapid and sustained increase in endogenous myogenin mRNA (108). In contrast, an antisense Myf5 oligomer abolished IGF2-induced myogenin transcription, creatine kinase elevation and cell fusion in L6 muscle cells (170). Defective myotube formation was also observed in C2C12 myoblasts that were targeted with Myf5 antisense oligonucleotides or shRNA (71, 224). These data suggest that Myf5 is necessary and sufficient for myogenic

differentiation. However, evidence from Myf5-null primary myoblasts implied an inhibitory role of Myf5 on myogenesis (184). These cells underwent precocious differentiation with attenuated proliferative ability, indicating that Myf5 was favorable for proliferation. In addition, Myf5 overexpression in C2C12 cells failed to increase the myotube fusion index, although higher expression of muscle genes including myogenin and MCK was observed in the transfected cells (31).

In our study, Myf5 was a positive mediator of C2C12 differentiation. Myf5 silencing impaired myotube formation with a decreased fusion index and decreased muscle gene expression (Fig 2.9) while forced Myf5 expression significantly promoted myogenesis (Fig 2.10). *In vivo* studies have indicated a redundant relationship between Myf5 and MyoD (41, 97, 105, 236), and we detected an increase in MyoD protein level upon Myf5 silencing during C2C12 differentiation (Fig 2.9C). However, up-regulated MyoD seemed to fail to completely compensate for the Myf5 loss in our study, because impaired myotube formation was still observed upon Myf5 silencing in C2C12 cells (Fig 2.9A), indicating that Myf5 plays an independent role in myogenic differentiation at least *in vitro*. We did not detect any significant change in MyoD expression in Map4k4-silenced C2C12 cells (Fig 2.4 and Fig 2.9C), but a preliminary experiment in mouse primary satellite cells showed that both Myf5 and MyoD expression increased upon Map4k4 silencing during muscle differentiation, associating with enhanced myotube formation observed in the Map4k4-silenced cells (Mengxi Wang, unpublished data). This finding indicates that both Myf5 and MyoD could be the downstream effectors of Map4k4 to regulate myogenic differentiation in primary muscle cells. In addition, although MyoD

expression was unaltered upon Map4k4 suppression, its transcriptional activity may increase to promote myogenic differentiation. Further investigations are required to examine Myf5 and MyoD transcriptional activities upon Map4k4 suppression or ectopic expression, especially in primary satellite cells.

Regulation of Myf5 expression by Map4k4 in C2C12 muscle cells

A transient increase in Myf5 expression was detected upon Map4k4 silencing in C2C12 muscle cells (Fig 2.8), suggesting that Map4k4 is a negative regulator of Myf5 expression during early myogenic differentiation. It is also supported by a correlation between the increased Myf5 expression (Fig 2.8) and reduced Map4k4 protein level as well as kinase activity (Fig 2.1, B and C) at earlier time points during muscle differentiation in the controlled cells. It is unclear why Map4k4 expression and kinase activity are decreased during C2C12 myogenic differentiation. Serum withdrawal might be one trigger for the expression down-regulation, because Map4k4 has been suggested to be a serum-response factor (SRF)-regulated gene in cardiac cell differentiation (303). Growth factors, cytokines and hormones that are abundant in fetal bovine serum are potential regulators of Map4k4 kinase activity, and further research is needed to find out mechanisms by which Map4k4 is regulated during skeletal myogenesis.

We performed double knockdown experiments and observed that Map4k4 silencing-induced myogenic differentiation was attenuated by Myf5 suppression (Fig 2.9), whereas the defective myogenesis caused by ectopic Map4k4 expression was rescued by Myf5 activation (Fig 2.10), supporting the involvement of Myf5 in Map4k4-mediated muscle

differentiation. Notably, RNAi-mediated Myf5 suppression failed to completely abolish Map4k4 silencing-promoted muscle differentiation because the myotubes with the double knockdown were bigger than the one resulted from Myf5 silencing only (Fig 2.9A). On one hand, other downstream effectors beside Myf5 are probably also involved in Map4k4 regulation of myogenic differentiation. On the other hand, Myf5 might not be suppressed as efficiently at the double knockdown condition as it was silenced alone. Satellite cells with complete deletion of Map4k4 and Myf5 will be better tools than C2C12 muscle cell line with siRNA-mediated gene suppression to determine the regulatory relationship between Map4k4 and Myf5 as well as their roles in muscle differentiation.

Myf5 expression was affected by Map4k4 at both the mRNA and protein levels. The mechanisms by which Map4k4 modulates Myf5 activation are important directions for future research. There are several hypotheses that we are testing or have tested to provide more information on the mechanistic aspects of the relationships among Map4k4, Myf5 and myogenesis.

Pax3 activates Myf5 transcription during skeletal muscle development (13, 242). We measured Pax3 expression during C2C12 differentiation in the Map4k4-silenced cells and scrambled controls. Pax3 mRNA levels were relatively low in the control myoblasts as suggested by high Ct numbers obtained from real-time RT-PCR and decreased further with myogenic differentiation. Map4k4 silencing did not significantly change Pax3 expression during myogenesis, indicating that Pax3 is probably not the cause of Map4k4 suppression-induced Myf5 transcription at the early stage of differentiation (Fig A.2). It

is still possible that Map4k4 regulates Pax3 transcriptional activity to affect Myf5 expression. Experiments such as luciferase assays would be needed to test this hypothesis.

Myf5 is also a direct target of canonical Wnt signaling, in which activated β-catenin binds to Tcf/Lef sequences in the regulatory region of Myf5 promoter to induce Myf5 expression *in vivo* and *in vitro* (29, 107, 122). A recent study discovered that R-spondin2 (RSPO2), a Wnt/β-catenin signaling activator, induced Myf5 expression and myogenic differentiation via the canonical Wnt pathway in C2C12 muscle cells (107). RSPO2-stimulated Myf5 up-regulation was impaired by treating the cells with DKK1, an antagonist for Wnt/β-catenin signaling that acts on LRP5/6 receptors (244). These findings implied a possible involvement of canonical Wnt signaling in Map4k4-mediated Myf5 transcription and myogenic differentiation. In fact, we detected an increase in the expression of RSPO family gene RSPO3 during the early stages of C2C12 differentiation upon Map4k4 silencing (Fig A.3A). Myoblasts treated with RSPO3 had enhanced Myf5 mRNA and protein expression (Fig A.3, C and D), and differentiated into bigger myotubes with higher MyHC expression (Fig A.3B). These data indicated a potential for Map4k4/RSPO3/β-catenin signaling that regulates Myf5 transcription. However, Map4k4 silencing in myoblasts failed to induce a further increase in Myf5 mRNA level upon RSPO3 treatment and vice versa (Fig A.2E), suggesting that Map4k4 does not regulate Myf5 transcription by targeting RSPO3. In addition, Map4k4 depletion did not activate a classic Wnt/β-catenin signaling target gene Axin2 expression in C2C12 myoblasts, while exogenous RSPO3 or another canonical Wnt signaling activator Wnt3a did (Fig A3.E), implying that Map4k4 may not regulate β-catenin activity. Consistently, no significant

changes of β-catenin nuclear accumulation were observed upon Map4k4 silencing in the basal, Wnt3a- or RSPO3- treated conditions (Fig A3.F). Based on these results, we believe that Map4k4-mediated Myf5 expression is Wnt/β-catenin signaling-independent. Although the RSPO3 transcripts were increased by Map4k4 silencing, RSPO3 protein levels were not measured. It is possible that RSPO3 protein levels remain unchanged in response to Map4k4 depletion, and other unknown effectors that negatively regulate Wnt/β-catenin signaling could be activated upon Map4k4 suppression, thus masking the promoting role of RSPO3 in the signaling pathway.

A calcineurin/NFAT-dependent pathway can regulate Myf5 expression in skeletal muscle reserve cells, a group of non-dividing satellite cell-like cells in myotube cultures. The calcium-activated phosphatase calcineurin dephosphorylates NFAT, resulting in the nuclear translocation of NFAT to activate Myf5 transcription in reserve cells (95). In addition to controlling Myf5 expression in reserve cells, calcineurin and its target NFATs have been implicated to positively regulate skeletal muscle differentiation and hypertrophy (73, 79, 94). Different NFAT family genes are expressed at distinct stages of myogenic differentiation and regulate various aspects of myogenesis (1) with NFATc3 expressed mainly in myoblasts promoting nascent myotube formation (180), while NFATc1 and NFATc2 are mainly found in myocytes and myotubes and are involved in myoblast-myotube fusion and muscle hypertrophy (117-118, 189, 245). We have preliminary data revealing a faster gel migration pattern of NFATc3 protein from C2C12 myoblast cytosolic and nuclear extracts upon Map4k4 silencing, suggesting that Map4k4 may mediate NFATc3 phosphorylation and thus nuclear translocation in myoblasts (Fig

A.4). However, this effect of Map4k4 silencing on NFATc3 phosphorylation and nuclear translocation may not be sufficient to explain the enhanced Myf5 transcriptional activation upon Map4k4 silencing, because NFAT has been shown to regulate Myf5 expression only in reserve cells, but not in proliferating myoblast cultures (95). The regulatory interaction between Map4k4 and NFATc3 shed light on another potential mechanism by which Map4k4 regulates skeletal muscle differentiation. More experiments are required to confirm the effect of Map4k4 in NFATc3 phosphorylation and determine its functional outcome in myogenesis. It would also be interesting to determine whether the decreased NFATc3 phosphorylation observed upon Map4k4 depletion is mediated through direct interactions between Map4k4 and NFATc3 or by regulating NFATc3 upstream modulators including the phosphatase calcineurin or kinases such as GSK3β, which phosphorylates and inactivates NFATc3 in C2C12 muscle cells (272). In addition, the relationship between Map4k4 and other NFAT genes (eg. NFATc1 and NFATc2) could be examined to determine whether Map4k4 regulates myoblast fusion and muscle hypertrophy in an NFAT-dependent manner.

We have observed a modest increase in Myf5 mRNA (~1.4 fold) but an abundant increase (~3 fold) in Myf5 protein in Map4k4-silenced myocytes. The discrepancy between Myf5 mRNA and protein levels suggests the possibility that Map4k4 may regulate Myf5 at the translational or post-translational level. Map4k4 silencing in cultured adipocytes promoted PPARγ translation and global protein synthesis through activating mTOR/4EBP1 signaling pathway (102), thus Map4k4 may regulate Myf5 in the same manner. We did detect a marked increase in Myf5 protein levels in Map4k4-

silenced C2C12 cells by western blotting (Fig 2.8). However, Map4k4 silencing in C2C12 myoblasts had no detectable effect on the mTOR signaling pathway during myogenic differentiation as determined by unaltered phosphorylation of downstream effectors mTOR p70S6K and 4EBP1 (Fig 3.3). Moreover, Map4k4 suppression did not enhance IGF/Akt/mTOR signaling in IGF1-treated C2C12 myoblasts compared with scrambled siRNA controls (Fig 3.2). These results suggest that Map4k4 may not be a regulator of the mTOR signaling pathway in skeletal muscle. It is still possible that Map4k4 mediates Myf5 translation through other mechanisms such as regulating translation initiation factor eIF2 activity, because a potential interaction between Map4k4 and one of the eIF2 regulators has been detected in cultured adipocytes (Adilson Guilherme, unpublished data). Further investigations are required to determine whether Map4k4 interacts with the eIF2 regulator in C2C12 myocytes, and global protein synthesis could be examined upon Map4k4 silencing. More importantly, [^{35}S] methionine incorporation studies that specifically measure radioactive amino acid incorporation into Myf5 protein should be conducted to ensure that the Myf5 protein level alteration is caused by Map4k4-mediated Myf5 translation, but not only due to the different amounts of Myf5 transcripts available for protein synthesis. Besides enhanced translation, the increased Myf5 protein content could also be because of reduced protein degradation. However, I could not detect a difference in Myf5 protein stability in Map4k4-silenced myoblasts compared with scrambled controls (Fig A.5).

Potential role of Map4k4 in skeletal muscle development and regeneration

To further understand the role of Map4k4 in skeletal muscle development, we are deleting Map4k4 using the Cre/loxP recombination system in mice. Distinct Cre mouse strains including Myf5-Cre and Myogenin-Cre mice will be used to delete Map4k4 in committed myoblasts and differentiating myocytes respectively during myogenesis. Because Map4k4 suppression in C2C12 myoblasts resulted in hypertrophic myotube formation (Fig 2.2), skeletal muscle mass is expected to be greater in Map4k4 flox/flox:Myf5-Cre mice compared with flox/flox controls. Map4k4 flox/flox:Myogenin-Cre mice would display increased myofiber diameter because of enhanced fusion ability upon Map4k4 silencing as demonstrated in cultured muscle cells (Fig 2.7).

The effects that Map4k4 exerts on fusion and Myf5 expression *in vitro* also suggest a potential role for Map4k4 in skeletal muscle regeneration, which is a rapid and extensive self-renewal process that relies on satellite cells. These cells are maintained quiescent in basal conditions and become activated in response to muscle damage or contraction. Activated satellite cells proliferate, migrate to the injury site and differentiate into myoblasts that fuse to restore skeletal muscle architecture. To determine the role of Map4k4 in skeletal muscle regeneration, Map4k4 flox/flox mice will be crossed with Pax7-Cre^{ERT2} mice, whose Cre recombinase expression will be induced by tamoxifen in Pax7-positive cells that represent satellite cells (187). Tamoxifen will be delivered by gavage to delete Map4k4 in satellite cells in adult Map4k4 flox/flox:Pax7-Cre^{ERT2} mice, and cadiotoxin will be injected into the right tibialis anterior (TA) muscle of these mice

to induce regeneration while uninjected left TA muscle will be used as the control. Myf5 up-regulation is associated with satellite cell activation (60) and Myf5 null mice display compromised regenerative myogenesis (96, 269), suggesting that Myf5 is a positive regulator of skeletal muscle regeneration. Because Map4k4 silencing enhanced myoblast fusion and Myf5 expression in C2C12 muscle cells, Map4k4 flox/flox:Pax7-Cre^{ERT2} mice with tamoxifen treatment would be expected to have improved skeletal muscle regenerative capacity compared with controls. However, satellite cell migration is an important step during muscle regeneration, and Map4k4 has been demonstrated to positively regulate tumor cell motility (58) and stimulate presomitic mesodermal cell migration in a nonautonomous manner during embryogenesis (293). Therefore, deleting Map4k4 in satellite cells may interfere with their proper migration to the injury site, resulting in impaired skeletal muscle regeneration. In addition, Map4k4 silencing was suggested to reduce cell proliferation in hepatocyte carcinoma cell lines (157). Although no effect of Map4k4 on proliferation was observed in C2C12 myoblasts (Fig 2.3), it is still possible that Pax7-Cre-mediated Map4k4 deletion during early regeneration would induce precocious differentiation of satellite cells, leading to depletion of the progenitor cell pool and increased formation of new muscle fibers (hyperplasia).

Mdx mouse model is another tool to study the role of Map4k4 in skeletal muscle regeneration. Mdx mice lack the X-linked dystrophin gene that encodes a cytoskeletal protein involved in plasma membrane stabilization and signaling transduction between extracellular matrix and intracellular cytoskeleton (67). These mice display a persistent and progressive cycle of degeneration and regeneration in skeletal limb muscles and

represent a mouse model for human Duchenne muscular dystrophy (DMD) (67). To study the function of Map4k4 in regenerative myogenesis, skeletal muscle specific Map4k4-deficient mdx mice will be generated by crossing mdx mice with Map4k4 flox/flox:Myf5-Cre mice, which have Map4k4 deletion in embryonic myoblasts and approximately 90% of satellite cells that are Myf5-postive (144). Parameters including skeletal muscle morphology, muscle fiber membrane integrity, fibrosis, immune cell infiltration, muscle strength and satellite cell proliferation and differentiation will be compared between the Map4k4 flox/flox:Myf5-Cre/mdx mice and the mdx controls. It is difficult to predict the phenotype of skeletal muscle specific Map4k4-deficient mdx mice considering the undetermined effects of Map4k4 on satellite cell migration and proliferation. I would expect to observe improved skeletal muscle regeneration and muscular dystrophy in the Map4k4 flox/flox:Myf5-Cre/mdx mice if Map4k4 deletion has no inhibitory effect on satellite cell migratory and replicative capacities.

Conclusion

This thesis provides evidence for a novel inhibitory function of Map4k4 in skeletal muscle differentiation. The repression is modulated in a Myf5-dependent manner, independently of Akt signaling or MAP kinases that have been identified to act downstream of Map4k4. Other regulators may be also involved in the Map4k4-mediated myogenic differentiation, and NFATc3 is proposed as a candidate. The mechanisms by which Map4k4 regulates Myf5 expression are not clear. Pax3 and canonical Wnt signaling pathway, which are well studied modulators of Myf5 transcription, appear not

to be involved in Map4k4-mediated Myf5 expression. It will be interesting to test whether Map4k4 regulates Myf5 translation in muscle cells as it affects PPARγ protein synthesis in adipocytes in the future. In addition, I propose that Map4k4 may regulate skeletal muscle differentiation, fusion and regeneration *in vivo*, implicating Map4k4 as a drug target for muscle disorders such as muscular dystrophy. I believe that the work described herein and my future aims will shed light on Map4k4 as a new signaling node in muscle development and function.

Appendix A

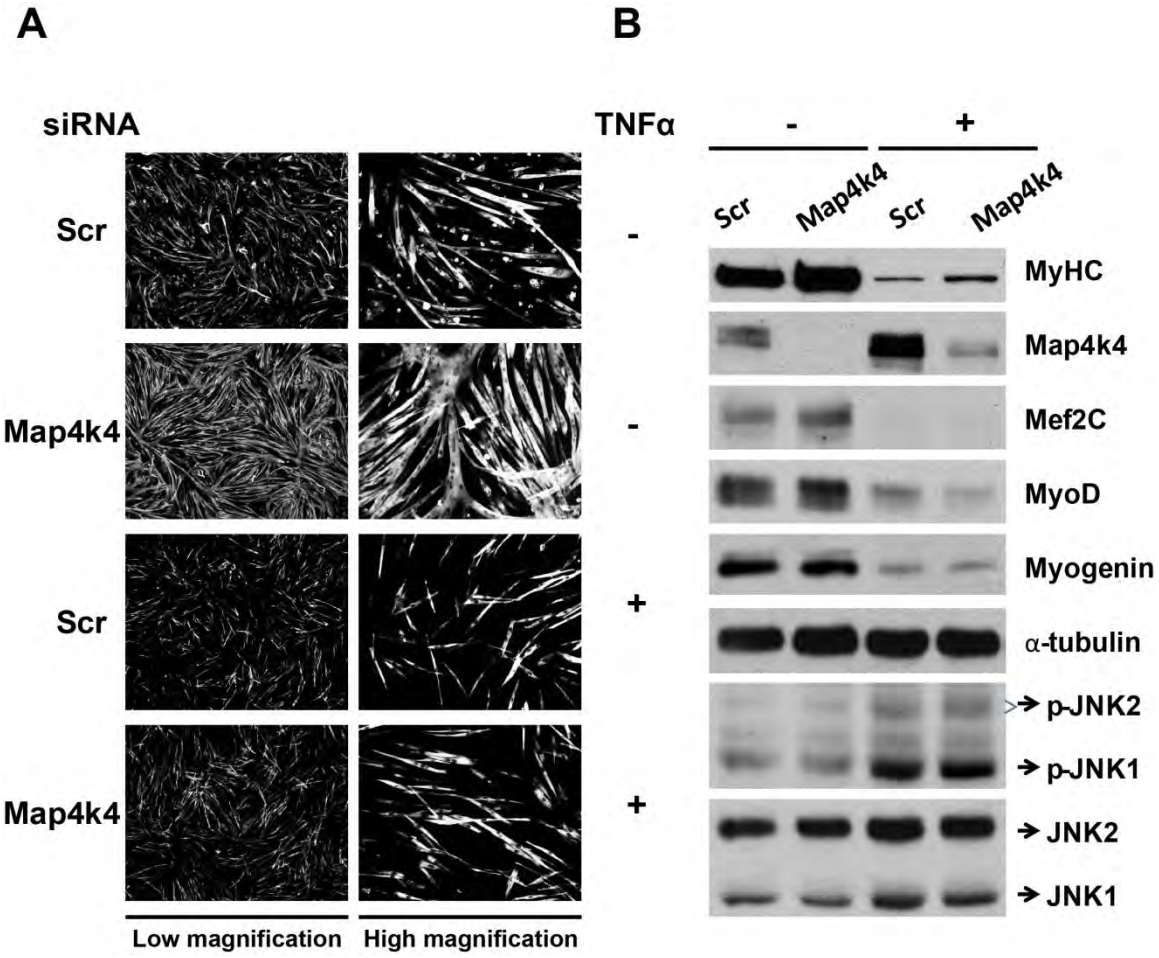

Fig A.1 **Map4k4 silencing marginally rescued the inhibitory effect of TNFα on C2C12 differentiation.** C2C12 myoblasts were transfected with scrambled siRNA or siRNA against Map4k4. 24 h later the cells were differentiated with TNFα (5ng/ml) in DM for 72 h. Untreated siRNA-transfected cells were used as controls. (A) Cells were fixed and immunostained for MyHC and myoblast differentiation was observed by fluorescence microscopy. (B) Immunoblot analysis of Map4k4, myogenic markers and JNK activation. Data is representative of three independent experiments.

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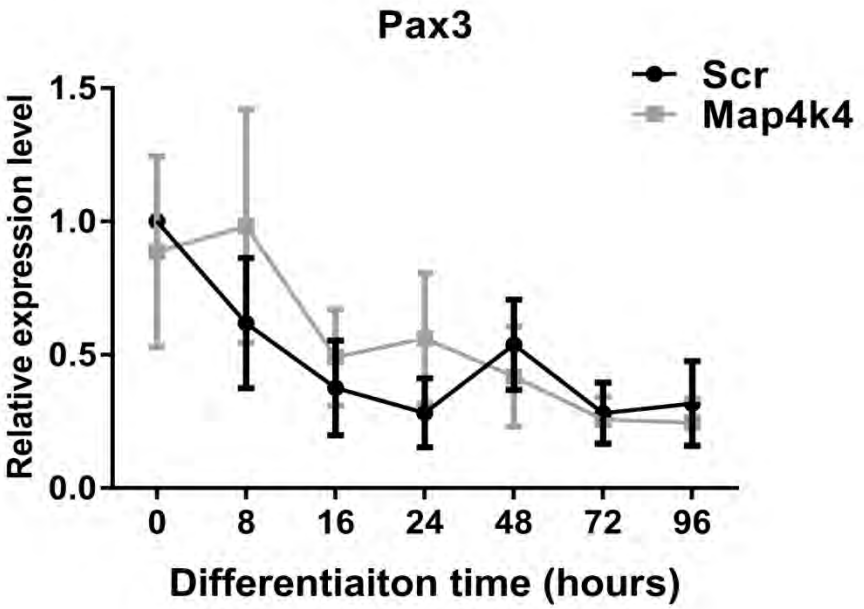

Fig A.2 **Map4k4 silencing does not change Pax3 transcription**. C2C12 myoblasts were transfected with scrambled or Map4k4 siRNA and transferred to DM for the indicated times 24 h post transfection. Pax3 mRNA level was determined by Real-Time PCR. Data is represented as the mean ± SEM of three independent experiments.

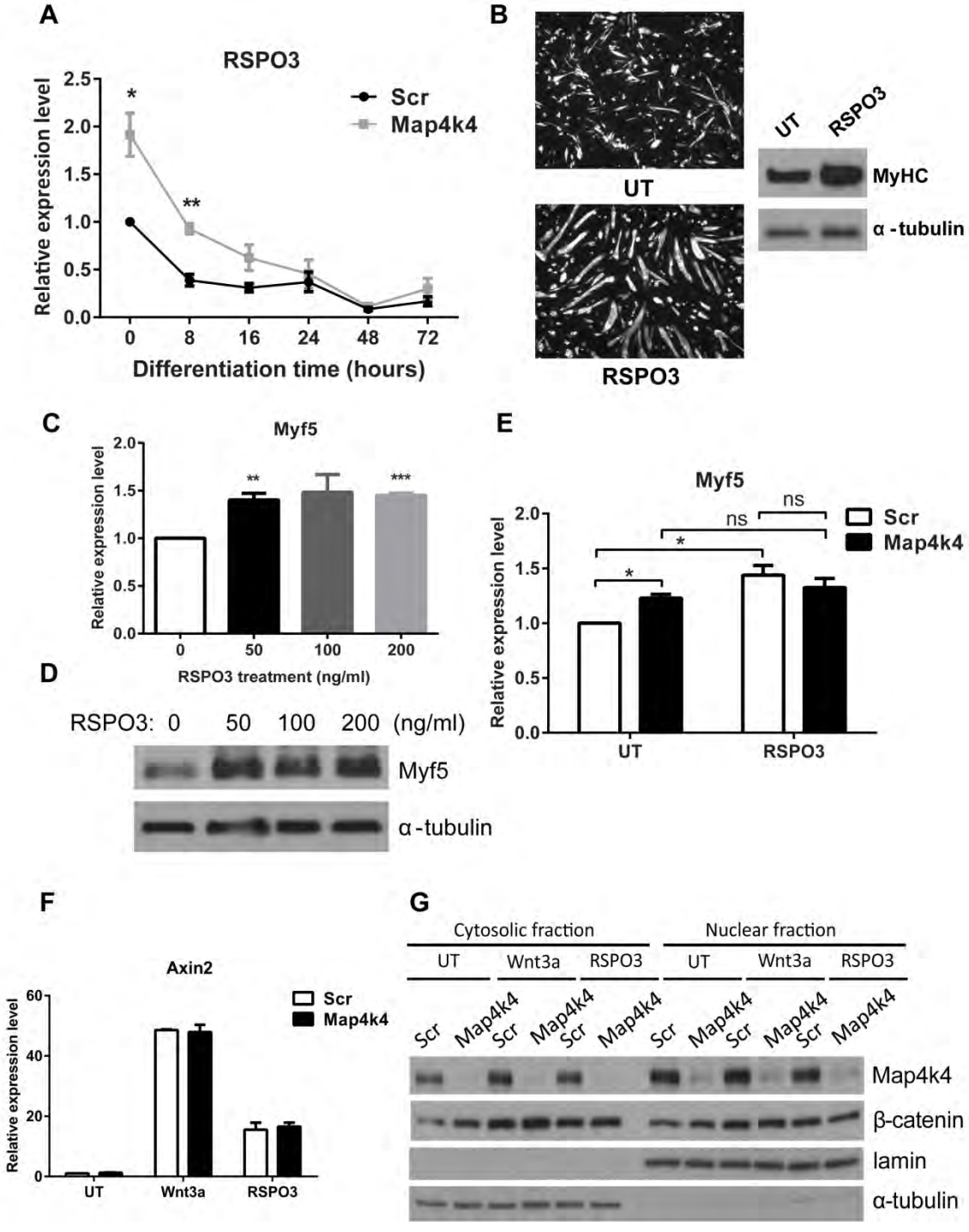
A
RSPO3
Relative expression level
Scr
Map4k4
Differentiation time (hours)
B
UT
RSPO3
MyHC
α -tubulin
C
Myf5
Relative expression level
RSPO3 treatment (ng/ml)
D
RSPO3: 0 50 100 200 (ng/ml)
Myf5
α -tubulin
E
Myf5
Relative expression level
Scr
Map4k4
ns
UT
RSPO3
F
Axin2
Relative expression level
Scr
Map4k4
UT
Wnt3a
RSPO3
G
Cytosolic fraction
Nuclear fraction
UT
Wnt3a
RSPO3
Map4k4
β-catenin
lamin
α-tubulin

Fig A.3 **Map4k4 silencing does not promote Myf5 transcription through activating RSPO3-mediated Wnt/β-catenin signaling pathway.** (A) RSPO3 expression during myogenic differentiation. C2C12 myoblasts were transfected with scrambled or Map4k4 siRNA and transferred to DM for the indicated times 24 h post transfection. RSPO3 mRNA level was determined by Real-Time PCR. (B) C2C12 myoblasts were treated with RSPO3 (50ng/ml) for 24 h in DM and cultured in DM without RSPO3 for another 48 h. Cells were fixed and immunostained for MyHC and myoblast differentiation was observed by fluorescence microscopy (100×). MyHC expression was also assayed by Western blotting. (C-D) C2C12 myoblasts were treated with different amounts of RSPO3 as indicated for 24 h in GM. Myf5 mRNA and protein levels were detected by Real-Time PCR and Western blotting respectively. (E-G) C2C12 myoblasts were transfected with scrambled or Map4k4 siRNA. 24 h later the cells were treated with RSPO3 (50ng/ml) or Wnt3a (25ng/ml) in GM for 24 h. Untreated siRNA-transfected cells were used as controls. Myf5 expression (E) or Axin2 expression (F) was examined by Real-Time PCR. (G) Nuclear and cytosolic factions were prepared and analyzed for Map4k4, β-catenin, lamin and α-tubulin protein amounts by Western blotting. Data is represented as the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

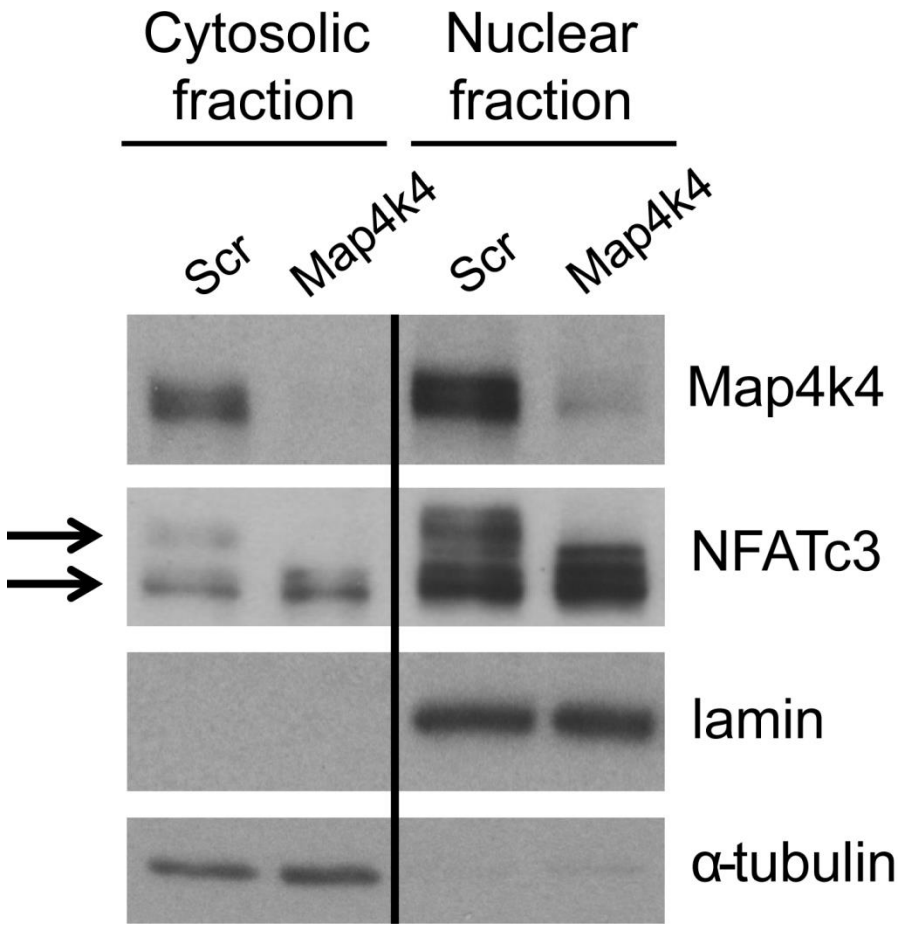

Fig A.4 **Map4k4 silencing results in NFATc3 dephosphorylation in C2C12 myoblasts.** C2C12 myoblasts were transfected with scrambled or Map4k4 siRNA. 48 h later cytosolic and nuclear protein extracts were immunoblotted with anti Map4k4, NFATc3, lamin and α-tubulin antibodies. Arrows on the left illustrate the change in NFATc3 migration due to phosphorylation status. Data is representative of two independent experiments.

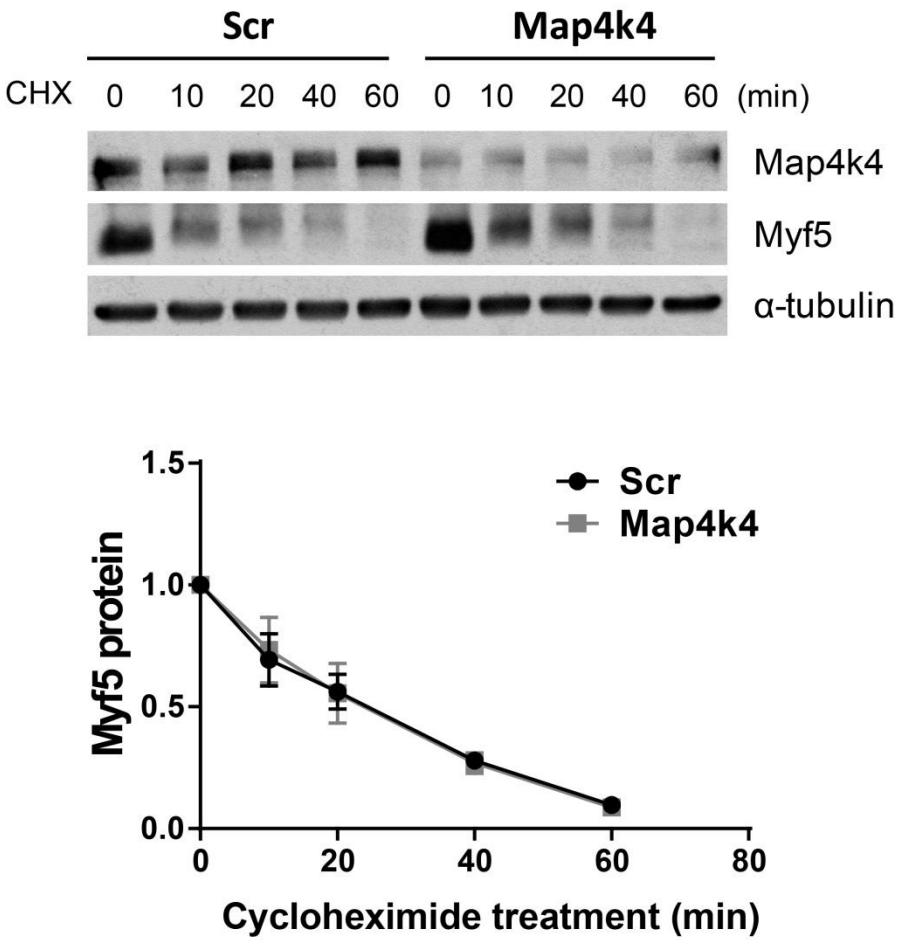

Fig A.5 **Map4k4 silencing does not change Myf5 protein stability.** C2C12 myoblasts were transfected with scrambled siRNA or siRNA against Map4k4. 24 h later the cells were treated with cycloheximide (5 μg/ml) for the indicated times. Cell lysates were examined by Western blot and densitometry analysis for Myf5. Densitometry is representative of three independent experiments.

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